The chemosensory ecology of a foraging hawkmoth

Dissertation

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“As it spread the net of its antennae to seine the night breeze, it caught the perfume of the primroses.
The scent became enmeshed in its plumes, became in truth a part of the moth,
and stirred strange energies in its frail body.
Slave to the wanton call, the moth spread its wings for the first time and bore off into the wind,
winding up the tangled skein of the perfume.”

Vincent Gaston Dethier, 1980,
“The moth and the primrose”

Contents

Introduction

"The world is a thing of utter inordinate complexity and richness
and strangeness that is absolutely awesome."
Douglas Adams

Sensory ecology aims to understand how an animal perceives its world and how this perception has been shaped by interaction of the animal with its environment (Stevens, 2013). However, the goal for us to perceive the world like another animal has often been considered impossible (Nagel, 1974). Already, it is difficult to imagine the world with the colour vision of a bee, where our blue has been turned to green and a yellow sunflower now appears red and it might even be impossible to guess how a bat 'sees' the world through its echolocation system or how a moth perceives an odour plume in flight (Dawkins, 1986). Even though we might ultimately be denied these final insights to the inner world of an animal, we can still aim to reconstruct this world using neurobiological and ethological methods (Hansson and Stensmyr, 2011). These methods form the corner stone of this thesis in which we have attempted to perceive the world like a foraging hawkmoth.

Figure 1 *Manduca sexta* foraging on a *Nicotiana alata* flower.

(Picture by Anna Schroll)

Due to their well-defined foraging behaviour hawkmoth have become leading model organisms within the fields of behavioural ecology and neurobiology (Kelber et al., 2002; Riffell et al., 2013; Sponberg et al., 2015). Within this thesis I have focused on the olfactory guided behavioural of the hawkmoth *Manduca sexta* aiming to relate the olfactory preference of the moth to its foraging decisions (Figure 1). Through this I have tried to generate some further insights into the ecological pressures, which might have directed the evolution of the olfactory system in hawkmoths and into their coevolution with the plants they pollinate.

In the succeeding paragraphs of this introduction I aim provide an overview of: (I) the ecology of this hawkmoth, (II) a short, historical overview of the resarch of olfaction in insects, (III) an introduction into the neurobiology of olfaction and (IV) the odour-guided behaviour of this model species, in oder to give a short background for the following thesis chapters.

I. The hawkmoth *Manduca sexta* and its nectar resources

Among the Lepidoptera, adult hawkmoths are probably the most adapted ones to an airborne life. Most of the about 1400 hawkmoth species known today are characterised by their long slender wings and bodies which are the most streamlined in any insect measured so far (Kawahara et al., 2009; Willmott and Ellington, 1997). These morphological features allow hawkmoths to migrate over long distances as well as exploiting nectar sources in a radius of up to 20 km, making them important pollen vectors for many rare plant species (Amorim et al., 2014; Raguso and Willis, 2003). This is particularly true for the approximately 70 species of the genus *Manduca*, a group of large conspicuous hawkmoth, that have their centre of diversity in middle and Central America (Kawahara et al., 2013). From this group of moths *M. sexta* has developed into a major model species for insect neurophysiology, biomechanics, behaviour and ecology (D. Kessler et al., 2015; Matsumoto and Hildebrand, 1981; Riffell et al., 2014, 2013a; Sane et al., 2007). Even though others species like *M. quinquemaculata* or *M. rustica* have also been studied occasionally (Contreras et al., 2013; Kessler and Baldwin, 2001; Kessler et al., 2010), the ecology of most other *Manduca* species is still largely unknown. In addition most functional studies so far have focused on interaction with flowers in the North American part of the *Manduca* distribution range while most of the biogeographic and ecological data is currently available from Central and South America (Moré et al., 2012), a fact which potentially limits the connection between physiological and ecological results in *M. sexta*.

In the semi-arid landscapes of the southwestern United States of America *M. sexta* mostly forages on large flowers, which show the usual characteristics of the so called “hawkmoth-pollination syndrome”: white corollas, slender corolla tubes and strong night-time emissions of aromatic and terpinoid odours (Riffell et al., 2013a) (Figure 2). However, if these preferred flowers are not available *M. sexta* also readily switches to other nectar sources like those of *Agave palmeri*, which are normally visited by nectar-feeding bats, but the moth returns to its innate preference, as soon as the innately preferred flowers become available again (Riffell et al., 2008b). Interestingly, many of the flowers preferred by *M. sexta* belong to the plants of the Solanaceae family such as *Nicotiana, Datura*, or *Petunia* (D. Kessler et al., 2015; Klahre et al., 2011; Riffell et al., 2008b), which are also the major food source for the *M. sexta* larvae (Kessler et al., 2010). This double edged interaction suggests that the relationship between moth and plant might not only be shaped by the pollination service of the adult moth, but may also be influenced by the antagonistic effects of the *M. sexta* caterpillar.

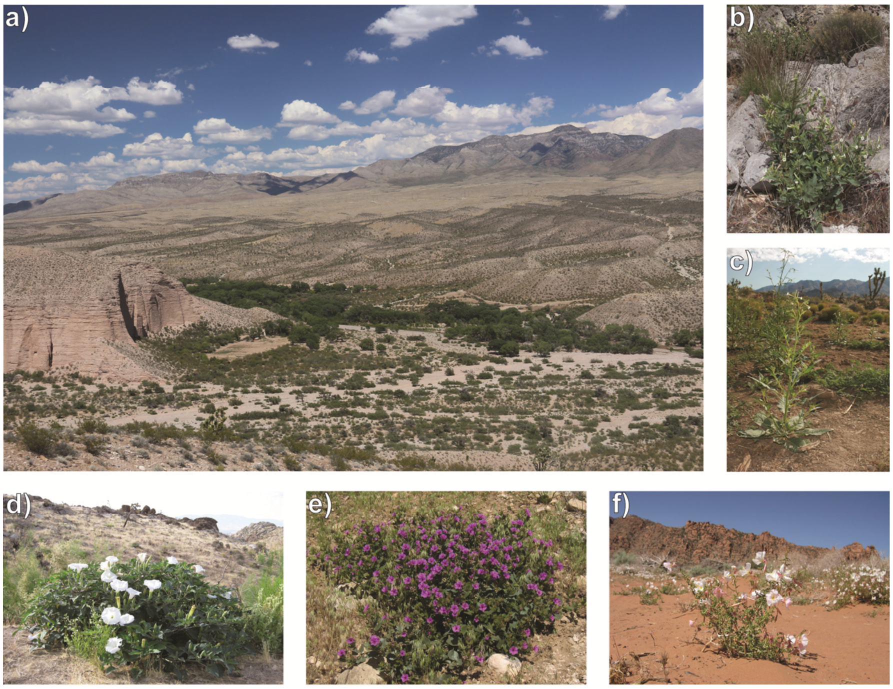

Figure 2 A typical foraging habitat of *M. sexta* in the south-western part of North America (a). The main nectar sources are: *Nicotiana obtusifolia* (b), *Nicotiana attenuata* (c), *Datura wrightii* (d), *Myrabilis* spp. (e) and *Oenothera spp.* (f). All pictures by Danny Kessler.

Additionally, *M. sexta* does share its habitat and thus also its flower resources with several other hawkmoth species such as *M. quinquemaculata* or *Hyles lineata* (Alarcón et al., 2008), which makes the competition with these species another likely factor which drives the specialisation of *M. sexta* towards specifically adapted flowers (Rodríguez-Gironés and Llandres, 2008).

In chapter 1, I explore the specific interactions between *M. sexta* and several *Nicotiana* flowers from both the South American as well as the North American range of the *M. sexta* distribution and investigate, to which extent these flowers could be adapted to hawkmoth pollination. In chapter 4 we manipulate the morphological match between *M. sexta* and *N. attenuata* to test how the interaction between multiple traits might alter the specificity of this plant- pollinator relationship.

II. Research on odour- guided behaviour and neurobiology in insects: a historical perspective from naturalistic observations to neurogenetics

The first steps into the olfactory world of insects were taken by the French naturalist Jean- Henri Fabré in the mid-19th century. Fabre (1879) - through simple experiments - demonstrated that male moth could find their mating partner based on the odorants released by the female (Schneider, 1992). With these observations Fabré was also among the first to explore the instinctive behaviour of animals in their natural environment, an approach, which was revived more than fifty years later by Nikolaas Tinbergen and Konrad Lorenz, the founding fathers of ethology (Tinbergen, 1951). These new studies also facilitated the research on odour-guided behaviour of insects, especially in the honey bee (Frisch, 1927). In parallel to these behavioural studies, Dietrich Schneider and his group, working alongside Konrad Lorenz at the Max- Planck institute for behavioural physiology, pioneered the application of electrophysiological techniques to examine the insect antenna and thereby greatly enhanced the understanding of physiological mechanisms by which odorants are detected (Schneider, 1957; Schneider and Boeckh, 1962).

These early neurobiological techniques were later extended to the central nervous system of different insects, where intracellular measurements of single neurons, as well as measurements using calcium sensitive dyes have greatly extended our knowledge of the central processing mechanisms of olfactory information (Joerges et al., 1997; Matsumoto and Hildebrand, 1981). Simultaneously, the greater availability of these neurobiological data also catalysed the developing fields of sensory ecology and neuroethology, which aimed to place the information on the sensory systems of different animals into their ecological and behavioural context (Hansson and Stensmyr, 2011; Stevens, 2013). Recently, the utilisation of genetic tools which allow the targeting of specific neuronal populations in the vinegar fly, *Drosophila melanogaster,* have further facilitated our knowledge on the processing of olfactory information (Su et al., 2009).

However, while much of the research has focused predominantly on neurobiological methods, less emphasis has been placed on the adaptation of behavioural and ecological concepts, which were developed based on the original ethological theories (Chittka and Thomson, 2001). Within this field of animal behaviour the focus shifted towards measuring the adaptive value of certain behavioural traits, leading to the development of optimal foraging models (Charnov, 1976; Schoener, 1971) and the concept of evolutionary stable strategies. These new approaches have then been summarised by Krebs and Davies providing the foundation of the field of behavioural ecology (Davies and Krebs, 1981). Although, these more ecological methods have so far been rarely adapted in relation to the neurobiology of different insects, the use of more natural stimuli and bioassays along with a greater evolutionary perspective has received growing attention with in sensory ecology and neuroethology (Chittka and Briscoe, 2001; Hansson et al., 2010). More recently, new video recording techniques allow the investigation of odour guided behaviour of many insects in greater detail and more complex environments, which might ultimately facilitate a stronger link between neurobiology and behavioural ecology (Breugel and Dickinson, 2014).

Throughout the different chapters of this thesis I aimed to combine these behavioural and neurobiological approaches, trying to establish a closer link between these two fields of research. With this I hoped to place behavioural traits and their underling neurobiological mechanisms into a framework, which allows a better quantification of the adaptive value of these traits for the animal.

III. The olfactory system of the hawkmoth *Manduca sexta*

Of all sensory stimuli, scent and taste are the hardest to grasp: The visual system detects light, which is defined by wavelength creating colour and photon density creating brightness or hue. Sound can also be precisely measured by wavelengths and intensity and finally touch senses temperature and pressure. The chemical senses, however, detect stimuli, which might vary on dozens of different scales, such as chain length, polarity, chirality and many more. This multitude of properties presumably explains why the olfactory system requires many more different receptor types, than for example the visual systems. The hawkmoth *M. sexta* has only three colour receptors, but approximately 139 chemosensory receptors comprised of three major classes (Koenig et al., 2015a; White et al., 2003).

These receptors, while mainly expressed in olfactory sensory neurons (OSN) on the antenna of the adult insect, are also found in the neurons of other body parts such as the labial palps (Su et al., 2009) and in chapter 3, a novel group of OSNs on the proboscis of the adult hawkmoth is introduced. All OSNs are housed in cuticular structures, the sensilla, which can take different forms, depending on the receptor type that their neurons express. The three main classes of sensilla are: 1.) the sensilla trichoidea, which commonly house pheromone detecting neurons, 2.) the sensilla basiconica which hold most of the neurons sensitive to flower and other plant odours and 3.) the sensilla coeloconica, which are thought to contain neurons mainly responsive to acids and amines (Shields and Hildebrand, 2001; Silbering et al., 2011). However, there is also a great number of additional chemosensory sensilla types in different insects species and in the different chemosensory tissues (Barth, 1991). Hawkmoth for example have also been shown to use CO_2 to detect freshly opened flowers at a distance (Goyret et al., 2008), which has so far been shown to be mainly detected by sensilla in the labial pit organ of *M. sexta* (Guerenstein et al., 2004a; Kent et al., 1986), but receptor genes homologue to the CO_2 receptors in *D. melanogaster* have also been found on the moth antenna (Koenig et al., 2015a), the female ovipositor (Klinner et al., 2016), on the proboscis and the tarsi of the male moth (Grosse-Wilde personal communication, April, 6st, 2016).

The antenna of the adult male hawkmoth contains more than 262,000 OSNs (Dieudonné et al., 2014; Homberg et al., 1988), which project from the antenna to the first processing centre in the brain - the antennal lobe (AL) (Figure 3). Here, all OSNs expressing a certain receptor type converge into structures called glomeruli. Within these glomeruli, the OSNs connect to local interneurons (LN) and projection neurons (PN). The LNs link between different glomeruli and are thought to establish a gain control on the OSN input (Martin et al., 2011). However, in *M. sexta* these ~340 LNs display a comparatively large morphological diversity and the function of these different LN classes is still largely unknown (Strausfeld, 2012). Within the glomerulus OSNs and LNs also connect to the 360- 400 PNs which transmit the olfactory information up to the higher brain centres such as the mushroom bodies (MB) and the lateral horn (LH) (Homberg et al., 1988). In the MBs olfactory and visual information become integrated and memory formation takes place (Stöckl et al., 2016a). In contrast to the MBs, the LHs receive exclusive input from the olfactory system and although the function of this neuropile is largely unknown in moth, it has been shown to be involved in innate behaviours in flies (Strutz et al., 2014).

Under natural conditions olfactory stimuli rarely occur as single chemical compounds but are most often a mix of many different molecules. The flower odour of *Nicotiana alata* for example comprises about 70 compounds (Raguso et al., 2003). In addition, odour blends might not only be defined by their chemical compounds, but also by the ratios at which these compounds occur (Webster et al., 2010). How and where in the nervous system this information about a certain odour blend is combined to form the specific ‘odour object’ or ‘Gestalt’ of a certain odour blend is not fully understood but it might already be established at the antennal lobe level (Riffell et al., 2009).

In chapter 2 of this thesis I approach this question on how odour blends are defined behaviourally, by analysing the impact of the odour blend composition and the blend concertation on the behaviour of the hawkmoth *M. sexta*.

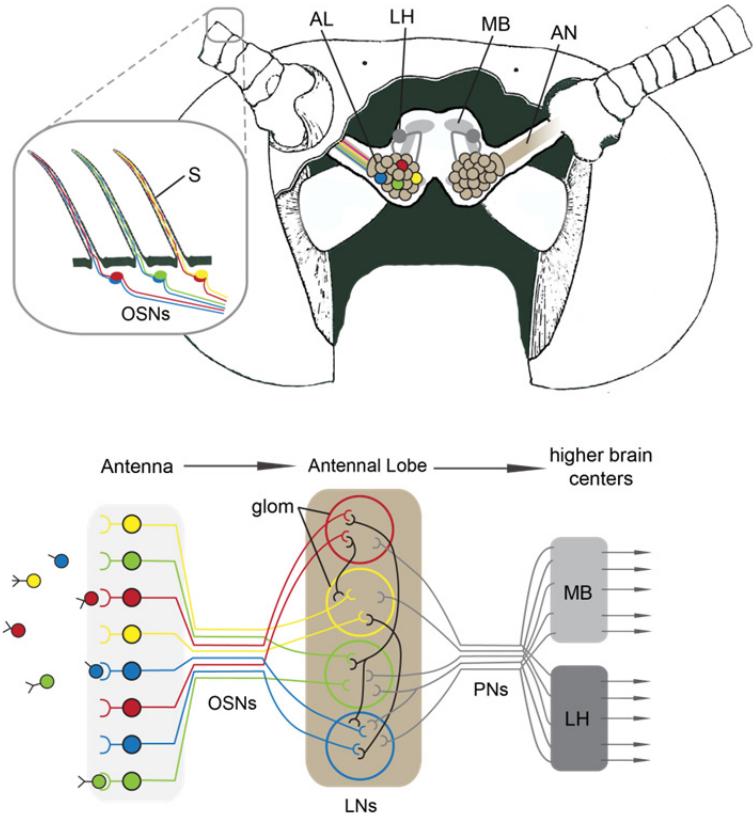

Figure 3 Principle organisation of the olfactory system in the hawkmoth *M. sexta*. (OSN) Olfactory sensory neuron; (Glom) Glomerulus; (LN) Local interneuron; (PN) Projection neuron; (MB) Mushroom body; (LH) Lateral horn. Adapted from Späthe (2013).

IV. Olfactory searching

Insects perceive their world in a way, which is particularly strange to us. Not only do they see the world through the multiple facets of their compound eyes, they also live in a world strongly guided by their chemical senses which are again differently organised than the chemo-sensory systems of most vertebrates (Schoonhoven et al., 2005). Even though the importance of odours for the orientation of insects was already recognised by Fabré, investigations into the physical and chemical properties of the olfactory landscape have so far remained scarce (Buehlmann et al., 2015; Riffell et al., 2014; Steck et al., 2009).

Volatile chemicals seldom defuse undisturbed, but are normally quickly broken up by the wind into distinct odour plumes. These wind forces also keep volatiles that are emitted simultaneously together in consistent odour packages or bursts, counteracting forces of molecular diffusion, which would otherwise drive volatiles apart (Riffell et al., 2008a). Due to this strong impact of the wind and other abiotic factors chemical gradients cannot give reliable information over a distance of more than a few centimetres (Murlis et al., 1992). Different to mammals, which detect odours while sniffing them into their nasal cavity when they are breathing, the insect antenna is directly in contact with the air, which allows the insect a near instantaneous perception of the volatile molecules. This direct perception enables the insect to resolve the time difference between individual odour bursts and to use this intermittency to estimate the distance to the odour source (Vickers, 2006).

However, finding an odour plume and following it to its origin in a moving and turbulent surrounding is not an easy task. First the insect has to find a way of efficiently locating the odour plume in the environment. Often extensive cross-wind runs or flights have been proposed to be the most effective searching strategy and direct observations of these searches in a natural environment have been made in ants and moths (Buehlmann et al., 2014; David et al., 1983). In addition, radar tracking of honeybees in the field has suggested that bees use Lévy flights[1] to locate the odour plume of a feeding side (Reynolds et al., 2009). Interestingly, up- and downwind flights might provide a viable alternative strategy in cases of highly turbulent environments, since as soon as wind directions change by more than 60°, odour plumes will meander to such a degree that the cross-wind drift of the odour plume will exceed its downwind drift (Cardé and Willis, 2008). However these turbulence- dependent strategies have so far only been investigated theoretically (Dusenbery, 1990).

As soon as the insect has found an odour plume and contacted the first filaments it usually turns upwind to follow the odour plume (Figure 4). To determine the upwind direction insects are thought to use their visual system to assess the drift which is created by the wind, a strategy thus known as optomotor-anemotaxis (Vickers, 2006). Although the exact physiological mechanisms underlying this behaviour are still unclear, this model has proven very consistent with most behavioural results so far. When the upwind course is established, most insects show

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[1] Lévy flights are a form of random movement in which the length of the search paths between two turning points is drawn from a distribution with a power-law tail. As a consequence of this, intervals of small scale local searches are interspaced with occasional long runs/flights into a new area. Lévy flights are thought to allow an animal a more efficient exploration of a large area than a random search based on a Gaussian distribution (Reynolds and Rhodes, 2009)

a straight upwind flight as long as they repeatedly encounter new odour filaments (Mafra-Neto and Cardé, 1994). In moths this straight upwind surge will change to a ‘zick-zack’ flight pattern known as casting, in which the animal turns up and down the wind line as soon as they do not encounter new odour filaments within a given time (Cardé and Willis, 2008). These counterturns are thought to arise from the action of so called ‘flip-flop’ neurons, which activate the steering muscles of the moth in an alternating fashion, but are normally suppressed during repeated odour stimulation (Kanzaki et al., 1991). With more time elapsing between odour stimulations these counterturns widen further until the moth will finally resort again to an extended cross-wind flight in search for a new odour plume. However, the length of the upwind surge and the suppression of the counterturning does not only depend on the time interval between different odour encounters, but also on the quality of the odour detected. Incomplete pheromone blends for example, are less likely to trigger an upwind surge than the full blend (Vickers and Baker, 1992). However, till now the optomoter-anemotactic response of the moth has only been examined in detail for different pheromones, but little is known on whether insects also use these mechanisms during food searching.

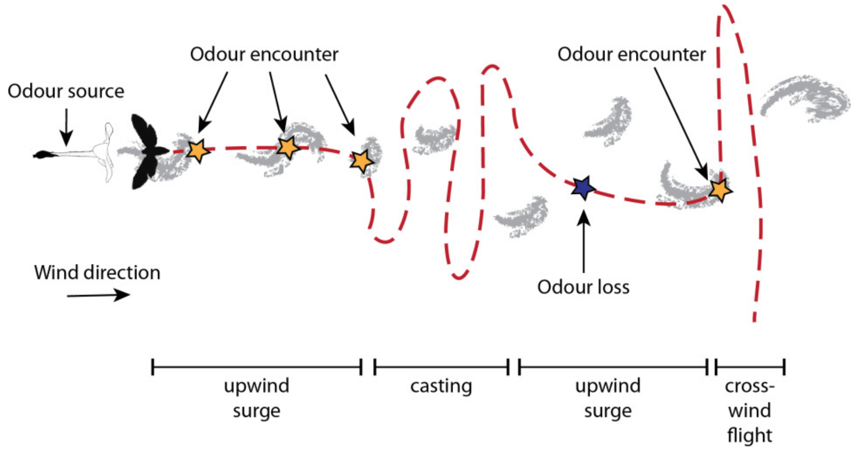

Figure 4 Schematic upwind flight of *M. sexta* approaching a floral odour source. Red dashed line indicates the moth flight path, grey sketches indicate odour pulses.

In the chapter 1 of this thesis it was investigated to what extent the odour plumes of *Nicotiana* flowers, which differ in their reward quality, are able to trigger upwind surges in the hawkmoth *M. sexta*. Through this I was aiming to link these odour-guided searching strategies with the general foraging ecology of the hawkmoth and its co-evolution with different flowering plants.

Upon arriving within a few centimetres of the source the odour filaments fuse into a continuous odour field and the insects has to relay on chemotactic mechanisms to finally locate its mating partner or food source (Cardé and Willis, 2008). Honeybees landing on a flower for example are fully immersed in the scent cloud of that flower and use the gradient between their two antennae or the pending movements of a single antenna to orientate towards the centre of the flower (Lindauer and Martin, 1963). In chapter 3 of this thesis we show how hawkmoth can use olfactory sensilla on their proboscis to evaluate single flowers, suggesting mechanisms by which moth detect nectar resources at a close range.

V. Plant-Pollinator coevolution

Insects and plants are the two kingdoms of multicellular organisms dominating the surface of our planet: insects by the number of species, plants by biomass (Schoonhoven et al., 2005). These two kingdoms are tightly linked through a long history of coevolution and a major step within this evolutionary *tête-à-tête* was the employment of insects by certain plants as pollen vectors (Barth, 1991). This development accelerated the rise of the flowering plants or Angiosperma, which dominate today's land fauna with regards to the number of know species (Schoonhoven et al., 2005). However, the evolution of flowers not only facilitated the number of plant species, but also induced the evolution of many new insect species, specialised on taking nectar and pollen (Misof et al., 2014). The Lepidoptera - moth and butterflies - have driven the adaptation to nectar feeding to an extreme, leading to the development of mouth parts several times the length of the insect's body, which they use to suck nectar from tubular flowers of a corresponding length, even at the expense that they can no longer feed from the flower pollen (Krenn, 2010). Moreover, the adaptations of certain Lepidoptera and in particular certain hawkmoth, to flower feeding are not only limited to their proboscis, but also their flight abilities and visual systems, as they are particularly suited to feed from specific types of flowers (Sponberg et al., 2015; Jordanna D. H. Sprayberry and Suver, 2011; Stöckl et al., 2016b).

In spite of the strong morphological adaptations of hawkmoth pollinators to the flowers they pollinate, the evolutionary pressure that the moth exert on these flower traits has been far more widely studied (Nilsson, 1988; Whittall and Hodges, 2007), than the effects which flowers have on the insect's fitness (Heinrich, 1976). Also the co-evolution of plants and pollinators has often been viewed as strongly biased towards the plant side: long-tongued hawkmoth for example appear to exert a selection pressure on flowers to evolve ever longer corolla tubes, while hawkmoth with a long proboscis have been found to forage opportunistically on flowers with both short and long corolla tubes, making them independent of specific flower types (Johnson et al., 2016). However, most pollinator species operate on the edge of energetic profitability, thus even small changes, like a proboscis which allows for a slightly more efficient use of flower resources, could have strong effects on the pollinator's fitness (Bauder et al., 2015; Heinrich, 1979a), although these might not become directly apparent by the absolute number of flower species a pollinator can visit. Optimal-foraging models additionally support the hypothesis that long-tongued hawkmoth should specialise on long-tubed flowers under the assumption that these flowers will provide a higher energy gain for these moth and that they allow them to avoid competition with short tongued hawkmoths species (Rodríguez-Gironés and Llandres, 2008). In chapter 1, we provide direct evidence that moth do indeed gain the highest net energy from flowers matching their proboscis even when nectar calories do not differ between flower species, supporting these model predictions (Haverkamp et al., 2016a).

Detecting and evaluating resources takes a key position in determining the foraging efficiency of different pollinators as this information form the basis for most of the behavioural decisions taken by the animal. Within the 'hawkmoth-pollination syndrome', for example, flowers mainly emit volatile blends composed of oxygenated aromatic and terpenoid compounds (Riffell et al., 2013a) and plants like *N. alata, Datura wrightii* or *Petunia auxillaris* which have all been studied intensively for their pollination by *M. sexta* show a remarkable overlap in the volatile compounds that they produce and which have been found to be highly attractive to *M. sexta* (Haverkamp et al., 2016a; Klahre et al., 2011; Riffell et al., 2013a). Interestingly, flower match not only the identity of their scent compounds to the preferences of the moth but in some cases they also match the time of volatile emissions to the main activity phases of their night time pollinators (Yon et al., 2015) and have even been shown to change the temporal rhythm of their emissions to select between different pollinators (Kessler et al., 2010).

Chemosensation, as the detection of olfactory and or gustatory cues, has been argued as an essential factor in the development of host plant specialization and thereby of insect biodiversity (Linz et al., 2013; Schoonhoven et al., 2005). Olfactory genes evolve relatively rapidly and are thus, often highly adapted to the specific foraging requirements of a certain insect (Hansson and Stensmyr, 2011). Due to this potential for rapid evolution, the olfactory gene set and thereby, the olfactory system in general, provides an important candidate for mediating the adaptation of *M. sexta* to the floral volatiles emitted by its preferred nectar sources (Koenig et al., 2015a). Additionally, flowers might also exploit an existing sensory bias in a certain pollinator, for example, by mimicking the sex pheromone of the pollinator (Schiestl, 2015). Interestingly, such a sensory exploitation could play an important role in moth-pollinated flowers, as here floral volatiles also resemble those compounds which are normally used as oviposition cues by the female moth (Reisenman et al., 2010).

Hawkmoths appear to be uniquely adapted to both the chemical and morphological properties of their nectar plants and I have investigated this adaptation in multiple traits throughout this thesis. In chapter 1, the impact of morphological adaptations on the feeding foraging performance of the hawkmoth *M. sexta* was examined and correlated to the behavioural response towards different floral volatiles. In chapter 4, I additionally tested the influence of flower orientation on the foraging success of *M. sexta*, which revealed a strong adaptation of the moth's motor skills to specific flower positions.

Overview of manuscript

Chapter 1

Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth

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Already Darwin had hypothesized that every hawkmoth species should be attracted to flowers matching its proboscis in length. In this 1st chapter, Darwin's hypothesis was tested using the hawkmoth *Manduca sexta* and seven moth pollinated *Nicotiana* species. We found that *M. sexta* was indeed most attracted by the volatiles of those flowers which most closely resembled its proboscis in length. It was furthermore shown that the moth energy balance was also most optimal when the moth was foraging on flowers matching the length of it proboscis. Through these results we provide further insights into the co-evolution of pollinators and plants and into the selective pressures which might have influenced the evolution of the olfactory system in hawkmoth.

Built on an idea conceived by A. Haverkamp, M. Knaden and B. S. Hansson.
Experimental design: A. Haverkamp (50%), J. Bing, E. Badeke, M. Knaden and B. S. Hansson.
Wind tunnel assays and analyses: A. Haverkamp (90%)
Respiratory measurements and analyses: J. Bing and A. Haverkamp (40%)
Photo-ion detector measurements and modelling: A. Haverkamp (50%) and E. Badeke (50%)
Wrote the manuscript: A. Haverkamp (60%), M.Knaden and B.S. Hansson

Chapter 2

Host plant odors represent immiscible information entities - Blend composition and concentration matter in hawkmoths

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In the 2nd chapter the influence of changes in the headspace composition and headspace concentration on the oviposition behaviour of *M. sexta* was tested. It was found that the animals did not discriminate between the unaltered and the diluted blend of attractive host plants. However, when the blends of two previously attractive host plants were mixed at equal proportions, the resulting mix was no longer attractive to the hawkmoth. These findings indicate that *M. sexta* uses a blend specific 'odour image' for host location, which is also robust against certain changes in the strength of the blend signal. Similar mechanisms could also be used by the hawkmoth to locate energetically profitable flowers.

Built on an idea conceived by all authors.
Experimental design: A. Späthe, A. Reinecke, M. Knaden and A. Haverkamp (5%)
Performance and analysis of wind tunnel assays: A. Späthe, A. Reinecke and A. Haverkamp (20%)
Performance and analysis Photo-ion detector measurements: A. Späthe and A. Haverkamp (30%)
Wrote the manuscript: A. Späthe, A. Reinecke, M. Knaden, B. S. Hansson and A. Haverkamp (5%)

Chapter 3

Hawkmoths evaluate scenting flowers with the tip of their proboscis

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Efficient foraging in hawkmoth relies on a precise evaluation of individual flowers during hovering flight. In this 3rd chapter we could demonstrate that *M. sexta* possesses olfactory sensory neurons on the tip of the proboscis, which detect floral volatiles and help to increase the time invested by the moth into handling an individual flower. This increase in time investment resulted in a greater foraging success for the moth and a greater cross-pollination for the flower, both in the wind tunnel and under semi- natural conditions in a free flight tent, highlighting the importance of this close range flower evaluation by the proboscis for pollinator- plant interactions.

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Wind tunnel assays and analyses: A. Haverkamp (80%) and F. Yon
Measurement of seed set and nectar uptake: F. Yon and A. Haverkamp (40%)
Single sensillum recordings: A. Haverkamp (50%) and I. W. Keesey
Scanning electron microscopy, immunohistochemistry and image analyses: C. Mißbach and A. Haverkamp (20%)
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Chapter 4

The right angle to plant-pollinator interactions: How Flower orientation influences plant fitness and hawkmoths' foraging efficiency

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Pollinators and the flowers they visit have coevolved in many different traits and for a successful interaction these traits need to occur in a coherent manner. In the 4th chapter the flower orientation was altered during the time of the peak volatile emissions from a natural upward or horizontal to a downwards position. At this downward orientation the moth were not able to gain nectar or to pollinate the flower. However, as this change in the flower orientation did not change the flower odour distribution, the moths were not able to detect the change and allocated the same amount of hovering time into these manipulated flowers. These results demonstrate the importance of floral volatiles for the foraging decision of the moth and shows how pollinators and plants might have co-adapted simultaneously within multiple traits.

Build on an idea conceived by all authors
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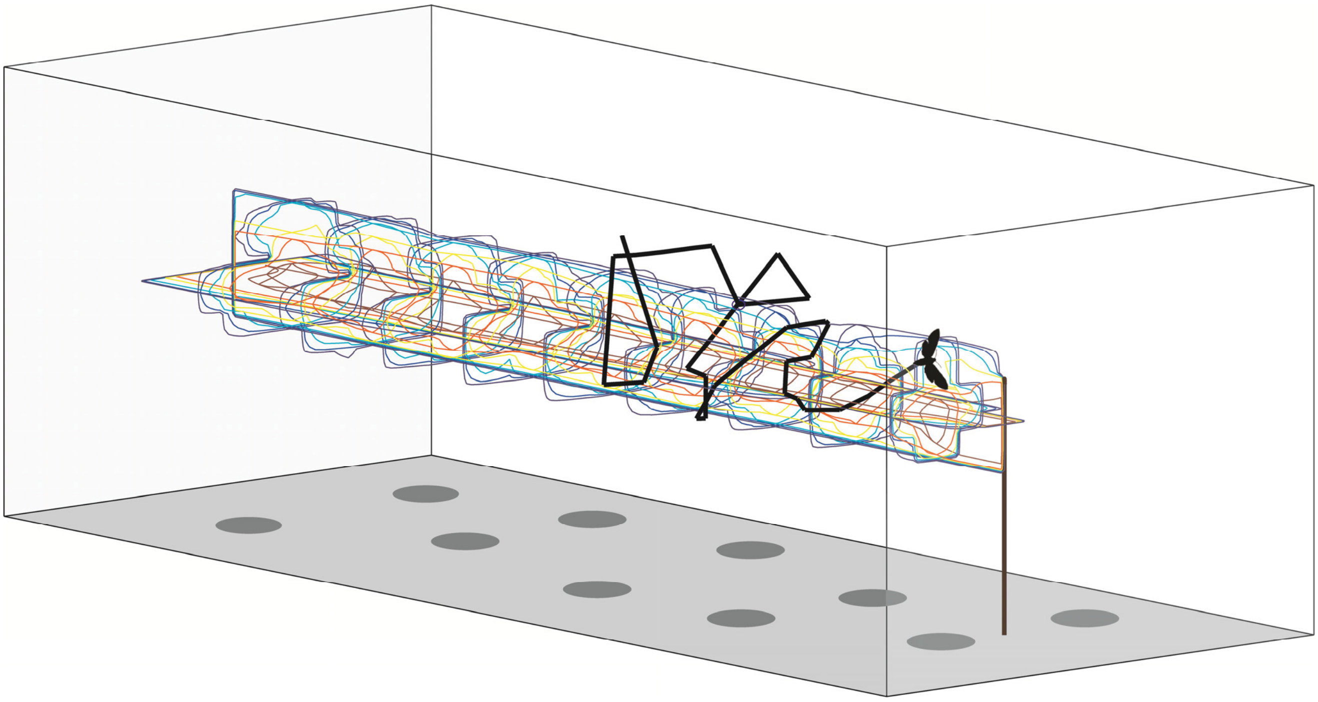
Wind tunnel assays and analyses: A. Haverkamp (60%) and F. Yon
Measurement of pollen transfer and nectar uptake: F. Yon and A. Haverkamp (40%)
Modelling of flower movement energetics: F. Yon and A. Haverkamp (10%)
Wrote the manuscript: A. Haverkamp (40%), F. Yon, M. Knaden, I.T. Baldwin, B. S. Hansson

Chapter 1

“I grew up watching star trek. I love star trek.
Star trek made me want to see alien creatures,
creatures from a far-distant world.
But basically, I figured out that I could find those alien creatures right on earth.
And what I do is I study insects.”

Michael Dickinson



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Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth

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Cost efficient foraging is of especial importance for animals like hawkmoths or hummingbirds that are feeding 'on the wing', making their foraging energetically demanding. The economic decisions made by these animals have a strong influence on the plants they pollinate and floral volatiles are often guiding these decisions. Here we show that the hawkmoth *Manduca sexta* exhibits an innate preference for volatiles of those *Nicotiana* flowers, which match the length of the moth's proboscis. This preference becomes apparent already at the initial inflight encounter, with the odour plume. Free-flight respiration analyses combined with nectar calorimetry revealed a significant caloric gain per invested flight energy only for preferred—matching—flowers. Our data therefore support Darwin's initial hypothesis on the coevolution of flower length and moth proboscis. We demonstrate that this interaction is mediated by an adaptive and hardwired olfactory preference of the moth for flowers offering the highest net-energy reward.

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When foraging, pollinators have to balance their energy expenditure against their energy gain to maximize their reproductive success[1,2]. Through these energetic economic decisions pollinators are not only influencing their own ecology, but do also alter their evolutionary relationship with the flowers they pollinate[3]. Darwin was among the first to propose a coevolution between pollinators and flowers, based on pollen transfer and increased nectar uptake[4]. On the basis of his initial observation that red clover flowers can only be pollinated by long-tongued bumblebees, Darwin suggested that the morphology of pollinators and flowers has coevolved ‘ [...] in the most perfect manner’. From this initial hypothesis, it has been argued that pollinators would forage most effectively on flowers matching their morphological, as well as their sensory requirements. Thereby pollinators would drive the evolution of floral traits, leading to the development of ‘pollination syndromes’, where phylogenetically unrelated flowers match the preference of their pollinators in form, colour and scent[4–6]. However, long-tongued hawkmoths and other pollinators with a specialized morphology have frequently been observed foraging on flowers not matching their morphological traits[7,8]. This generalization among pollinators raises the question how specialized pollination systems can evolve and persist.

Floral volatiles have been argued to inform a pollinator about the potential energy gain to be obtained from a flower, allowing the pollinator to recognise a most rewarding flower among others and thereby facilitating a coevolution of plants and pollinators[9]. Pollinators are faced with the dilemma that they cannot infer directly about the nectar amount of a certain flower[10] and even less about the specific foraging costs associated with that flower. Hence, pollinators have to rely on indirect cues, such as odour or colour to predict the reward value of a flower. The hawkmoth *Manduca sexta* has been shown to innately prefer flowers emitting oxygenated aromatic compounds and certain terpenes[11,12]. However, even among these flowers, corolla morphology and nectar energy varies greatly[13] and studies correlating olfactory preferences of a pollinator and the reward value of different flowers are still scarce. Interestingly, flowers not emitting volatiles have been shown to receive less pollination service by *M. sexta* than those emitting volatiles, but lacking nectar, indicating that floral scent might indeed be a more important predictor for a nectar reward, than the reward itself[14]. In those cases, in which pollinators do strongly rely on volatiles to predict the energy gain of a flower, volatiles might indeed support the establishment and stabilization of specialized plant–pollinator interactions.

Floral volatiles typical for a ‘hawkmoth pollination syndrome’ are often associated with a long slender shape of the corolla, seemingly fitting the proboscis of a hawkmoth[15]. However, evidence that individual pollinators might be foraging more efficiently on flowers matching their proboscis length has rarely been presented. In a competitive situation between several bee species, each one utilizes mainly those flowers matching its proboscis, suggesting that each bee species might have a foraging advantage on its matching flower[16]. Moreover, it has been shown that long-tongued individuals sometimes have longer handling times than short-tongued individuals, indicating that simply increasing the proboscis length might not be an evolutionary stable strategy[2,17]. Similarly, a study on hummingbirds using artificial flowers found that birds foraging on matching flowers exhibited the shortest handling times[18]. Although these studies provide a good indication that foraging on matching flowers is energetically advantageous for a pollinator, a full energy balance on natural flowers might be required to draw further conclusions. Most studies on energy use have inferred energetic cost only indirectly, for example, by measuring foraging time[19,20]. Hence, the question whether the need to optimize foraging has indeed influenced the coevolution of matching proboscis and flowers, still remains controversial.

On the basis of these previous findings, we aimed to test whether the hawkmoth *M. sexta* does exhibit a behavioural preference towards volatiles emitted by flowers matching its proboscis and whether this preference is indeed adaptive for the moth.

M. sexta has a close association with different plant species of the *Nicotiana* genus, thereby offering an attractive system for the study of plant–pollinator interactions[21,22]. We selected seven *Nicotiana* species that overlap in geographic range with the distribution of *M. sexta* and are known to be moth pollinated[23–25], but vary in corolla lengths (Fig. 1). Using wind tunnel assays and three-dimensional (3D) video tracking, we tested whether *M. sexta* has an innate preference for those flowers most closely matching its proboscis in length. Furthermore, we performed inflight respiration measurements and nectar calorimetry to investigate whether *M. sexta* does indeed forage most optimally on the one flower directly matching its proboscis. Taken together, our results demonstrate that *M. sexta* exhibits an innate preference for *Nicotiana* flowers matching its proboscis, and only these flowers contribute significantly to the energy gain of the moth during foraging. Through these results, we show how coadaptation mediated by floral volatiles can arise even in apparently generalized pollination systems, supporting Darwin’s hypothesis on the coevolution of pollinators and flowers.

Results

Morphological match. The flowers of all tested *Nicotiana* species emit volatiles that can be detected by *M. sexta* and have previously been argued to be associated with moth pollination[25,26] (Supplementary Fig. 1). However, while the corollas of six species are either significantly shorter (four species) or longer (two species) than the proboscis of

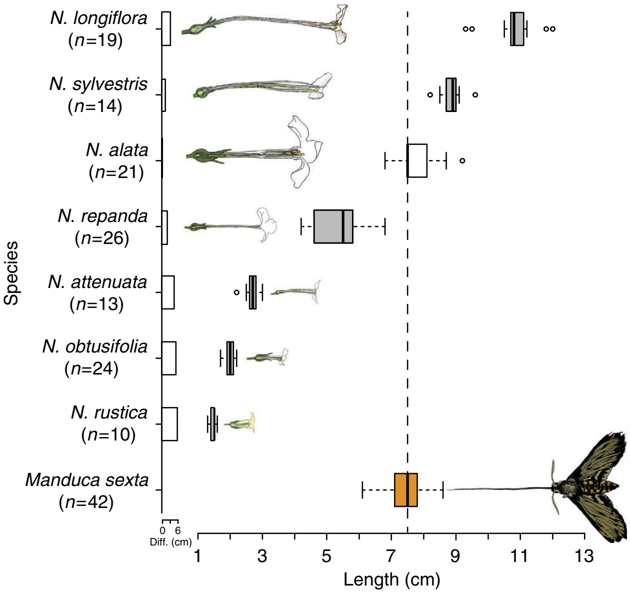

Figure 1 | Corolla and proboscis lengths of seven *Nicotiana* species and the potential pollinator *Manduca sexta*. Grey boxes indicate corolla lengths that significantly differ from the length of the moth proboscis (orange box, dotted line indicates proboscis median length; $P < 0.05$, Kruskal–Wallis test followed by Holm corrected Wilcoxon rank-sum test), while the length of the *N. alata* corolla (white box) does not differ from the proboscis length ($P > 0.05$). Bar plots next to the flower names indicate absolute differences between corolla length and the moth’s proboscis (cm).

M. sexta, only the corolla of *Nicotiana alata* matches the proboscis length (median *N. alata* = 7.5 cm, $n = 21$; median *M. sexta* = 7.5 cm, $n = 42$; corrected Wilcoxon rank-sum test, $P = 0.2568$; Fig. 1).

Flight behaviour and olfactory preference. By characterizing the odour filament distribution within the plume (Fig. 2a), using a photoionization device (200 A miniPID, Aurora) and by analysing the moths' flight patterns with a custom-built 3D tracking system, we were able to estimate the odour encounter rate for each moth and flight (Supplementary Movie 1). As the odour encounter rate was highest in the core of the plume, only animals approaching the source on a direct path would experience a high encounter rate (Fig. 2a). The quantification of odour encounters by a flying moth, hence, informs about the moth's motivation and performance to focus on the core of the plume. When we calculated the odour encounter rate per second of flight, most flowers differed significantly from the no-flower control ($n = 22$–27, Kruskal–Wallis test, $P < 0.0001$), with the highest odour encounter rates being observed for *N. alata* (median = 1.60%, $n = 27$, corrected Wilcoxon rank-sum test, $P < 0.001$) and *N. sylvestris* (median = 1.49%, $n = 27$, corrected Wilcoxon rank-sum test, $P < 0.001$; Fig. 2b). Interestingly, the longest as well as the shortest flower did not differ from the control (*N. longiflora*: median = 0.32%, $n = 25$, corrected Wilcoxon rank-sum test, $P = 0.9875$; *N. rustica*: median = 0.24%, $n = 25$, corrected Wilcoxon rank-sum test, $P = 1.0$).

We found such rapid upwind surges when presenting the headspace of *N. alata* to hungry moths (as compared with a no-flower control, median = 16.38 cm s^{-1}, $n = 12$, corrected Wilcoxon rank-sum test, $P = 0.037$; Fig. 2c), while none of the other tested flower odours induced significantly increased upwind speed. Finally, the flower odour plumes of *N. alata* and *N. sylvestris* were the only ones that resulted in significantly more animals, reaching the source as compared with the control (*N. alata*: median = 48.15%, $n = 27$, corrected Fisher's exact test, $P = 0.0043$; *N. sylvestris*: median = 37.04%, $n = 27$, corrected Fisher's exact test, $P = 0.044$). Consequently, we conclude that although most of the tested *Nicotiana* species triggered behavioural responses, only the odours of *N. alata* and to a lesser extent *N. sylvestris*, that is, those species whose corollae exhibit the best fit to the *M. sexta* proboscis, provoked behaviour that finally guided *M. sexta* moths to the source.

Nectar amount and calorific value. Since not nectar volume, but energy content is of ultimate importance for the moth, we analysed the calorific value of the nectar in each tested species, using a gas chromatography–mass spectrometry (GC–MS)-based approach (Fig. 3a). Although the nectar volume was related to the flower length (Supplementary Fig. 2a), the nectar of longer flowers was often less concentrated (Supplementary Fig. 2b). Therefore, the energy provided by flowers of different length was mostly similar and unrelated to flower length. Hence, nectar energy on its own did not explain the moths' foraging preference.

Nectar gain. Knowing the nectar energy content for each *Nicotiana* species, we next analysed how much of this nectar was consumed per moth and visit. We then estimated the species-specific gross-energy gain attained per moth and flower visit (Fig. 3c). While the moths gained significant amounts of energy from visiting the flowers of the two attractive species *N. alata* (median = 42.17 J, $n = 10$, corrected Wilcoxon rank-sum test, $P = 0.0059$) and *N. sylvestris* (median = 16.68, $n = 6$, corrected Wilcoxon rank-sum test, $P = 0.0359$), visiting four out of five of the less attractive flowers resulted in a gross-energy gain not significantly different from zero. In case of the shorter flowers, this reduced energy gain was mainly due to a low success rate in flower handling: in case of the shortest provided flower type (*N. rustica*) only 20 per cent of the moths succeeded in collecting the nectar. Interestingly, moths easily inserted their proboscis into the non-attractive *N. longiflora*. However, due to the too long corolla of this flower species the nectar could not be reached by the moths, resulting in no energy gain whatsoever from this otherwise nectar-rich species.

Energy balance. Although the gross-energy gain of the moth did already explain the behavioural preference of the moth to a certain degree, the choice for *N. alata* became even more understandable when we in addition took into account the energetic costs a moth faced per flower visit. To do so, we measured the respiration of the moth while foraging on different flowers, using a custom-built set-up (Fig. 3b; Supplementary Movie 2), which allowed us to measure the ambient CO_2 increase during a single-flower approach in free flight. When we calculated the net-energy gain (that is, balanced the gross-energy gain per flower visit against the rate of energy spent), the moth's preference for *N. alata* turned out to be highly adaptive. Only feeding on flowers from *N. alata* resulted in a significantly positive net-energy gain (median = 28.27 J, $n = 10$, corrected Wilcoxon rank-sum test, $P = 0.014$; Fig. 3d), indicating that *N. alata* would indeed be the optimal and most reliable foraging choice.

Discussion

Pollinators and plants have coevolved with respect to certain traits such as morphology, colour or scent, leading to so-called 'pollination syndromes'[27]. This coadaptation within multiple traits has often been argued to lead to a specific advantage for a suiting pollinator, driving the evolution of such specialist pollination systems[6]. In the present study, we show that *M. sexta* has an innate olfactory preference for those flowers whose corolla length matches the length of the moth's proboscis. When analysing foraging costs and gains of naive moths at flowers with differing corolla lengths, we found that only matching flowers significantly contributed to the moth's net-energy gain. Therefore, this study provides new evidence on how specialized pollination systems can be stabilized through physiological advantages and how this specialization is strengthened by the sensory system of the animal.

Floral volatiles are of particular importance to night-active pollinators such as *M. sexta* to effectively locate a suitable flower[28,29]. However, the mechanisms by which pollinators use volatiles to find and assess flowers are still far less understood than the use of visual cues in plant–pollinator interactions[9]. Flying insects following an odour plume usually navigate, using the frequency of odour encounters (that is, the percentage of time during which the moth encounters the odour) rather than the odour concentration[29,30]. Notably, odour filaments maintain the specific volatile composition emitted by the flower and would thereby allow the pollinator to recognise the identity of a flower also at a distance[30]. Our results highlight the importance of the odour encounter rate for *M. sexta* to navigate towards a flower in the absence of informative visual cues. Moreover, we found that already the first encounter with the odour plume did enhance the upwind movement of the moth. The extent of the upwind movement depended on the presented flower, indicating that flower-specific differences in the detection and/or valence of floral volatiles were already established at a distance of >1 m. These results are coherent with pheromone-induced flight

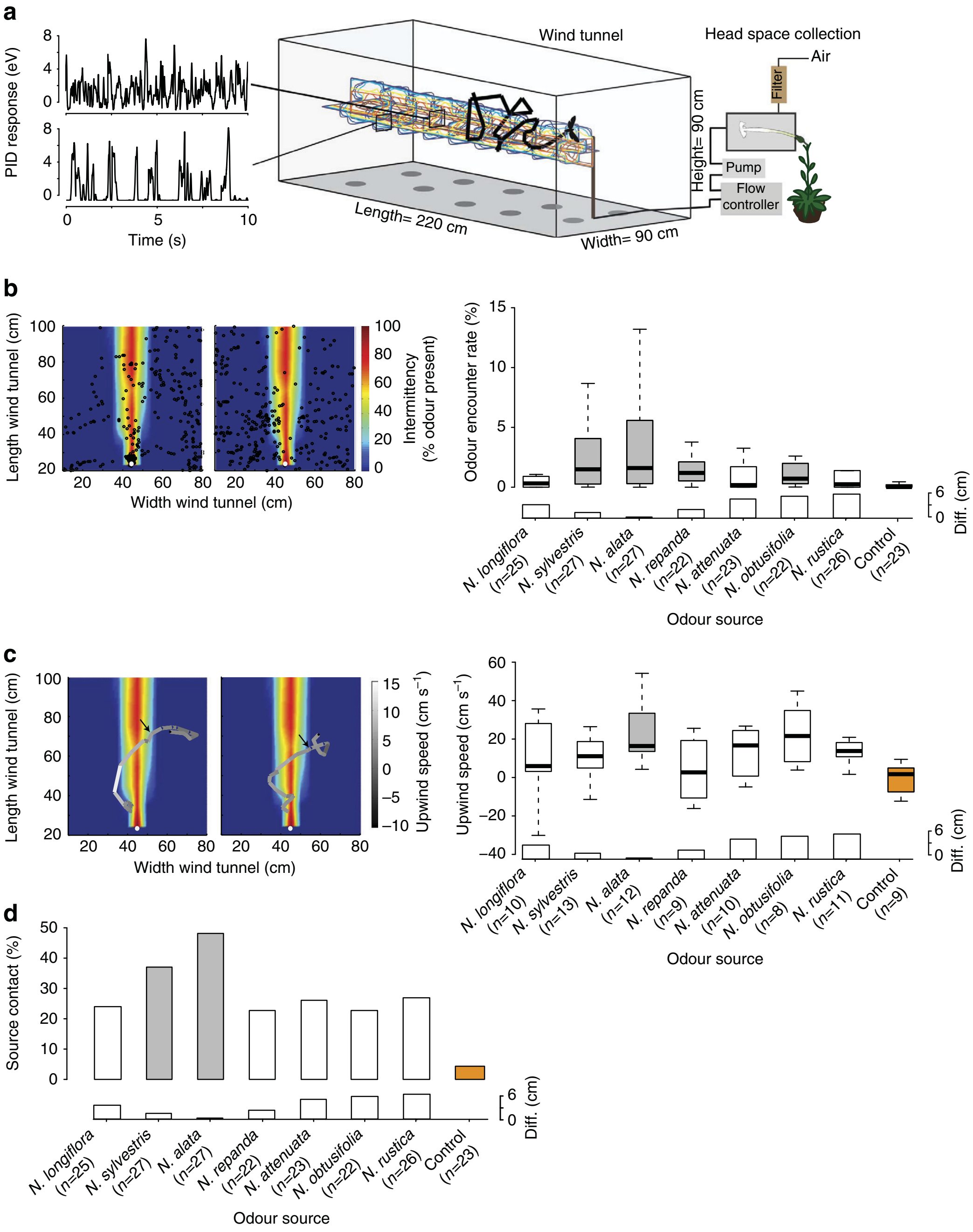

Figure 2 | Behavioural responses of *Manduca sexta* towards headspaces of different *Nicotiana* flowers. (**a**) Experimental set-up. Bold black line, example trace of a moth approaching the odour source; coloured pattern in tunnel, representation of odour intermittency within a plume as derived from Photo-Ionization Detector (PID) measurements. Left: PID recordings in central (top panel) and peripheral (lower panel) positions of the plume. Each amplitude denotes a filament. (**b**) Moth's encounter rate with odour plumes of different *Nicotiana* species. Few but long filaments at source result in a high odour encounter rate (% of time the moth encounters the odour at a specific location in the wind tunnel) as well as short, but many filaments downstream of the source. Grey boxes, $P<0.05$ to no-flower control (Kruskal–Wallis test ($P<0.0001$) followed by corrected Wilcoxon test). Outliers not shown. Bar plots above flower names indicate absolute differences between corolla length and the moth proboscis (cm) as shown in Fig. 1. Left panel: example flight recordings with *N. alata* (left) and *N. obtusifolia* (right) as stimuli, superimposed on the reconstructed odour plume. Black circles represent the last 400 individual tracking events (tracking rate 10 Hz) of two individual flights. Colours indicate the intermittency (that is, the per cent of time odour was measured at that position in the tunnel). Light grey dots indicate position of the odour source. (**c**) Median upwind speed during 0.5 s after first contact with odour plume. Grey boxes, $P<0.05$ to no-flower control (Kruskal–Wallis test ($P<0.0001$) followed by corrected Wilcoxon test). Grey boxes, $P<0.05$ to no-flower control (Kruskal–Wallis test ($P=0.0432$) followed by corrected Wilcoxon test). Outliers not shown. Bar plot as above. Left panels: example flight tracks (*N. alata* (left) and *N. attenuata* (right) as stimuli) reconstructed from individual tracking events similar to those shown in **b** 1 s before to 2 s after plume encounter. Flight tracks are superimposed on the reconstructed odour plume. Black–white colour scale indicates upwind speed of moth (cm s^{-1}). Heat map as above. Black arrow indicates the point of first plume contact. (**d**) Mean per cent of source contacts when different flower headspaces were provided. Grey colour, $P<0.05$ to no-flower control (Bonferroni-corrected Fisher's exact test).

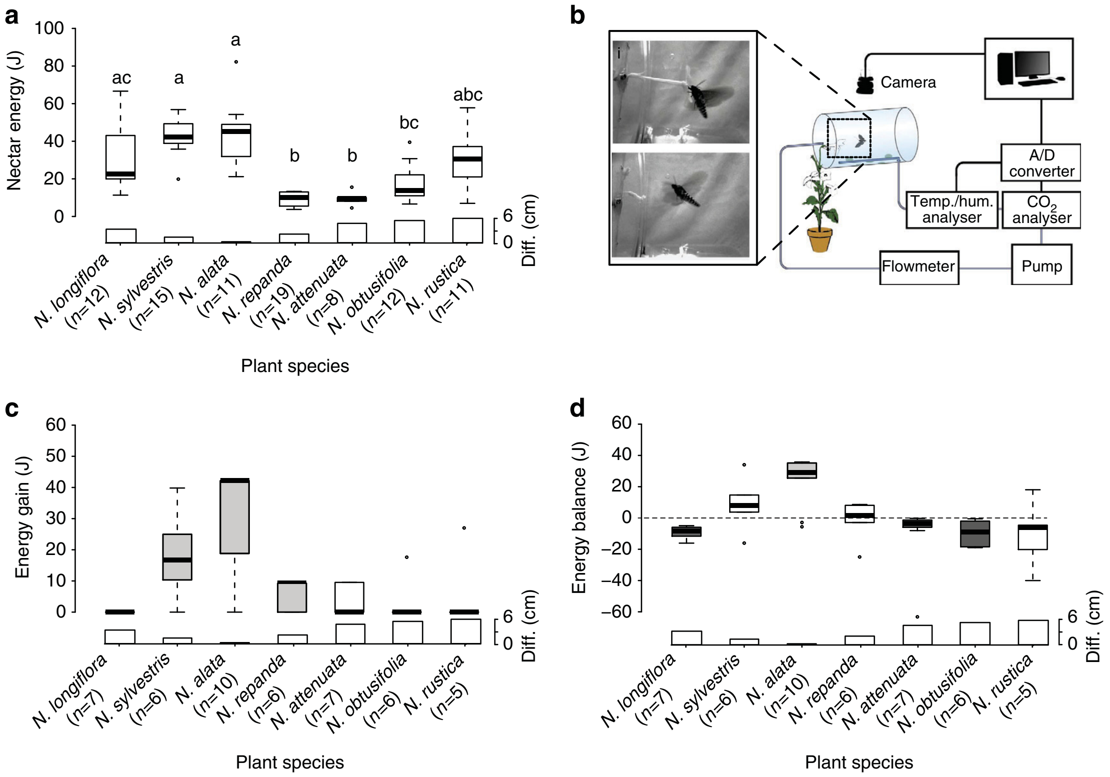

Figure 3 | Cost-benefit analyses of *Manduca sexta* flower handling. (**a**) Total amount of energy per flower provided by the different plant species. Letters indicate significant differences ($P<0.05$, Kruskal–Wallis test ($P<0.0001$) followed by corrected Wilcoxon test). Black points indicate outliers. (**b**) Schematic drawing of the set-up used for measuring foraging efficiency. Pictures show *M. sexta* foraging on *N. alata* and below *N. obtusifolia*. (**c**) Gross-energy gain (J) of *M. sexta* when foraging on different flowers. Grey colour, $P<0.05$ (one-sided Wilcoxon test against zero). (**d**) Energy balance (J) of *M. sexta* after foraging on different *Nicotiana* plants. Light grey colour indicates flowers significantly greater than zero; dark grey indicates flowers significantly smaller than zero ($P<0.05$, two-sided Wilcoxon test against zero).

behaviour, where a single encounter with the pheromone plume does already trigger a rapid upwind surge of a male moth[31,32]. *M. sexta* responds to the loss of an odour plume with casting flights, that is, zig-zag flights perpendicular to the wind direction[33]. Correspondingly, we found a fast increase in upwind speed upon plume encounter, which was sustained for ~2 s. Rapid turns and sudden increases in flight speed are complex behavioural tasks, which involve sensory feedback from the antenna[34] and the wings[35]. Nonetheless, the interaction between flight control and the olfactory system is not fully understood[36], and further work might be needed to test the adaptations of *M. sexta* to track the complex odour plumes of flowers in flight.

Similarly, to pheromone communication between male and female moths, a strong preference towards certain floral volatiles could lead to the development of a ‘private channel’ between a pollinator and a flower, which would then further increase coadaptation. However, such a development is often limited by phylogenetic constraints both on the evolution of olfactory genes in the moth, as well as on the development of genes regulating volatile production in the flower[25,37]. Most of the physiologically active compounds that were emitted by the flowers tested in our study (for example, benzyl alcohol or different aldoximes) derive from the amino acid phenylalanine[26,38], indicating that many of these plants use similar pathways for the production of floral volatiles. Nevertheless, all plants also emitted unique compounds, which should allow the moth to discriminate between the different plants (Supplementary Fig. 1). Interestingly, *Petunia axillaris* another flower that has repeatedly been found to be pollinated by *M. sexta* does match the moth’s proboscis in a similar way as *N. alata* and also emits a similar volatile profile[39,40]. In *Petunia*, the synthesis of these volatiles is mainly regulated by a single transcription factor[41]. Correspondingly, recent work on the *M. sexta* olfactory genes has revealed several lineage specific expansions[42]. These genetic duplication events might have increased the sensitivity of sphingid moths towards specific floral volatiles, parallel to what has been suggested for the *Drosophila* genus[43]. Thus ‘private channels’ between pollinators and plants could evolve based on the relatively small genetic changes, which would then allow a coadaptation to take place.

Given the high spatial and temporal variability of floral resources, it has been a longstanding question under which circumstances pollinators should have developed a behavioural preference, that is, become specialized[8]. Most flowers in our study provided a similar amount of nectar energy, suggesting that the interaction between *M. sexta* and the different *Nicotiana* flowers was not determined by any flower-specific differences in nectar gain. We therefore asked, whether flower-specific foraging costs might play a role in shaping the interaction between *M. sexta* and the different plants. Foraging costs are particularly high for species feeding ‘on the wing’ such as bats, hummingbirds and especially hawkmoths[3,44]. In addition to this, foraging costs may vary considerably depending on the nectar load or environmental conditions[45,46]. Hence, direct energy measurements were needed to fully determine the impact of foraging costs, especially on the behaviour of hovering pollinators. In our study, we found hovering costs ranging from 0.26 W g^{-1} for matching flowers to

$0.54\,\mathrm{W\,g^{-1}}$ for too long or too short flowers. Hovering costs at flowers with a corolla lengths similar to the length of the moths' proboscis were not different from the reported energy expenditure of *M. sexta* during hovering without flower handling of $0.23\,\mathrm{W\,g^{-1}}$ (ref. 47). However, the energy consumption was significantly higher at the two shortest and the longest flowers (Supplementary Fig. 3). While high costs at the longest flower, *N. longiflora,* might have resulted from the constant attempt to reach the nectar source, high costs at the too short flowers might arise because these flowers provided less stabilization for the inserted proboscis and thereby a less stable hovering flight, which has been shown to increase the energy consumption due to a constant need for acceleration and deceleration[48]. In addition, it has to be taken into account that on the shorter flowers moths had a success rate of only 20%, which again increased foraging costs, as the moths were forced to invest several costly attempts before reaching the nectar. Together, the high hovering costs and the low success rates significantly influenced the foraging outcome of the moths when visiting the different flowers. However, we only tested naive hawkmoth during their first contact with a flower and as it has been shown that experience can improve flower handling performance[49], one can assume that repeated contact even with the morphologically less fitting flowers could result in a positive energetic outcome. Therefore, the preference for matching flowers should not prevent the moth from visiting other flowers, in case matching flowers are absent in a habitat or so far apart that travelling costs exceed flower handling costs. Indeed, in the absence of innately preferred flowers, *M. sexta* has been shown to flexibly widen its foraging scope, but also returns to its innately preferred flower when these become again available[11,12]. However, as experience could also improve the handling efficiency on matching flowers, one would assume that the relative order of the flower preference and the net energy gained from the different flowers would remain also for experienced moth or would even be further strengthened, as it has been shown that the learning rate of insect pollinators also depends on the gained energy reward[50].

As Waser *et al.*[8] pointed out, a flower preference in pollinators becomes beneficial when travel costs are low compared with the costs for flower handling. Willmott and Ellington[46,51] calculated the cost of forward flight for *M. sexta* at travelling speed ($3\,\mathrm{m\,s^{-1}}$) to be $0.13\,\mathrm{W\,g^{-1}}$. Hence, different from the bumblebee where the cost of hovering and forward flight have been found to be similar[52], hawkmoths experience 2–4.2 times higher costs while handling a flower than while travelling between flowers. Taken together, the energetic characteristics of the interaction between *M. sexta* and the different *Nicotiana* species might indeed facilitate the development and maintenance of a strong receiver bias by *Manduca* towards volatiles of matching flowers.

Our results suggest that the difference in net-energy gain of *M. sexta* obtained from different flowers has influenced the coevolution between *Nicotiana* flowers and one of their main pollinators, *M. sexta*. However, energy gain might not always translate into direct fitness advantages, since other factors such as predation and mating success might further influence the life history of an animal[53]. Aerially foraging bats, present a major predation threat to nectar-foraging moths during the search for suitable flowers[54]. A reduced searching time due to an increased detectability or valence of floral volatiles, such as we found in this study might therefore not only increase the energy gain, but could also reduce predation risk. In addition, nectar feeding in hawkmoths leads to a fivefold increase in female fecundity[55] and it has even been suggested that carbon availability limits the life history of nectar-feeding lepidopteran to an even greater extent than nitrogen limitations[56]. Hence, behavioural preference towards flowers with a higher energy gain could indeed lead to fitness differences for hawkmoth foraging on different flowers, even though such a preference might also render this pollination system more vulnerable to environmental and climatic changes[57].

In *On the Origin of species* Darwin wrote: ‘Thus I can understand how a flower and a bee might slowly become, either simultaneously or one after the other, modified and adapted to each other in the most perfect manner, [...].’, suggesting for the first time a coevolution of plants and pollinators. Our study shows that, although *M. sexta* might potentially forage on a variety of flowers, only those flowers matching the specific morphology of this pollinator contribute significantly to their energy gain during nectar foraging, supporting Darwin's initial hypothesis. We furthermore demonstrate that this interaction of moth and flower is mediated by the olfactory preference of the moth. Potentially, this reciprocal interaction between morphological fit and chemical communication of flowers and moths was the precursor of the evolution of numerous specialist pollination systems. Our results therefore stress the importance of chemical communication for pollination and conservation ecology.

Methods

Insect rearing. All animals used were reared at the Max Planck Institute for Chemical Ecology, Jena, Germany, as described in detail by Koenig *et al.*[42] Eggs were obtained from female moths, which were kept under ambient conditions and provided with *N. attenuata* plants for oviposition. Larvae were subsequently maintained on artificial diet at 70% relative humidity and 27 °C with a light:dark cycle of 16:8. Fifth-instar caterpillars were individualized for pupation and left till 1 week before adults enclosed from the pupae at the same climate conditions. Pupae were sexed, and male and female pupae were transferred to separated flight cage with a light:dark cycle of 16:8, 70% relative humidity and 25 °C during the light phase, and 60% relative humidity and 20 °C during the dark phase. Only adult male moths between 75 and 80 h after enclosing from the pupae were used for all experiments.

Plant breeding and headspace collection. All plant species were grown at the Max Planck Institute for Chemical Ecology, Jena, Germany, for several generations. Plants were grown in the greenhouse at 23–25 °C, 50–70% relative humidity and a light:dark cycle of 16:8 h (Philips Son-T Agro 400 W Na vapour bulbs, $350–500\,\mu\mathrm{mol\,m^{-2}\,s^{-1}}$ photosynthetic photon flux at plant level) until elongation. At least 1 week before the experiment plants were transferred to a climate chamber with the same settings as the moth flight cage. Plants were watered daily with 100 ml tap water supplemented with $0.12\,\mathrm{g\,l^{-1}}$ fertilizer (Peters Professional Allrounder, Planta Düngemittel, Germany, nutrient composition: 20% N, 20% P_2O_2, 20% K_2O, 0.015% Cu, 0.12% Fe, 0.06% Mn, 0.01% Mo and 0.015% Zn). For all experiments we used flowers from 5 h till 9 h after the start of anthesis.

To provide a natural headspace of a single flower to the moth in the wind tunnel, we used a set-up as depicted in Fig. 2a. The plant was contained in a separately ventilated compartment behind the wind tunnel. Individual flowers were then carefully placed into a custom made plastic (polyoxymethylene) collection chamber through a small opening without detaching the flower from the plant. The opening was further sealed with cotton wool just behind the sepal leafs. The collection chamber had a fixed volume of 200 ml. To actively collect the floral headspace, we pushed charcoal-filtered air at a rate of $0.9\,\mathrm{l\,min^{-1}}$ into the chamber, while simultaneously pulling $0.7\,\mathrm{l\,min^{-1}}$ out of the chamber and into the wind tunnel using teflon tubing. In the wind tunnel, the plant headspace was released through a small opening (Ø 4 mm). The slight overpressure was applied to exclude contamination with green leave volatiles from the plant. All flowers were placed at least 1 h before the start of the experiments. Through this set-up, we aimed to present the most natural olfactory stimulus possible to maintain the exact blend composition and emission rate of every flower. Although a previous study using a similar set-up has highlighted the importance of the blend composition over the blend strength[58], we cannot conclude whether the here-described preference for *N. alata* is solely based on the blend composition or also on the emission rate. Nonetheless, our data show a clear preference just based on the headspace of a single flower with its natural characteristics.

Odour plume reconstruction. Odour distribution and pulse dynamics in the wind tunnel were measured using a Photo-Ionization Detector (200 A miniPID, Aurora Scientific, Canada) and acetone as a tracer gas. In the close vicinity of the source (0–20 cm), we measured the concentrations in steps of 5 cm along the *x*, *y* and *z* axis. With greater distances from the source, we decreased the measuring steps to 20 cm along the three axes. At each point in space we recorded for 2 min. Data acquisition and storage was done via LabVIEW (National Instruments, USA), further analyses were performed using Matlab (Mathworks, USA). To determine odour filament frequency and the percentage of odour presents, we set a threshold of 1 eV and counted the number of signals above this threshold. On the basis of these values, we derived an odour intermittency value for every point in the wind tunnel by linear interpolation.

3D tracking of moth flights. Hawkmoth inflight response to floral volatiles was analysed in a wind tunnel (plexiglass, $220 \times 90 \times 90\,\text{cm}^3$) set at a constant airflow of $0.4\,\text{m}\,\text{s}^{-1}$, 0.5 lux light, 25 °C and 70% humidity. Flight paths were captured using a custom-built tracking system. Initial images were recorded at 30 Hz by four cameras (Logitech), two positioned on top and two at the side of the wind tunnel. Cameras were set to a resolution of 800×600 pixels, with each pixel having a size of $\sim 0.3\,\text{cm}^2$. The position of the moth was calculated at a rate of 10 Hz based on a background subtraction algorithm implemented in C. Further analyses of tracking data were performed using custom-written Matlab scripts. Individual hawkmoths were kept in small mash tubes (diameter, 13 cm; height, 15 cm) in a pre-exposure chamber at the same temperature, humidity and light as in the wind tunnel for about 1 h before the experiment. For testing, the opened mash tubes were inserted into the wind tunnel onto a take-off platform. Each moth was given 5 min to initiate wing fanning to be considered for analyses. After taking flight moths were recorded for 4 min. All experiments were performed within the last 2 h of the moth's scotophase.

Flights within plume. By aligning the odour filament distribution within the plume with the 3D tracked flight patterns, we estimated the number of odour signal contacted for each moth and flight. As the odour signal was highest in the core of the plume, only animals approaching the source on a direct path would have encountered a high number of odour encounters. The quantification of odour signal detected by a flying moth, hence, informed about the moth's motivation and performance to focus on the core of the plume.

Plume-induced upwind flights. We analysed the upwind speed of the moth shortly after the encounter of the odour plume. For this, the recorded flight tracks were combined with the reconstructed odour plume. We first determined the first point at which a moth based on the reconstructed plume would have encountered the odour signal above 1 eV (noise threshold), during the 4 min of free flight. Starting from this point, we calculated the mean upwind speed over the next 0.5 s, resulting in a single value for each animal. We then compared the upwind speeds of moths tested with flower plumes with those of moths tested with an empty control.

Nectar analyses and net-energy calculation. The energetic outcome of the moth foraging flight is affected by the moth's energy invested and the energy gained through the nectar. Therefore, we investigated the nectar energy provided by the flowers of different plant species, using a GC–MS-based approach. First, we collected nectar from flowers 5–9 h after the onset of anthesis, using 3–5 plants per species. To do so, all flowers of one plant were collected and the corolla tube was cut at half length. We then placed the flower part containing the nectaries upside down in a 50-ml reaction tube and centrifuged for 2 min at 1,000 r.p.m., which caused the nectar to accumulate at the bottom of the reaction tube. Subsequently, the fresh weight of the nectar was noted and the sample was freeze-dried overnight at −80 °C and 0.014 mbar. Dried samples were weighted again and dissolved in a ratio of 1 mg:1 ml in pyridine. To increase the nectar volatility, we derivatized our samples by taking a 40 µl aliquot of the sample and adding 50 µl BSTFA (*N*,*O*-bis(trimethylsilyl)-trifluoroacetamide) supplemented with 1% TMCS (trimethylchlorosilane). In addition, 10 µl phenyl-ß-D-glucoside (Fisher Scientific) dissolved in 1 mg:1 ml pyridine was added as internal standard. The sample was then shaken for 90 min at 37 °C and 225 r.p.m., before being further diluted by adding 900 µl pyridine. After derivatization, we injected 1 µl of our sample into the GC–MS (Agilent Technologies 7890 A, Aglient, USA), using a non-polar HP-5 column (30 m, 0.25 mm ID and 0.25-µm film thickness; Agilent, USA) and operating in split-less mode, with the injection port set to 240 °C. Helium was used as a carrier gas ($1.1\,\text{ml}\,\text{min}^{-1}$). The program started at an initial temperature of 60 °C for 3 min and was increased by 4 °C per min to a final temperature of 300 °C. To identify and quantify the outcome of the GC–MS analyses, we used concentration curves of pure sugars (fructose, glucose and sucrose at 1, 2.5, 5, 10, 15 and 20 µl). We then calculated the total energy per mg fresh mass of nectar for each plant species by summing the amount of all sugars multiplied with their specific energy content[59], and by multiplying this value with the dry to fresh mass ratio of each species. We calculated the mean total energy content for each species by taking the mean amount of nectar fresh mass from 20 additional flowers of each species and multiplied this value by the mean energy value of 1 mg of nectar from that species. The net energy of every foraging flight was derived by subtracting the energetic costs measured by respirometry from the mean energy content calculated for the specific flower species. For those flowers, where there was still nectar remaining in the flower after foraging, we subtracted the amount of the remaining nectar from the mean nectar value of the plant species.

Respiration measurements. Energy expenditure by the pollinator is a crucial factor when addressing the outcome of a flower–pollinator interaction. We therefore measured the CO_2 exhaled by *M. sexta* during flower handling via a flow-through respirometry system. Previous studies have often measured O_2 in addition to CO_2 to determine the substrate of the energy production by the animal[60–62]. However, as O_2 is present at a high background concentration, it is often difficult to be measured accurately for small animals, such as *M. sexta* at a high temporal resolution[63]. Since our study aimed to analyse energy expenditure at a relatively high temporal resolution, we have focus on measuring CO_2 emissions. The system was set up within a fully controllable climate chamber and consisted of a sealed glass cylinder (49 × Ø34 cm) from which the air was pumped into a closed loop through a non-dispersive infrared analyser (Li-820, Licor GmbH, Germany) and back into the cylinder at a rate of $2\,\text{l}\,\text{min}^{-1}$. Since temperature and humidity might influence the CO_2 measurements, we simultaneously recorded these two parameters in the air stream ahead of the CO_2 measurement (Sensirion SHT 75, Switzerland). Both sensors were connected to a PC outside the chamber and operated via LabVIEW (National Instruments, USA). Before every experiment, we placed an intact flower inside the chamber in such a way that the flower remained attached to the plant, but no leaf tissue was enclosed. Previous studies on *Datura wrigthii* flowers have shown that these flowers emit considerable amounts of CO_2 directly after opening, which then decline until ~4 h after anthesis[64]. To exclude any influence of the flower respiration on our measurements, we used only flowers 5–9 h after anthesis and analysed the emissions of several single flowers of each species and a resting moth. However, these measurements did not show any CO_2 emissions detectable by our system (Supplementary Fig. 4). After setting up the system in this way, we allowed the open cylinder and the surrounding air to equilibrate. As soon as the CO_2 concentration within the chamber remained constant, a moth was placed into the cylinder and the front was resealed. The moth was then allowed 5 min to initiate wing fanning and 4 min to approach the flower. During these periods the moth behaviour was constantly monitored using two cameras (Logitech C615, USA), at a resolution of 800×600 pixel and 30 frames per second (FPS). Videos were streamed and recorded using Noldus Media Recorder (Noldus, The Netherlands). Animals, which did not start wing fanning within this time limits, were regarded as non-responders and excluded from the statistical analyses. In case the moth fed from the flower before the 4 min had elapsed, the measurement was stopped as soon as the moth had left the flower. Hence, we recorded CO_2 production of each moth during a single flower visit. Directly after the experiment the length of the moth proboscis was measured. The length of the corolla tube for each flower was measured as the distance between corolla base and corolla disc. To check for the amount of remaining nectar, the base of the flower was carefully opened, the nectar was sucked out using a capillary and the amount of nectar was determined with an electronic balance. The remaining nectar amount in visited flowers was compared with the amount of nectar in non-visited flowers (see above) to calculate the nectar uptake per visit.

Volatile collection. For volatile collection, we used a push–pull system modified from Kessler *et al.*[21] Individual flowers were carefully placed into a custom made plastic (polyoxymethylene) collection chamber through a small opening without detaching the flower from the plant. The opening was further sealed with cotton wool just behind the sepal leafs. The collection chamber had a fixed volume of 200 ml. To actively collect the floral headspace, we pushed charcoal-filtered air at a rate of $0.5\,\text{l}\,\text{min}^{-1}$ into the chamber, while simultaneously pulling $0.4\,\text{l}\,\text{min}^{-1}$ through a glass tube (ARS, USA); packed with glass wool and 20 mg of Super Q (Alltech, Germany). The slight overpressure was applied to ensure that no leaf volatiles would be collected. All volatiles collections using SuperQ filters lasted for one full scotophase (8 h). Finally, volatiles were eluted rinsing SuperQ filters three times with 100 µl dichloromethane(DCM). Samples were then stored at −20 °C till further analyses. For each plant species, we sampled 3–5 flowers from different plant individuals.

Previous to each volatile sampling, the collection chamber and tube connectors containing plastic parts were soaked overnight in Labosol (neoLab, Germany). All parts were then rinsed with distilled water and ethanol, before heating them at 55 °C for 2 h. Holders for SuperQ filters were custom made from polyether ether ketone (PEEK) and always washed with DCM, and subsequently heated at 200 °C for 2 h.

SuperQ filters were washed shortly before use in a series of methanol, chloroform, acetone, DCM and hexane. PDMS tubes were rinsed in ethanol and heated for 1 h at 200 °C under a constant flow of nitrogen.

Volatile analyses. Collected headspace volatiles were analysed using a gas chromatograph-coupled mass spectrometer (Agilent 6890 GC & 5975C MS, Agilent, USA). The GC was used with a non-polar HP-5 column (30 m, 0.25 mm ID and 0.25-µm film thickness; Agilent, USA), operating in split-less mode at a constant flow of $1.1\,\text{ml}\,\text{min}^{-1}$ with helium as carrier gas. The inlet port was set to a temperature of 240 °C and injection volume of 1 µl. The GC oven was set to an initial temperature of 50 °C, which was held for 2 min. Thereafter, the temperature was increased at a rate of $13\,°\text{C}\,\text{min}^{-1}$ to 250 °C, which was again held for 5 min. The MS transfer line was maintained at 280 °C and the MS operated in electron impact mode (70 eV, ion source: 230 °C, quadrupole: 150 °C, mass scan range: 33–350 *m*/*z*, scanning rate 4.42 scan per s). Compounds were identified by comparing mass spectra against synthetic standards and NIST 2.0 library matches.

Electrophysiology. Gas chromatography coupled with electro-antennographic detection (GC–EAD) was used to identify those compounds in the volatile blends of the flowers that are perceived by the moth antenna. For this, we clipped the antenna of a 3-day-old male *Manduca* directly above the scapulum and before the third last flagellum. The two tips of the cut antenna were then inserted into two-glass electrodes filled with haemolymph–ringer. EAD signals were recorded via Ag–AgCl and pre-amplified (10 ×) by a probe connected to a high-impedance d.c. amplifier (EAG-probe, Syntech, The Netherlands). The signals were fed into an analog/digital converter (IDAC-4, USB, Syntech, The Netherlands) and transferred to a PC. GC stimulation was done by injecting 1 µl of the sample into the GC (Agilent 6890, HP-5 column, 30 m, 0.25 mm ID and 0.25-µm film thickness; Agilent, USA). The inlet port was set to 250 °C; the initial oven temperature of 50 °C was raised by 13 °C min^{-1} to a maximum of 250 °C that was held for 5 min. The gas stream leaving the GC was split 1:1 by a 4-arm effluent splitter (Gerstel, Germany), using N_2 (30.3 kPa) as a compensatory gas. One part of the gas stream was directed to the flame ion detector of the GC, whereas the other part was inserted into a humidified air stream (200 ml min^{-1}) leading to the antenna preparation. Data from the flame ion detector and EAD were visualized and recorded simultaneously, using Syntech GC/EAD32 Software (Version 4.6; Supplementary Fig. 1). For further analyses, we exported the data in ASCII format. Voltage amplitudes were determined manually using Matlab (MathWorks).

Data availability. The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files.

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Author contributions

A.H., M.K. and B.S.H. designed the study; A.H. conducted and analysed wind tunnel assays. A.H. and J.B. performed the respiratory measurements. A.H. and E.B. measured the odour filaments and reconstructed odour plume; A.H. wrote the first draft of the manuscript and all authors contributed significantly to the revisions.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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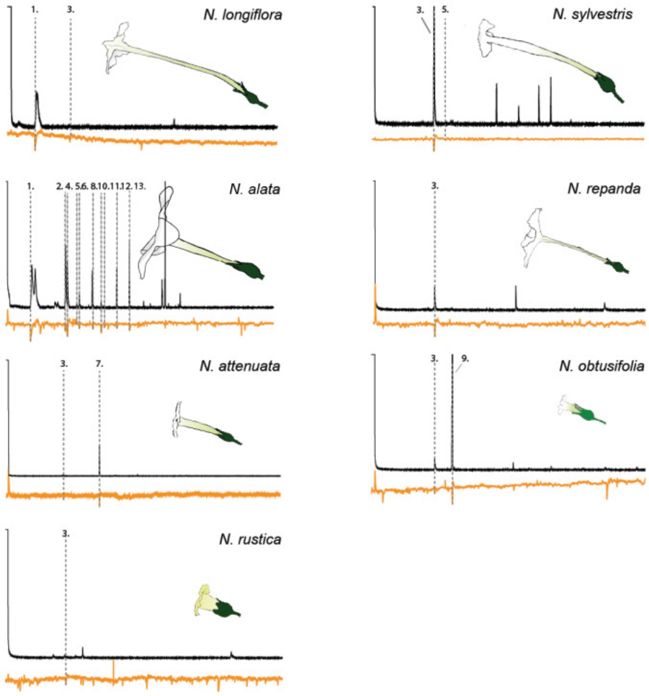
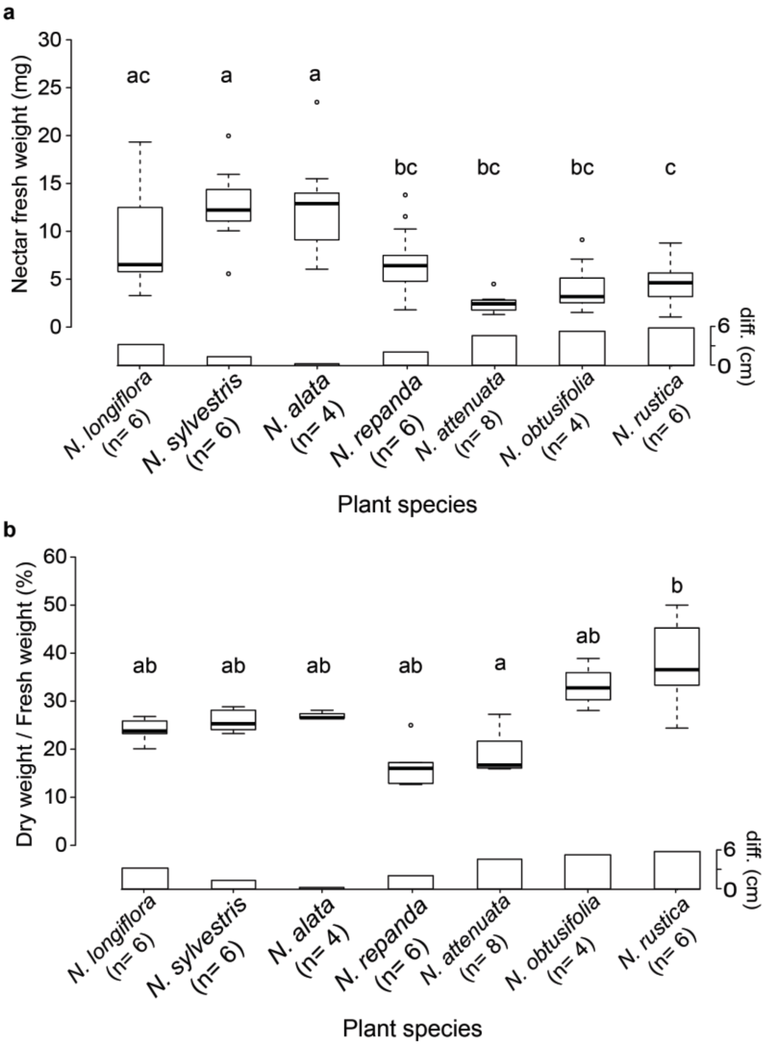
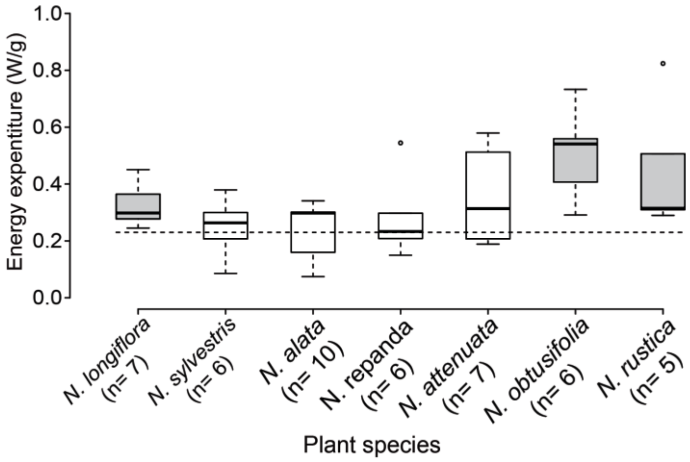


Supplementary Figure 1| Physiological response of *Manduca* towards volatiles from the tested *Nicotiana* species.

Traces show representative FID traces (blue) and corresponding EAD responses (yellow) for every flower species. Numbers depict the following compounds 1: 2 and 3- Methylbutyl aldoxime; 2: Eucalyptol; 3: Benzyl alcohol 4: Benzeneacetaldehyde; 5: Linalool; 6: Phenylethyl alcohol; 7: Unknown; 8: Methylsalicylate; 9: Geraniol; 10: 4-Methylbenzaldehyde; 11: Eugenol; 12. Isoeugenol. Each compound was verified by coelution with a synthetic standard.

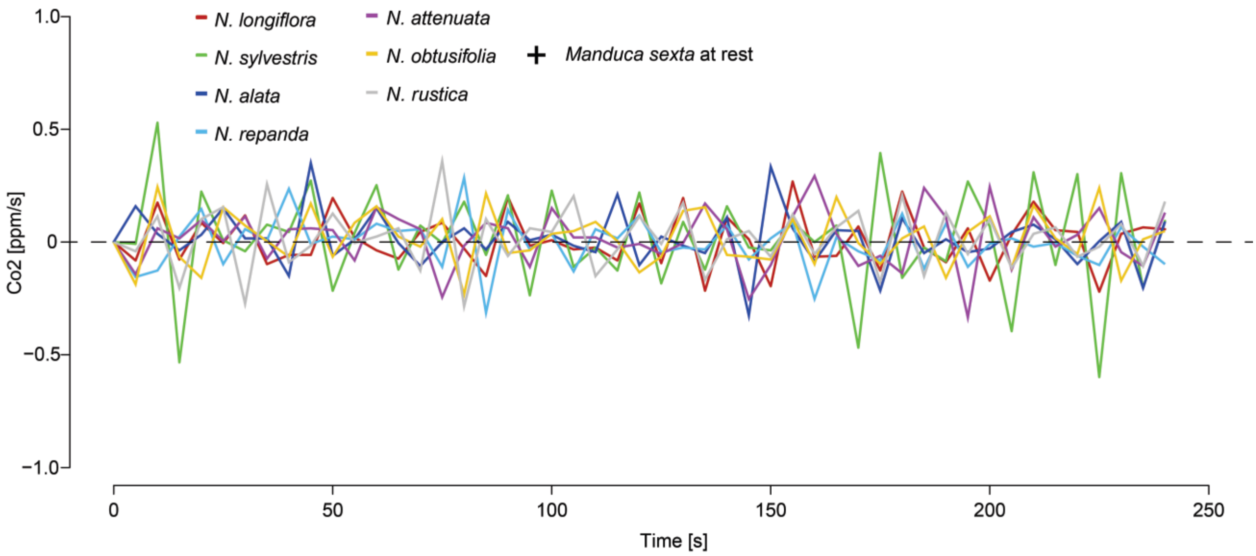


Supplementary Figure 2| Nectar amount and dry by fresh weight ratio of different *Nicotiana* species.
(a) Boxplot shows total amount of nectar (mg) per flower provided by the different plant species. Letters indicate significant differences ($P < 0.05$) according to Kruskal- Wallis test ($P<0.0001$) followed by Wilcoxon rank sum test with Holm correction for multiple comparisons. Black points indicate outliers. (b) Dry weight by fresh weight ratio shown as boxplot for the tested *Nicotiana* species. Letters indicate significant differences ($P < 0.05$) according to Kruskal-Wallis test ($P<0.0001$) followed by Wilcoxon rank sum test with Holm correction for multiple comparisons. Circles indicate outliers.



Supplementary Figure 3| Energy expenditure during foraging

Boxplot indicates energy expenditure (W/g) for *Manduca sexta* foraging on different *Nicotiana* species. Dotted line indicates energy expenditure hovering *Manduca* without flower contact [1]. Grey colour indicates energy expenditures significantly greater ($P<0.05$) than those of hovering moth without flower contact according to Wilcoxon rank sum test. Circles indicate outliers.



Supplementary Figure 4| Single flowers and resting moth did not result in a detectable CO2 emission rate. Different lines represent CO_2 emission rates [ppm/s] of single flowers and a moth at rest simultaneously enclosed in the respiration chamber for 4 min.

Supplementary References

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Chapter 2

Led by an instinct far more unerring than the practised eye of the botanist,
she recognizes the desired plant the moment she approaches it;
and upon this she places her precious burden.

W. Kirby and W. Spence, An introduction to entomology, 1863

Picture by Anna Späthe

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Host Plant Odors Represent Immiscible Information Entities - Blend Composition and Concentration Matter in Hawkmoths

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Abstract

Host plant choice is of vital importance for egg laying herbivorous insects that do not exhibit brood care. Several aspects, including palatability, nutritional quality and predation risk, have been found to modulate host preference. Olfactory cues are thought to enable host location. However, experimental data on odor features that allow choosing among alternative hosts while still in flight are not available. It has previously been shown that *M. sexta* females prefer *Datura wrightii* compared to *Nicotiana attenuata*. The bouquet of the latter is more intense and contains compounds typically emitted by plants after feeding-damage to attract the herbivore's enemies. In this wind tunnel study, we offered female gravid hawkmoths (*Manduca sexta*) odors from these two ecologically relevant, attractive, non-flowering host species. *M. sexta* females preferred surrogate leaves scented with vegetative odors form both host species to unscented control leaves. Given a choice between species, females preferred the odor bouquet emitted by *D. wrightii* to that of *N. attenuata*. Harmonizing, i.e. adjusting, volatile intensity to similar levels did not abolish but significantly weakened this preference. Superimposing, i.e. mixing, the highly attractive headspaces of both species, however, abolished discrimination between scented and non-scented surrogate leaves. Beyond ascertaining the role of blend composition in host plant choice, our results raise the following hypotheses. (i) The odor of a host species is perceived as a discrete odor 'Gestalt', and its core properties are lost upon mixing two attractive scents (ii). Stimulus intensity is a secondary feature affecting olfactory-based host choice (iii). Constitutively smelling like a plant that is attracting herbivore enemies may be part of a plant's strategy to avoid herbivory where alternative hosts are available to the herbivore.

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Introduction

For insects that do not support their offspring after oviposition, the choice of host plant plays an essential role in ensuring reproductive success. To provide its offspring with an optimal environment, the parent needs to take into account several aspects, e.g. palatability, nutritional quality and shelter from enemies. The influence of these factors on host plant preference has been studied intensely [1-3], but the role of olfaction in mediating host choice is still unclear.

Here we investigate olfaction-guided host choice in gravid hawkmoth, *Manduca sexta*, females. Adult *M. sexta* forage on nectar from plants of several plant families, whereas oviposition occurs almost exclusively on solanaceous plants [4,5]. In the Great Basin Desert of Utah, *M. sexta* feed and oviposit on wild tobacco, *Nicotiana attenuata*, and jimsonweed, *Datura wrightii*; *Datura* is preferred both for nectar feeding [6] and oviposition [7]. Both species, in intact and non-flowering state, have been shown to emit overlapping but distinct volatile bouquets. Aside from qualitative differences, the less preferred *N. attenuata* is characterized by a total emission rate of volatile organic compounds that is 5 times greater than that of *D. wrightii* [7].

We ask (i) whether the preference for *D. wrightii* persists with the presentation of cues exclusively from the headspace. As the plant volatiles emitted by *D. wrightii* and *N. attenuata* differ both in quality and quantity [7], we also ask (ii) whether stimulus intensity, i.e. total volatile concentration, contributes to the differential attractiveness and (iii) whether the complete host blend, potentially perceived as an olfactory host image, is mediating host choice.

Materials and Methods

Insect and plants

M. sexta larvae were reared in laboratory as described in [8]. Pupae and adults were kept under an inverse 16 h : 8 h light/ dark regime. Naïve females were mated the second night after emergence and tested during the subsequent night. Adults were supplied with sugar solution as desired. All plants were grown in a greenhouse as described [7]. Plants used for experiments were not yet flowering. Approximately 10 days before being used, plants were transferred into a climate chamber (23-25°C, 60-80% RH) with an inverse 16 h : 8 h light regime.

Behavioral experiments

The preference of gravid moths for plant headspace, including odors, CO_2 and humidity, emitted by the host species *D. wrightii* and *N. attenuata* was assessed in wind tunnel experiments (Figure 1A). Headspace from each species was compared individually and in a mixture against a clean air control (Figure 1A-I, II). The effects of signal intensity were tested within (Figure 1A-III) and between both species (Figure 1A-IV); see 'stimulus delivery' below.

Wind tunnel assay

The wind tunnel (Plexiglas, L x H x W 2.5 x 1 x 1 m) was set to an airflow of $0.4 ms^{-1}$, 25°C, and 70% RH. LED stripes (peak wavelength white: 448 nm, infrared: 846 nm) fixed at an angle to the side walls of the wind tunnel chamber provided continuous, indirect illumination at 0.3 Lux. Mated females were tested individually during the first 3 h of scotophase. 1 h before the experiment, females were transferred individually into mesh tubes (13 x 15cm). These served to place the females without further handling at the start of each experiment on a release platform. Females that did not show wing-fanning behavior within 3 min were gently prodded. Unlike no-choice wind tunnel experiments, where time until activation can be a critical parameter, activating the females does not bias the results in choice-based experiments. We observed the females for 4 min after take-off, noting every event involving physical contact with legs or ovipositor to the surrogate leaves at the odor sources. Since the choice between scented leaf dummies was the experimental criterion, only females that contacted the source were evaluated. Females that did not show wing-fanning behavior within 5 min or did not contact the odor sources were regarded as non-responders and excluded from the statistical analysis (see Table 1). Females showing proboscis extension, i.e. nectar-foraging, were also excluded. We evaluated first choices and preference indices based on the total number of repeated contacts to the sources: ((contacts A – contacts B) / total contacts) ranging from 1 (absolute preference of source A) to -1 (absolute preference of source B).

Stimulus delivery

All experiments were performed with plant headspace volatiles produced in real time delivered from two sources 40 cm apart at the upwind end of the tunnel (Figure 1A). 1 h before the experiment, plants were placed in glass boxes (L x W x H: 40 x 40 x 60 cm; glass panes fixed in aluminium frames) outside the wind tunnel. Two aluminum panes had V-shaped, guillotine-like front ends that fit into the notches of the lower frames of the glass box. Gently adjusted around the stems of experimental plants, they served to exclude rhizosphere volatiles from the headspace of the shoot. Active charcoal-filtered air (1.2 L/min) was introduced through a diffuser at the top of the glass boxes. Volatile-laden headspace air was removed close to the stem at the base of the box, pumped into the wind tunnel (0.8 L/min) and released continuously during the experiment below an artificial leaf (approximately 6 x 8 cm) made of light green tissue paper (Figure S1).

The total volatile emission of *N. attenuata* was found to be five times higher than that of *D. wrightii* [7]. To investigate the importance of stimulus intensity, i.e. plant headspace concentrations for ovipositing hawkmoths, AC-filtered air was added at a 1:4 (vol/vol) ratio via a y-connector. The efficient dilution of a reference headspace applying the experimental methods as described has been shown in a series of control experiments; see supplement. We asked whether (i) a diluted stimulus would be as attractive as the original plant headspace and (ii) a volatile concentration of a *N. attenuata* headspace adjusted to *D. wrightii* concentrations would reveal concentration effects in host species preferences.

To investigate whether the preference for *D. wrightii* might be linked to an olfactory host image that reflects the volatiles emitted by the plant, we superimposed the headspace of *N. attenuata* on that of *D. wrightii*, mixing them 1:1 (vol/vol) via a y-connector, and tested this mixture against clean air.

Results

Plants versus controls

For their first contact, females significantly preferred the headspaces of *D. wrightii* and *N. attenuata* over those of clean air controls (Figure 1B; *D. wrightii*: $p<0.001$; *N. attenuata*: $p<0.05$; Binomial Test). The total number of contacts with plant odor sources was significantly higher compared to the number of contacts with the control source (Table 1), resulting in a significant preference index (Figure 1C). When the two host blends were mixed, any preference for plant headspace was completely abolished (Figure 1 B, C; Table 1). Consequently, the preference index of the superimposed host blend experiment differed significantly from the indices of the experiments testing the species singly against clean air (Figure 1C; $p<0.0001$; Kruskal-Wallis Test; dF=2).

Signal attenuation: intraspecific

No preference was observed when headspace from *N. attenuata* or *D. wrightii* was presented against diluted headspace from the same species for both first contacts

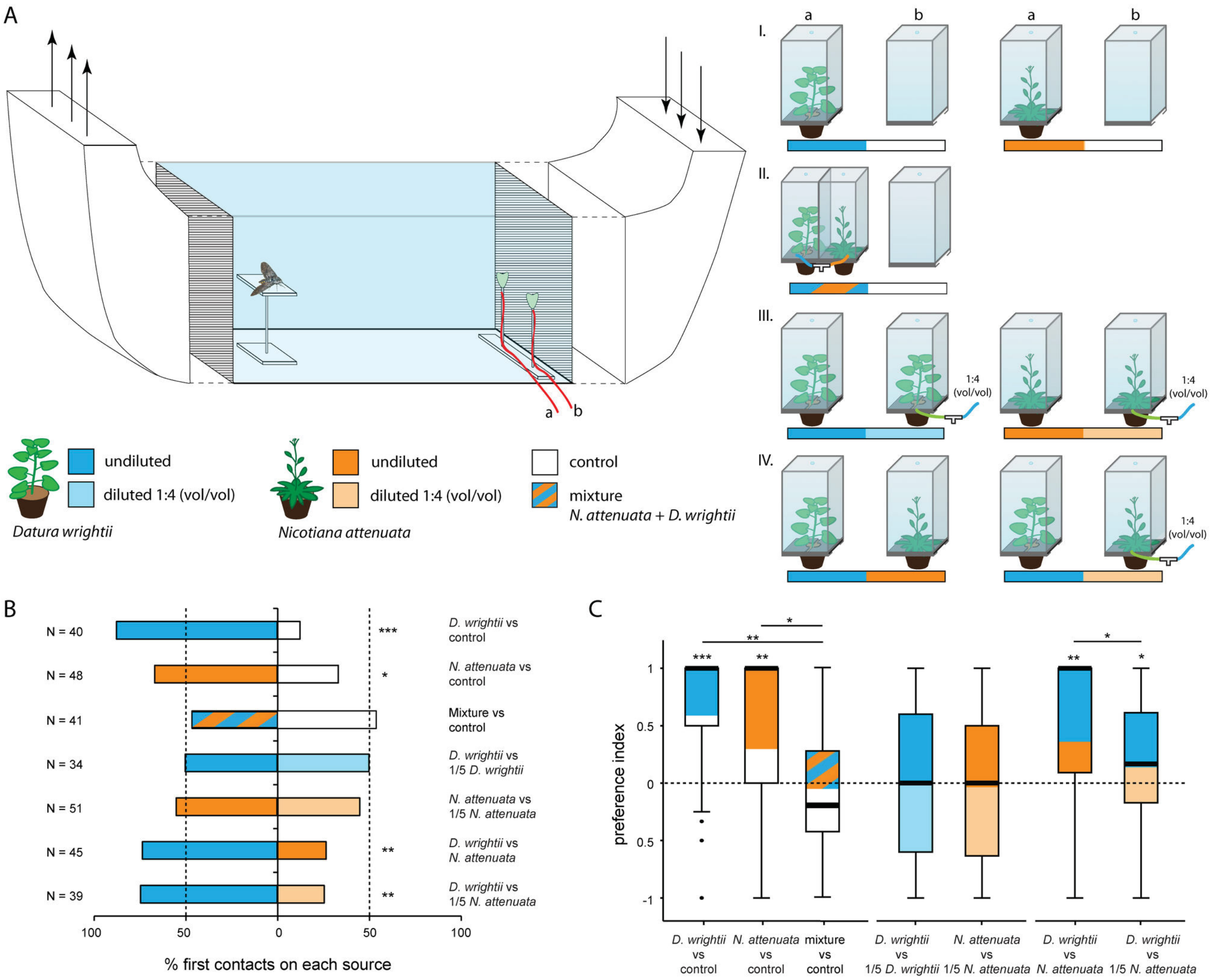

Figure 1. Effects of host blend composition and intensity on host choice in *M. sexta*. (A) Choice experiments with gravid *M. sexta* females were performed in a wind tunnel. Plants were placed in glass boxes outside the wind tunnel where they could not be seen by the moths. Pumps delivered plant headspace to two surrogate leaves serving as visual stimuli inside the wind tunnel. Two host plants, *D. wrightii* and *N. attenuata*, were tested (I) against a clean air control, (II) with their plant headspaces mixed together 1:1 against a clean air control, (III) against a conspecific plant whose headspace was diluted with clean air, and (IV) against each other, with *N. attenuata* headspace either not manipulated or diluted with clean air. Plant headspace and clean air were mixed in a 1:4 (vol/vol) ratio resulting in a 5-fold dilution. (B) The percentage of first choices made in the corresponding experiments. Sample size is given next to each experiment. Asterisks denote significant differences between sources (Binomial Test, *** $p<0.001$; ** $p<0.01$; *$p<0.05$). (C) Boxplots depict preference indices calculated from the number of contacts to each source. Values close to 1/-1 represent a high preference for one source; 0 means no preference. The black line delineates the median; color distribution within the box represents the percentage of contacts to each source. Asterisks above the boxes denote indices significantly different from 0 (Wilcoxon Signed Ranks Test, *** $p<0.001$; ** $p<0.01$; * $p<0.05$). Preference indices resulting from experiments in which the plant headspace of both species is offered superimposed or separately against clean air differed significantly (Kruskal-Wallis Test, $p<0.0001$, and Dunn's post hoc test, ** $p<0.01$, * $p<0.05$). Furthermore, preference indices derived from interspecific choice experiments were significantly different from each other (Mann-Whitney U Test, $p<0.05$).

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(Figure 1B) or for the total number of contacts (Figure 1C; Table 1; $p>0.05$; Wilcoxon Signed Ranks Test).

Signal attenuation: interspecific

Comparing non-manipulated plant headspaces, females showed significantly more first contacts (Figure 1B) and a significantly higher total number of contacts (Figure 1C; Table

Table 1. Numbers of responders, non-responders and animals excluded from analysis due to their behaviour of extending their proboscis during the experiment are given for each experiment.

		excluded	non-responders		responders			
Experiment	**total**	**Proboscis**	**no take off**	**no contact**	**contact**	**number of contacts (± SD)**		**Significance$^{a.}$**
D. wrightii						*D. wrightii*	2.9 ± 2.9	**dF=38**
vs	83	3	1	39	40			
control						control	0.7 ± 1.1	**p < 0.001**
N. attenuata						*N. attenuata*	2.1 ± 2.4	**dF=43**
vs	122	6	0	68	48			
control						control	0.8 ± 1.4	**p < 0.001**
mixture						mixture	3.0 ± 2.4	dF=37
vs	77	10	19	7	41			
control						control	3.9 ± 2.9	n.s.
diluted *N. attenuata*						1/5 N. *Attenuata*	2.5 ± 2.1	dF=46
vs	72	5	5	11	51			
N. attenuata						*N. attenuata*	2.4 ± 2.6	n.s
diluted *D. wrightii*						1/5 D. *wrightii*	2.2 ± 2	dF=30
vs	56	4	9	9	34			
D. wrightii						*D. wrightii*	2.6 ± 2.8	n.s.
N. attenuata						*N. attenuata*	1.1 ± 1.5	**dF=44**
vs	81	5	0	31	45			
D. wrightii						*D. wrightii*	2.5 ± 3	**p < 0.01**
diluted *N. attenuata*						1/5 N. *Attenuata*	2.7 ± 2.3	**dF=33**
vs	55	3	10	3	39			
D. wrightii						*D. wrightii*	4 ± 3.3	**p < 0.05**

Average number of source contacts (± SD) was tested with Wilcoxon Signed Rank Test.

a Wilcoxon Signed Rank Test

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1; p<0.01; Wilcoxon Signed Ranks Test; dF=44) to *D. wrightii*-scented sources than to *N. attenuata*-scented sources. Diluting the *N. attenuata* headspace in a ratio of 1:4 (vol/vol) with clean air to a volatile concentration similar to that of *D. wrightii* did not abolish the previously observed bias of first contacts and total number of contacts towards *D. wrightii* (Figure 1C; Table 1). Nevertheless, comparing preference indices between the experiments revealed that diluting the *N. attenuata* blend significantly weakened the preference for *D. wrightii* (Figure 1C; p<0.05; Mann-Whitney U Test).

Discussion

We show that volatile airborne stimuli alone are sufficient to elicit a differential preference of host plant species in mated *M. sexta* females. Headspace compounds from both species are attractive when compared to clean air, and the attractiveness is robust against a reduction in stimulus intensity. When spatially separated sources of *N. attenuata* and *D. wrightii* headspaces were presented simultaneously, insects showed a strong preference for the latter. This olfaction-mediated host choice corresponds to oviposition preferences previously demonstrated in *Manduca* [7].

N. attenuata plants emit a 5-fold higher amount of volatiles compared to *D. wrightii* [7]. Although *D. wrightii* headspace was still significantly preferred when volatile concentrations in the *N. attenuata* headspace were reduced to the level of the former, the comparison of preference indices revealed a significant attenuation of the host preference. Thus, stimulus intensity modifies olfactory host preference at the species level in a repeated choice setting.

The observed hierarchy of preference, which is consistent with what has previously been reported [7], underlines the prominent role played by *D. wrightii* as a host plant for *M. sexta*. The attractiveness of this species has been associated with the presence of large, night-blooming and intensely scented flowers that provide large amounts of nectar to the moth [6,9]. However, to uncouple host from foraging cues, we presented volatiles from non-flowering plants and excluded from analysis those animals that extend their proboscis during flight. Our results demonstrate that, as flower-derived odors do [10,11], vegetative plant volatiles play an important role in mediating host choice.

Carbon dioxide (CO_2) emissions serve *M. sexta* to assess nectar abundance in flowers [12,13]. CO_2 is also a close-range oviposition attractant in other insects, e.g. [14,15]. Plant respiration during the scotophase results in CO_2 emissions and *M. sexta* sense fluctuations in CO_2 concentration [16]. It is therefore conceivable that this gas and humidity serve to attract *M. sexta* females to host plants or help the moths to assess plant vigor. Preliminary data gathered in a different context indicate that individual *D. wrightii* and *N. attenuata* plants as used in this experiment emit CO_2 at 39.7 ± 30.40 (SD) and 26.5 ± 21.02 (SD) ppm above ambient levels, and relative humidity 9.8 ± 3.79 (SD) and 6.5 ± 1.32 (SD) percent above ambient levels, respectively. In our experiments, diluted plant headspace was as attractive as the non-diluted conspecific headspace. Mixing *N. attenuata* and *D. wrightii* headspaces abolished the preference female *M. sexta* had shown for each individual headspace when compared to clean air. We cannot exclude that CO_2 and humidity may co-attract insects or help them assess plant vigor when choosing among different plants. But neither of them overrode the combined effect of all natural plant headspace compounds.

Insects may extract information about a volatile-emitting plant through characteristic compounds or compound classes [17-19], and species-specific plant volatile blends [17,20]. Stimulus concentration has to our knowledge not been addressed as part of the mechanism that codes host information. A comparison of the volatile profiles of *N. attenuata* and *D. wrightii* revealed qualitative as well as quantitative differences: in undamaged plants, the volatile emissions of *N. attenuata* were 5 times higher than those of *D. wrightii* (total emission rate of *D. wrightii*: 1.8 ± 0.3 ng/min; total emission rate of *N. attenuata*: 10.5 ± 0.5ng/min [7]), and *N. attenuata* headspace was composed of many typical herbivore-induced plant volatiles (HIPVs), i.e. compounds whose emissions are up-regulated after herbivore attack [21]. It has been shown that HIPVs mediate avoidance of damaged plants in ovipositing moths [22,23]. Thus, the preference for *D. wrightii* compared to *N. attenuata* may be co-mediated by volatiles associated with herbivore-damaged plants. Consequently, reduced HIPV-concentrations might lead to the shifted preference indices observed in our experiments, and conversely enhanced HIPV emissions may protect the emitter as long as the herbivore may choose among alternative hosts in a field setting.

When we presented moths with different concentrations of two blends of identical compositions, they did not discriminate between full and diluted plant headspaces. In a turbulent environment distance from a stimulus source has been shown to be mainly coded by stimulus intermittency [24], differences in plant volatile concentration could also have been interpreted by the moths as a function of distance to the plant [25]. However, our results give no indication that a moth would prefer a host-plant blend at a higher concentration. Hence, ovipositing moths seem to rely predominantly on blend composition rather than on concentration.

The picture changed when we diluted the *N. attenuata* blend, bringing its total concentration to the level of *D. wrightii*, and presented both simultaneously. While *D. wrightii* is usually strongly preferred over *N. attenuata*, this preference became less accentuated when the odor of *N. attenuata* was diluted (Figure 1C). Thus, stimulus intensity may become a supplemental behaviorally relevant feature of an odor stimulus when different, i.e. species specific odor blends are available in a repeated choice setting, which is the standard case in the field. In support of these lines, *M. sexta* females preferred to oviposit on inbred horse nettle plants, which emit considerably fewer volatiles compared to outbred plants [26].

The preference shown for single plant blends rather than clean air disappeared when *N. attenuata* and *D. wrightii* blends were presented as a mixed stimulus. Clearly, *M. sexta* females could no longer evaluate the olfactory information provided by this combined species mixture, suggesting that the composition of the species-specific blend contains crucial information, an olfactory ‘Gestalt’. In Colorado beetles, adding single host volatiles [27] or a non-host plant [28] to the blend of a host plant has been shown to neutralize the host’s attractiveness. Host recognition in *M. sexta* females is very likely also dependent on ratio specificity. Several studies have reported that the emissions of host plants can be masked by repellent or neutral blends [29]. However, the reciprocal neutralization of two attractive blends from two naturally preferred host plants shown in this study has to our knowledge never been reported.

We showed that for a *Manduca sexta* female, the olfactory information emitted by host plants is sufficient to mediate its choice among alternative hosts. Furthermore, our results highlight that species-specific host odours represent information entities that loose their positive valence upon blending, and that stimulus intensity is a supplemental feature involved in choosing among alternative hosts. Constitutively emitting HIPV at a higher rate contributes to an herbivores preference for the alternative host and may, thus, be advantageous to the smelly plant.

Supporting Information

Figure S1. Experimental manipulation of plant headspace. (A) Experimental setup to generate headspace dilution and mixtures. Plants were placed in glass boxes (GB) and provided with active charcoal (AC) filtered air from the top. Two metal plates (MP) with a central opening that were located on metal slides beneath the box enclosed the plant stem close to the pot, thereby excluding roots and soil material from the box. Rotary vane vacuum and pressure pumps (RP; G12/01 EB, Gardner Denver, Inc., Puchheim, Germany) delivered plant headspace out of the glass box, and the resulting headspace flow was controlled with mechanical valves. To establish plant headspace of 1:4 dilutions and 1:1 mixtures, both flows were adjusted to the aimed flow ratio and merged in a Y-shaped connector. During the experiment, flow rates of the source flows (S1, S2) as well as the total flow (TF) were repeatedly checked with a digital flowmeter. (B) Differences in acetone concentration resulting from the flow ratios used during the experiments were measured with a fast response Photo-Ionization detector (PID) (miniPID 200A, Aurora Scientific Inc., Ontario, Canada) for a time window of 60 s. The internal offset and gain of the PID had been set to 0 and 1, respectively. Measurements were transferred to a personal computer (Pentium 4, 2.8GHz, Fujisu Siemens, Munich, Germany) via an analog-digital converter (National Instruments, Austin, Texas, US). Data acquisition within the entire system was adjusted to the PID’s maximum sampling rate of approximately 330Hz. During the test a 50% aqueous acetone solution was placed in the glass box to simulate plant headspace. Acetone headspace was pumped out of the box and regulated with valves to simulate non-manipulated (dark green), 1:1 mixed (green) and

1:4 diluted (light green) headspace. Clean air pumped out of a glass box served as a control measurement (blue line) and a second source for the 1:1 mixture. 1:4 dilutions were established directly with AC-filtered air. Non-manipulated headspace was measured before (solid line) and after (dotted line), adjusting the valves to ensure that the acetone concentration in the glass box did not drop. (C) Boxplots show the average acetone concentration in V resulting from PID measurements. By adjusting the flow ratio the non-manipulated acetone concentration (100%) reached the hoped-for concentration levels of 50 and 20% (dotted lines), representing the 1:1 mixture and 1:4 dilution, respectively.
(TIF)

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Author Contributions

Conceived and designed the experiments: AS AR MK BSH. Performed the experiments: AS AR AH. Analyzed the data: AS. Wrote the manuscript: AS AR MK BSH.

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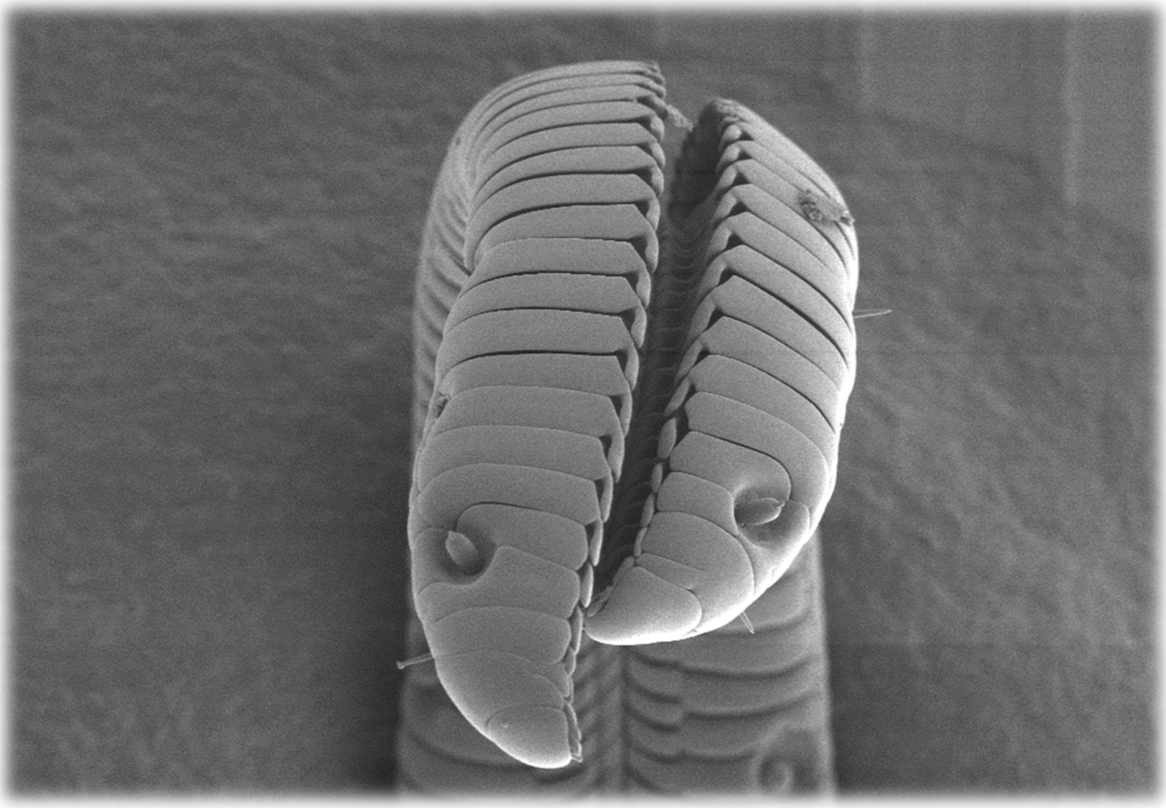
Chapter 3

"[…] if we were to view the fly as one successful solution to the problem of maintaining a working interface between the cosmos of life and the greater world, as a solution to the basic problems that are common to all, we might, just might gain some insights to multiple fundamental phenomena."

Vincent Gaston Dethier,
The Hungry Fly, 1976



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RESEARCH ADVANCE

Hawkmoths evaluate scenting flowers with the tip of their proboscis

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Abstract Pollination by insects is essential to many ecosystems. Previously, we have shown that floral scent is important to mediate pollen transfer between plants (**Kessler et al., 2015**). Yet, the mechanisms by which pollinators evaluate volatiles of single flowers remained unclear. Here, *Nicotiana attenuata* plants, in which floral volatiles have been genetically silenced and its hawkmoth pollinator, *Manduca sexta*, were used in semi-natural tent and wind-tunnel assays to explore the function of floral scent. We found that floral scent functions to increase the fitness of individual flowers not only by increasing detectability but also by enhancing the pollinator's foraging efforts. Combining proboscis choice tests with neurophysiological, anatomical and molecular analyses we show that this effect is governed by newly discovered olfactory neurons on the tip of the moth's proboscis. With the tip of their tongue, pollinators assess the advertisement of individual flowers, an ability essential for maintaining this important ecosystem service.
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Introduction

Floral scent has been associated with insect pollination since the 18th century (**Sprengler, 1793**); however, the complex functions of floral volatiles have been only recently investigated in more detail, due to the availability of new molecular and analytical techniques (**Raguso, 2008**). Floral scent not only attracts pollinators (**Klahre et al., 2011**), but also manipulates them through chemical mimicry (**Stökl et al., 2010**) and repels herbivores (**Junker and Blüthgen, 2010**), altogether increasing plant fitness (**Kessler et al., 2008**). However, research studying the function of floral scent has been divided along two themes with little cross-fertilization: 1) studies examining the fitness effects of floral scent without the causal behavioral responses of pollinators (**Kessler et al., 2008**), or 2) studies examining the sensory physiology of pollinators, neglecting the ecological consequences for the plant (**Raguso, 2008**). Here, we meld these approaches and show that floral scent increases the fitness of individual flowers not only by increasing their detectability (**Raguso and Willis, 2002**), but also by enhancing the pollinator's foraging motivation, and demonstrate that this is mediated by olfactory receptors on the tip of the moth's proboscis which detect floral scent.

The hawkmoth *Manduca sexta* (**Figure 1A**, **Video 1**) is a major pollinator of the wild tobacco *Nicotiana attenuata* in the Great Basin Desert (USA) (**Kessler et al., 2008**; **2010**; **2015**). *N. attenuata* emits a relatively simple floral scent dominated by a single compound: benzyl acetone (BA) (**Euler and Baldwin, 1996**). In spite of this simplicity, producing BA might come at considerable metabolic but especially ecological cost, as BA might attract not only nectar thieves and florivores, but also female hawkmoths in search of oviposition sites (**Baldwin et al., 1997**; **Kessler et al., 2010**). Plants might therefore reduce the amount of floral volatiles released as much as possible without losing their pollination services. Field experiments using plants in which the emissions of BA had been silenced by RNAi of the biosynthetic gene *NaChal1* (CHAL) have shown that BA is required to maximize pollination success (**Kessler et al., 2008**; **2015**). It was suggested that lacking this scent made plants in nature 'invisible' to hawkmoth pollinators. However, the precise mechanisms by which odors of single flowers influence pollinator behavior and thereby plant fitness have rarely been examined in the direct interaction between plant and pollinator (**Klahre et al., 2011**; **Riffell et al., 2008**). Hence, how floral scent emitted by individual flowers functions in this mutualistic interaction remained unknown, particularly as it is unclear how pollinators detect single volatile compounds within complex natural environments (**Hansson and Stensmyr, 2011**; **Riffell et al., 2014**).

Results and discussion

We investigated the function of floral scent in the context of individual flower-moth interactions, by offering individual male moths the choice between BA-emitting flowers (i.e. empty-vector transformed flowers (EV)) and non-emitting flowers (i.e. CHAL) in a free flight tent (24 m × 8 m × 4 m, 10 CHAL and 10 EV plants, spaced 50 cm apart). The flight tracking revealed that moths chose to visit the same number of emitting and non-emitting flowers in a random sequence (**Figure 1B**, **Figure 1—figure supplement 1A**, probability of changing between EV or CHAL flowers during consecutive visits: 0.47). In a second bioassay conducted in a wind tunnel (2.4 m × 0.9 m × 0.9 m, moonlight [0.5 lux of sunlight spectrum]), we presented plants with either emitting or non-emitting flowers to individual moths and analyzed their flight patterns, approaches and flower contacts. In none of these analyses, we found any difference between plants with emitting and non-emitting flowers (**Figure 1—figure supplement 1B,C**). These results suggest that visual cues and general vegetative plant odors provided sufficient information for the moths to locate flowers, consistent with previous work using artificial flowers (**Raguso and Willis, 2002**) and clearly showing that non-scenting flowers are not 'invisible' to moths.

If non-scenting flowers are found by moths, why is plant fitness reduced? Does BA emission change the pollination probability? To test this, we loaded the moth's proboscis with a standardized number of pollen grains using a fine brush. When such pollen-enhanced moths were allowed to forage freely on antherectomized *N. attenuata* flowers, seeds produced per flower of EV and CHAL plants differed significantly (**Figure 1C**). Scentless flowers matured very few seeds, reflecting the inferior pollination services provided by the moths despite a similar number of visits. This result highlights the importance of BA emission for the fitness of individual flowers and confirms the results of previous studies, which investigated the effects of BA emission on plant fitness at a population level (**Kessler et al., 2008**; **2015**). But if the flowers were equally detectable by the moths, what behavioral mechanism was responsible for the plant fitness consequences?

To analyze the effect of BA emission on moth behavior in greater detail, we quantified the time invested by a moth at individual flowers in a wind tunnel assay. The moths spent significantly more time at emitting than at non-emitting flowers (**Figure 2A**) particularly while trying to insert their proboscis, so even before tasting the floral nectar (**Figure 2—figure supplement 1A**). However, having successfully inserted their proboscis, the time of nectar uptake was similar between them (**Figure 2—figure supplement 1B**). This suggests that BA emission increased the motivation of moths to forage when individual flowers were evaluated at a close range, possibly because BA emissions, are closely linked to the physiological state and thereby also to the potential nectar amount of a flower (**Bhattacharya and Baldwin, 2012**; **Yon et al., 2015**; **Kessler et al., 2015**). By increasing the probing time in BA-emitting flowers, moths increased their success rate at their first as well as at consecutive flower visits and, therefore, collected more nectar per flower visit in tent (**Figure 2B**, **Videos 1** and **2**, **Figure 2—figure supplement 1C**) and wind tunnel assays (**Figure 2—figure supplement 1D**). These results agree with a study using different *Petunia* lines which found that although flower scent aided navigation, increased nectaring was the most consistent effect of floral scent (**Klahre et al., 2011**).

The large fitness consequences of floral volatiles for both moth and plant beg the question: how do moths evaluate the headspace of individual flowers? The wide spread of the antennae and their distance from the flower resulting from the moth's long proboscis which is fully extended during nectaring suggests that the olfactory spatial resolution of the antennae might be too low to resolve individual flowers in an inflorescence or even between neighboring plants (**Willis et al., 2013**). Hence, we inferred that the moth's proboscis might play a role in flower perception (**Goyret and**

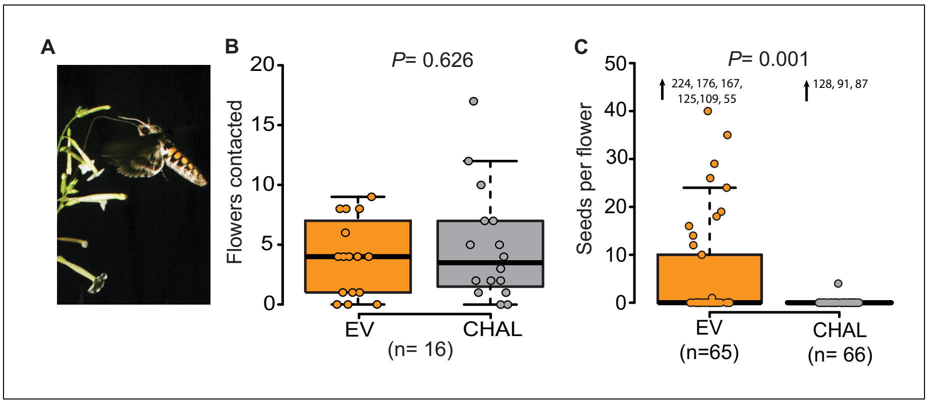

Figure 1. Even though *M. sexta* visited the same number of benzyl acetone (BA)-emitting (EV) and non-emitting flowers (CHAL), BA-emitting flowers received superior pollination services, increasing seed production. (**A**) *M. sexta* feeding from *N. attenuata* flowers. (**B**) Number of EV and CHAL flowers visited per moth on each foraging flight when 10 randomly placed plants per line were presented in a two-choice, free-flight tent assay (Wilcoxon signed rank test). (**C**) Seeds matured per antherectomized flower after visitations by a moth experimentally loaded with pollen (Wilcoxon rank sum test). Extreme values are shown as numbers.

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The following figure supplement is available for figure 1:

Figure supplement 1. Free flight response of *M. sexta* towards flowers emitting and not emitting BA.

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Raguso, 2006). Using reverse transcription PCR, we qualified the accumulation of transcripts of olfactory genes in the proboscis of *M. sexta*. Similar to the mosquito *Aedes aegypti*, but contrasting with predictions for nectar feeding insects (***Jung et al., 2015***), we found that the olfactory co-receptor Orco was expressed in the tip region of the proboscis along with the ionotropic co-receptor, IR25a (***Figure 3A***). Notably, Orco was only expressed in the first centimeter of the proboscis whereas the ionotropic co-receptor, IR8a, was only found in upper sections. This heterogeneous distribution of olfactory genes is consistent with the idea that the moth proboscis plays a more complex role in chemoreception than previously thought (***Reiter et al., 2015***). Screening the proboscis tip by scanning electron microscopy (***Figure 3B***, ***Figure 3—figure supplement 1A,B***) we found a sensillum type that was not previously described for *M. sexta* (***Reiter et al., 2015***). This sensillum resembled the known sensillum styloconicum, but instead of a single tip pore, had a multiporous cone (***Figure 3B$_4$***). Similar sensillum types have been described in other lepidopteran species (***Faucheux, 2013***), but their function remained unknown, although the presence of odorant-binding proteins suggested a role in olfaction (***Nagnan-Le Meillour et al., 2000***). We used an antibody raised against Orco (Nolte et al., unpublished), and found a single Orco- positive cell only in the first multiporous sensilla styloconica (mSt) at the tip of the proboscis (***Figure 3C***, ***Figure 3—figure supplement 1***).

Given the presence of potential olfactory sensilla on the proboscis of *M. sexta*, we wondered whether neurons housed in these sensilla play a role in the detection of BA and could thus help explain the pollination differences of EV and CHAL flowers. We performed single sensillum recordings and tested the response of neurons present in all sensillum types occurring at the tip of the proboscis to an air puff of BA at an ecologically relevant concentration (0.1 mM ***Kessler and Baldwin, 2007***). Only neurons in the Orco-positive sensillum reacted to this compound (***Figure 4B***, ***Figure 4—figure supplement 1A***). In a further test with 41 other

Video 1. *M. sexta* foraging on EV flowers emitting BA in a free-flight tent.

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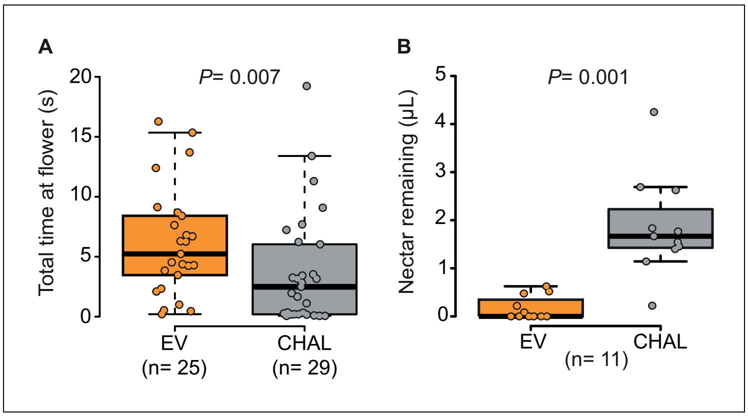

Figure 2. Moths spent more time and removed more nectar from BA-emitting (EV) flowers than from scentless (CHAL) flowers. (**A**) Time spent by moths at single flowers (Wilcoxon rank sum test) in a wind tunnel assay. (**B**) Nectar remaining in flowers after moths attempted to feed in a two choice tent assay (Wilcoxon signed rank test).
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The following figure supplement is available for figure 2:

Figure supplement 1. Behavior of *M. sexta* while foraging on EV and CHAL.
DOI: 10.7554/eLife.15039.006

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ecologically relevant odorants, the first mSt was found to be more sensitive to BA and the structurally related benzylacetate (***Figure 4C***, ***Figure 4C—source data 1***). Though these results show that neurons in the proboscis tip of *M. sexta* can detect volatile BA, it remained unclear whether the moth would also respond behaviorally to this compound based only on the input from neurons of the proboscis sensilla.

To disentangle the proboscis input from other chemosensory organs, we devised a behavioral experiment in which the corolla tube of a flower was replaced by a Y-maze choice assay (***Figure 4D***). Each arm of the Y-maze was either connected to a source of humidified air or humidified air scented with BA (0.1 mM). By drawing air directly behind the entrance of the Y-maze, the experimental set-up excluded antenna-based olfaction. Hence, as soon as a moth entered the flower aperture during free hovering flight, only the proboscis experienced the air stream containing either a solvent control, or BA (***Video 3***). During their first and subsequent insertions, the moths chose both Y-tube arms with equal frequency (***Figure 4F***, ***Figure 4—figure supplement 1B***), but inserted their proboscis for a significantly longer time into the arm containing the BA-scented air (***Figure 4E***), demonstrating that the moth was able to detect BA with only the proboscis. Moths seem to use the olfactory input from the proboscis not for orientation on the corolla, but rather to assess the specific quality of an individual flower, consistent with the notion that the close-range orientation of the proboscis on the flower can be informed by mechanical and visual cues (***Goyret and Raguso, 2006***; ***Sponberg et al., 2015***).

Our findings show that hawkmoths are well adapted to visit and detect volatiles of single flowers. Floral volatiles, such as BA, not only function as navigational cues (***Haverkamp et al., 2016***), but also inform pollinators about the identity and the physiological state of individual flowers (***Bhattacharya and Baldwin, 2012***; ***Yon et al., 2015***). Only BA-emitting flowers encourage the moth to visit a flower long enough to lead to successful pollination. Our results show that floral scent is an essential chemical feature for hawkmoths to gain nectar from, and pollinate, a single flower. Interestingly, many flowers require that their pollinators

Video 2. *M. sexta* attempting to forage on Chal flowers not emitting BA in a free-flight tent.
DOI: 10.7554/eLife.15039.007

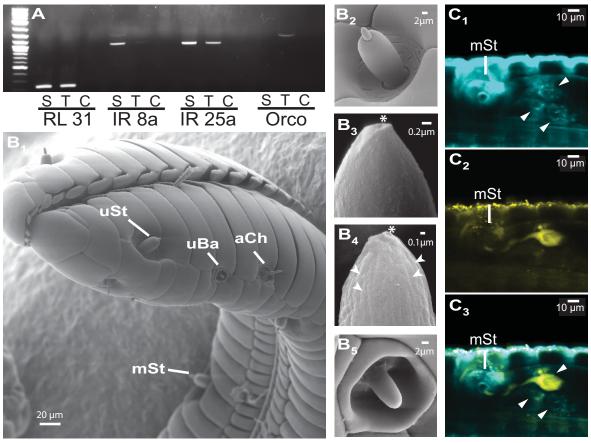

Figure 3. The *M. sexta* proboscis harbors sensilla, which house sensory neurons expressing olfactory genes. (**A**) Reverse transcription PCR using either the proboscis shaft (S), the first centimeter of the proboscis tip (T) or a water control (C) with primers for the three major olfactory co-receptors (IR8a, IR25a, Orco). The transcripts of the ribosomal gene RL131 and water were included as positive and negative control. (**B1**) Scanning electron microscopy images of the *M. sexta* proboscis tip show three types of potential chemosensory sensilla: sensilla styloconica (**B2**) with a uniporous (uSt (**B3**)) and multiporous (mSt (**B4**)) cone and uniporous sensilla basiconica (uBa (B5, ***Figure 3—figure supplement 1B***)) as well as aporouse sensilla chaetica (aCh, ***Figure 3—figure supplement 1A***). Asterisks mark tip pore, arrowheads indicate side pores. Neuronal labeling using anti-bodies against horseradish peroxidase (**C1**) and against Orco(**C2**) indicate three neurons close to the first mSt sensillum (arrows), of which one expresses the olfactory co-receptor Orco (**C3**, ***Figure 3—figure supplement 1***).

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The following figure supplement is available for figure 3:

Figure supplement 1. Different sensillum types were found in tip region of the *M. sexta* proboscis, but only multiporous sensilla styloconica (mSt) were determined to be Orco positive.

DOI: 10.7554/eLife.15039.009

acquire particular handling skills on their first visits, before the insects are able to use the flower efficiently (***Laverty, 1994***). This energy investment by the pollinator not only helps ensure outcrossing for the plant, but provides the insect with a more exclusive nectar source (***Heinrich, 1979***). In a recent study, inexperienced *M. sexta* were found to sometimes expend more energy on handling flowers than they gained from the nectar; if additional experience increases foraging efficiency, this would compel the moth to visit additional flowers of the same species (***Haverkamp et al., 2016***). Notably, the ability to smell BA with the tip of the proboscis may not only increase the motivation of *M. sexta* to invest energy into BA emitting flowers, but also strengthen the moth's learning of these flowers, as the nectar reward becomes associated with the presence of BA. Such a BA-conditioned learning rate might help to ensure a positive energy balance for the moth while at the same time ensuring cross-pollination for the plant (***Heinrich and Raven, 1972***). However, the question to what extent the interaction of moths and plants relies on the moths' learning ability requires additional attention in future studies.

Although both – metabolically costly and risky in terms of herbivory – thousands of plant species actively emit floral scent (***Wright and Schiestl, 2009***). These emissions might be a consequence of the physiological requirement for scent compounds by certain pollinator guilds when collecting nectar, even when visual cues would be sufficient to attract pollinators to a plant. The ongoing evolutionary interaction between plants and pollinators relies heavily on floral scent (***Parachnowitsch et al., 2012***; ***Schiestl and Johnson, 2013***), and may explain the absence of scent-free plants in native *N. attenuata* populations (***Kessler et al., 2015***). To pollinators with the appropriate sensory system, floral scent provides a wealth of information, highlighting the importance of chemical communication in this mutualism, on which many of our crops rely (***Radera et al., 2015***).

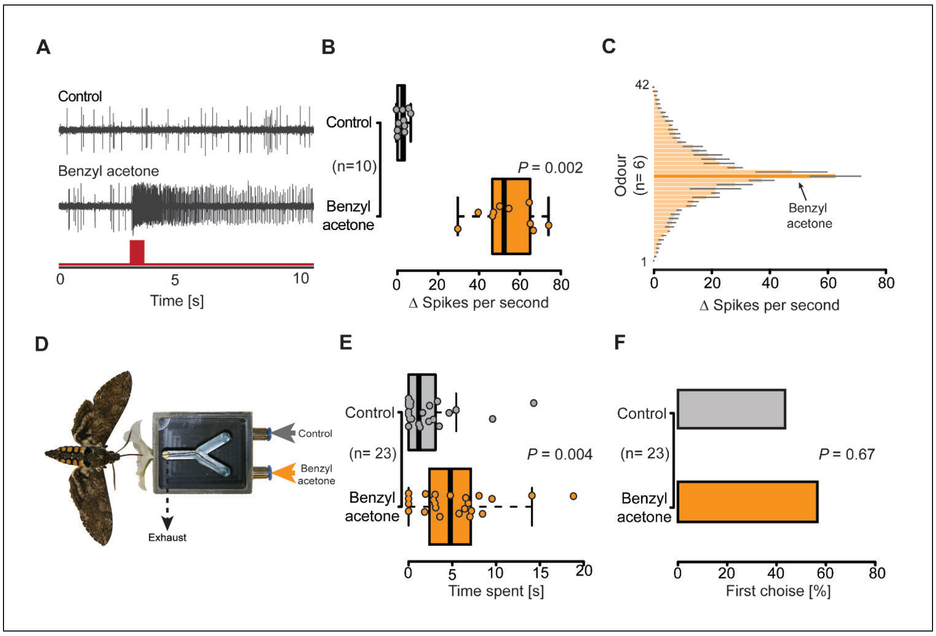

Figure 4. Olfactory sensory neurons housed in proboscis sensilla respond to BA and are sufficient for flower evaluation. (**A**) Single sensillum recordings from the first multiporous sensilla styloconica. Upper trace depicts a characteristic response to the water control; lower trace shows a response to BA from the neuron in the same sensillum. Red bar indicates time of stimulus. (**B**) Boxplot shows Δ spikes per second recorded from the first mSt when stimulating with water control or BA (0.1 mM) for 0.5 s. Neurons responded with a significantly higher spike rate to BA than to the water control (Wilcoxon signed-rank test). (**C**) Response profile of the first mSt to 42 different odorants. Black bars indicate S.E.M. Names and spike rate of each odorant can be found in ***Figure 4C—source data 1***. (**D**) Behavioral assay to test the response to either humidified air with BA (0.1 mM) or humidified air only. Exhaust excludes antennal olfactory input. (**E**) Moth inserted their proboscis significantly longer into the arm in which BA was present (Wilcoxon signed-rank test). (**F**) Moths chose equally often between Y-tube arms containing BA-scented air or solvent control at the first approach (Exact binominal test).

DOI: 10.7554/eLife.15039.010

The following source data and figure supplement are available for figure 4:

Source data 1. Response spectrum of the first multpourus sensillum to ecological relevant odors.

DOI: 10.7554/eLife.15039.011

Figure supplement 1. BA does not influence all types of sensilla on the proboscis of *M. sexta* and does also not enable the moth to navigate actively towards BA.

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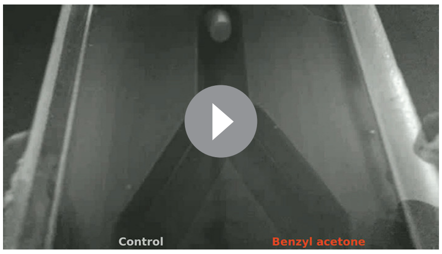
Materials and methods

Plants

We used two transgenic *Nicotiana attenuata* Torr. (Solanaceae) lines derived from *Agrobacterium tumefaciens* (strain LBA 4404) transformation of wild type *N. attenuata* plants which were collected in a native population at the DI Ranch (Santa Clara, UT, USA) in 1988 and subsequently inbred for 22 generations (***Krügel et al., 2002***). Both lines have been described earlier, empty vector control plants (EV) transformed with pSOL3NC (line number A-04-266-3) (***Bubner et al., 2006***), as well and plants silenced by RNAi in the production of floral scent, CHAL line (*N. attenuata* chalcone synthase; pRESC5CHAL, line number A-07-283-5) (***Kessler et al., 2008***). Seeds were sterilized and germinated on Petri dishes with Gamborg's B5 media as described in ***Krügel et al. (2002)***. Petri dishes with 30 seeds were maintained under LD (16 hr light and 8 hr dark) conditions in a growth chamber (Percival, Perry, Iowa, USA) for 10 days, and seedlings were transferred to small pots (TEKU JP 3050 104 pots, Pöppelmann, Germany) with Klasmann plug soil (Klasmann-Deilmann, Germany) in the glasshouse. After 10 days, plants were transferred to 9 cm × 9 cm pots for wind tunnel experiments or to 1 l pots for tent experiments.



Video 3. Y-maze of the proboscis choice assay, BA-scented air is provided at the right arm and the solvent control in the left arm. Odors were removed by applying a vacuum at the entrance of the Y-maze.
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The glasshouse growth conditions are described in **Krügel et al. (2002)**. For use in the wind tunnel, plants were transferred to a York Chamber (Johnston Controls, USA) with an inverted day/night cycle; daylight time was from 17–9, maintained at 25˚C and night time temperatures were 22˚C, with a humidity of 60–80%. Plants used in the tent experiment were cultivated in the Max Planck Institute for Chemical Ecology (MPICOE), Jena, Germany, main glasshouse. After attaining the rosette stage of growth, plants were transferred to a second glasshouse, maintained at the same conditions, located in Isserstedt, Germany, where the plants were cultivated until used for the tent experiments.

Insects

Moths used in the wind tunnel experiments were obtained from a colony maintained at the in Jena, Germany. Animals were reared as previously described (**Koenig et al., 2015**). Eggs were collected from female *M. sexta* moths, which could freely oviposit on *N. attenuata* plants. For the tent experiments, eggs from native *M. sexta* populations at the Utah field station were collected and shipped to Germany. After hatching, caterpillars were maintained on artificial diet (wind tunnel) or on *Nicotiana tabacum* plants (tent) at an ambient temperature of 27°C, 70% relative humidity and a light regime of 16:8 (light: dark). Fifth instar caterpillars were transferred into individual wood chambers for pupation. One week before hatching, pupae were sexed and male pupae were transferred to a flight cage with 15.5 hr daylight and 7.5 hr dim light (0.5 lux). Temperature and relative humidity were set to 25˚C and 70% during day-light phase and to 20˚C and 60% during the dim light phase. A transition phase of 30 min was used between phases. Animals were used for experiments 72–76 hr after hatching.

Wind tunnel

No-choice assays were performed in a Plexiglas wind tunnel (220 cm × 90 cm × 90 cm). Charcoal-filtered air was pushed through the tunnel at a speed of 0.37 m/s. Air temperature and relative humidity was adjusted to 25˚C and 70%. Plants were transferred to the wind tunnel chamber at least 1 hr before the experiment; to avoid contamination, plants were cultivated in a separated compartment with an additional charcoal air filter. Directly before each trial, a single plant was placed at the front of the wind tunnel with the flower positioned 70 cm above the tunnel floor, 20 cm from the tunnel front and 45 cm from each tunnel side.

Moths were placed individually in mesh-cages (15 cm × Ø13 cm) 1 hr before the experiment and transferred to a pre-exposure chamber set to the same light and climate conditions as the wind tunnel. For each trial, a single *M. sexta* moth was placed on a platform 35 cm above the tunnel floor, 20 cm from the tunnel end and 45 cm from each tunnel side. After placement, every animal was given 5 min to innate wing- fanning. Animals which did not start wing-fanning (63%) within this time frame were considered as non-responders and excluded from subsequent analyses. After take-off, the behavior of each moth was recorded for 4 min using a custom-made 3D video tracking system. The tracking system consisted of four cameras (Logitech C615, USA, infrared filter removed) recording at 30 Hz and a resolution of 800 × 600 pixels (each pixel 0.3 cm^2). Using a background subtraction algorithm implemented in C, the 3D position of the moth was calculated at a rate of 10 Hz. Based on these tracking data, we analyzed the flight pattern of the moth during the last two seconds before encountering the flower in the wind tunnel using costume-written Matlab scripts (Mathworks, USA). All recorded flight tracks were cross-checked with video data and only complete recordings were used for further analyses. In order to avoid learning effects, we only considered the first flower approached by each moth.

Moth behavior at the flower was recorded at a rate of 30 Hz by a fifth camera (Logitech C615, infrared filter removed), which had been placed into the wind tunnel at a distance of about 30 cm from the flower. Recordings were automatically started by a custom-written movement detection algorithm. Flower probing times and total contact time were measured by manually analyzing the individual video files. Similar to the flight analyses, we only considered the first flower contact in the statistical analyses.

Tent

To emulate a natural environment, we conducted pollination experiments in a large tent (height, 4 m; width, 8 m; length, 24 m) with an enclosed roof to protect from rain and lateral mesh for natural airflow (***Kessler et al., 2015***). Plants were moved from the glasshouse to the tent which was located directly adjacent to the glasshouse. Experiments in the tent were conducted between August 27 and September 8 2014. Ten plants of each of the two lines EV and CHAL were aligned in rows at the central section of the tent. EV and CHAL plants were positioned directly next to each other, even touching inflorescences. The position of the EV and CHAL plants were changed after each moth, to minimize potential position effects. In order to use only freshly opened flowers, all open flowers were removed each morning, before experiments were conducted. Six to eight male *M. sexta* moths were released sequentially per night. A new moth was released only when the previous had stopped flying and every moth was only used once. Single flower visitations were observed, the genotype and time at a single flower was noted, and after the visitation, the approached flowers were removed to measure the remaining nectar. Each moth had up to 10 flower encounters and for each moth the mean nectar gain across all flower encounters was calculated.

Nectar and pollen analysis

Directly after each experimental wind tunnel trial, both the moth and the plant were removed. The remaining nectar in the flower was measured by carefully removing the flower base and removing the nectar with a pre-weighed capillary. The nectar amount was determined by reweighing the capillary and subtracting the two weights. In the tent trials, nectar volume was quantified directly using a BLAUDBRAND graduated capillary with a volume of 25 µL (Brand, Germany) by gently removing the corolla (***Kessler et al., 2007***).

The pollen load on the moth proboscis was determined by rinsing the proboscis three times in 1 mL of 1% Tween solution. 10 µL of 0.5% safranin (Sigma Aldrich, Germany) were added to each sample to stain the pollen outer layer. The samples were vortexed and centrifuged for 2 min at 10000 rpm. Thereafter, the supernatant was discarded without disturbing the pollen pellet and 100 µL distilled water was added to each sample. The samples were then vortexed and 10 µL were pipetted into a four-field Neubauer-counting chamber to determine the pollen number in each sample. Every sample was counted twice independently, and the mean value was used for statistical analysis.

Cross pollination experiment

To measure pollination rates in EV and CHAL plants by *M. sexta* in the wind tunnel, fully developed flowers were emasculated in the previous corresponding daylight morning cycle to avoid self-pollination (***Kessler et al., 2008***). For this, 3–4 flowers per plant of each line (EV and CHAL) were used in the wind tunnel, one plant and one moth at each time. Fresh pollen was collected in the corresponding morning from plants not being used for pollination. EV pollen which had been collected the previous night was rubbed on the hawkmoth proboscis using a fine brush prior to its release in the wind tunnel, in order to measure the pollen delivery to experimental flowers. If moths did not take flight voluntarily within 3 min after being placed in the wind tunnel they were excluded from the study. Moths, which took flight (73%), were allowed to do so for four minutes in each wind tunnel trial. The numbers of matured capsules, as well as the seeds produced from each capsule were counted after ripening. Capsules were collected shortly before opening, approximately 14 days after the experiment, dried in a desiccator, and once opened, the seeds were counted in petri dishes. After each trial the pollen from each *M. sexta* was collected by washing the proboscis to ensure that similar amounts of pollen had been placed on the proboscis. For pollen counts, the same procedure as for the pollen retrieval was used. On average, we found that 548.75 (n= 41, SEM= 88.85) pollen grains had been placed on a single proboscis. No difference was found between moths tested with EV (n= 21, mean= 569, SEM= 92.3) or CHAL (n=20, mean= 500, SEM= 159.3) plants (Student’s t-test, p= 0.71).

Scanning electron microscopy

M. sexta proboscises were cut 1 cm from the tip and fixed in 4% glutaraldehyde at 4°C overnight. Proboscises were then dehydrated in an ascending ethanol series (70%, 80%, 90%, 96%, 3x 100% ethanol, 10 min each), critical point dried (BAL-TEC CPD 030, Bal-Tec Union Ltd., Liechtenstein), mounted on aluminium stubs with conductive carbon cement (Agar Scientific, UK) and sputter coated with gold on a BAL-TEC SCD005 (Bal-Tec, Liechtenstein). Specimens were examined in a LEO 1530 Gemini scanning electron microscope (Zeiss, Germany) set at 8 kV and 11 to 15 mm working distance.

Immunohistochemistry and confocal laser scanning microscopy

The tip region of 20 *M. sexta* proboscises were carefully dissected into three small parts, cutting behind the first, before the fourth and behind the fifth sensillum styloconica. Directly after dissection, the proboscis parts were fixed in 4% paraformaldehyde (ROTH, Germany) in 1 M NaHCO3 (Sigma Aldrich, pH 9.5) overnight at 4°C. Subsequently, the samples were washed six times for 30 min in 1× phosphate-buffered saline containing 0.1% Trition X (PBS-T) (Sigma Aldrich, USA) and thereafter blocked for 3 hr in normal goat serum (NGS). The primary anti-body against Orco (kindly provided by Prof. Jürgen Krieger, University of Halle-Wittenberg, Germany) was applied at a 1:500 dilution in 2% NGS- PBS-T and incubated for 5 days at 4°C. Detection of the Orco antibody was performed by incubating in a goat-anti rabbit antibody linked to Alexa 488 (Invitrogen, USA) at a dilution of 1:200 in 2% NGS-PBS-T for 3 days at 4°C. In addition, we added an goat anti-horseradish peroxidase antibodies conjugated to Cy3 (Jackson Immuno Research, USA) at a dilution of 1:50 in 2% NGS-PBS-T to visualize neuronal tissue. For visualization, the samples were mounted in 50% glycerol on a microscope slide and scanned using confocal laser scanning microscopy (LSM 880, Zeiss, Germany). Alexa 488 was exited using the 488 nm line of the microscopes Argon laser, while a Helium Neon 543 laser was used to activate Cy3. Signals were detected by a spectral detector (quasar: 490–553 nm and 555–681 nm). All pictures were taken using a 20× air objective (N.A. 0.8). Scanning resolution was set to 1024 × 1024 pixel.

Total RNA isolation

Proboscises of ten male *M. sexta* were dissected and were cut 1 cm from the tip. Each tissue sample (tips and rest) was directly transferred to Tri-reagent (Sigma-Aldrich, USA). The samples were then homogenized with two 3 mm steel beads (Qiagen, Germany) using a TissueLyser (Qiagen, Germany) for 5 min at 50 Hz. Samples were stored at -20°C. Finally, RNA isolation was performed using TRI-Reagent (Sigma-Aldrich, USA) according to the manufacturer's instructions.

cDNA synthesis

RNA samples were treated with TurboDNAse (Ambion, USA) according to the manufacturer's instructions. DNAse was removed using Tri-reagent following the instructions of the producer. RNA was dissolved in 25 µL RNA storage solution (Ambion, USA). For cDNA synthesis 1 µg total RNA per sample was used as template for the Super Script III kit (Invitrogen, Canada).

Reverse transcription-PCR

For RT-PCR dNTPS (Thermo Fisher Scientific, Lithuania), cDNA, gene-specific primers and the Avantage 2 Polymerase mix (Clontech, Canada) were used following the manufacturer's instructions. Primers were designed according to Koenig et al. (2015): (RL31: GGA GAG AGG AAA GGC AAA TC and CGG AAG GGG ACA TTT CTG AC; MsexIR8a: CAA CCC CGA CGC GTA TCC GTA TCC and TTA CGG CCT ATA TTC ATT TTT AGG AAA AAC GCT TAT ATA TG; MsexIR25a: GGA GTC CGT ATA GCT ATC AGA ATA ATC GAG and TCA AAA TTT AGG TTT CAA ATT AGA TAA ACC TAA ATT TCT GGA TC; MsexORCo: ATG ATG GCC AAA GTG AAA ACA CAG G and CTA TTT CAG CTG CAC CAA CAC CAT G). Reaction was done in a thermocycler (GeneAmp PCR System 9700, PE Applied Biosystems, USA) with 95°C for 1 min, followed by 35 cycles of 95°C for 30 s, 60°C (for MsexIR25a: 62°C) for 30 s and 68°C for 90 s. The final step was incubation at 68°C for 3 min. The samples were loaded on a 1.5% agarose gel.

Electrophysiology

For electrophysiological recordings, moths were placed into a 15 mL reaction tube, from which the tip had been cut; in such a way that only the proboscis would extend from the tube. Eachanimal was then mounted on a microscope slide, and the proboscis was fixed with dental wax. Next we unrolled the first centimetre of the proboscis and fixed this part upside down on a small wax pedestal, so that most of the sensilla were approachable for electrophysiological recordings. Subsequently, the preparation was positioned under a microscope (BX51W1, Olympus, Japan) and a tungsten reference electrode was inserted into the proboscis shaft. The recording electrode was then inserted into the target styloconic sensillum via a motorized, piezo-translator-equipped micromanipulator (DC-3K/PM-10, Märzhauser, Germany). A constant air stream of humidified air was applied to the preparation. For stimulus delivery either 0.1 mg BA diluted in distilled water or distilled water only was loaded onto a filter paper, inserted in a glass pipette and puffed onto the proboscis using a Syntech stimulus controller (CS- 55, Syntech, The Netherlands). For the odor screen individual compounds were diluted in hexane (10^{-2} v/v) and 10 µL were loaded on to a filter paper and puffed as described before. A single puff lasted for 0.5 s. The recorded signal was then amplified (UN-06, Syntech, The Netherlands), digitally converted (IDAC-4, Syntech, The Netherlands), and recorded at a rate of 2400 Hz using AutoSpike v3.2. (Syntech, The Netherlands). Traces were exported as ASCII files and manually analyzed using R. Spikes were counted 2 s before the stimulus onset and 2 s thereafter. The number of spikes before the stimulus was then subtracted from the spikes counted after the stimulus onset. The resulting number of Δ spikes was then divided by the number of seconds analyzed. In all experiments, three day old male moths were used.

Proboscis choice

Olfactory preference of the proboscis was tested in a custom-built Y-maze (5 cm × 3 cm × 0.5 cm). Previous studies had found a 0.1 mM suspension of BA in nectar to be ecologically relevant in the interaction between *M. sexta* and *N. attenuata* (***Kessler and Baldwin, 2007***). Here, we tested 10 µL of 0.1 mM BA suspension in distilled water against the same amount of distilled water only. Both stimuli were pipetted onto a small filter paper discs and placed into 50 mL glass bottles. Bottles were connected to the Y-tube arms via Teflon tubing (Ø 6 mm). Charcoal-filtered air was pushed into the bottles so that the air flow at each Y-tube arm reached 0.1 L/ min. To prevent the moths' antenna from contacting BA headspace and assure a homogenous flow, air was removed from the opening of the Y-tube at a rate of 0.2 L/ min. The movement of the moth proboscis was recorded via a video camera (Logitech C615, infrared filter removed) at 30 Hz. Videos were captured using the software package Media recorder (Noldus, The Netherlands) and subsequently viewed and manually analyzed using EthoVision (Noldus, The Netherlands). For tests, the Y-maze set-up was placed into the wind tunnel described above and moths were allowed to forage freely for 4 min. In order to attract the moths to the Y-maze, we attached the corolla of a freshly cut *Nicotiana alata* flower, which does not release BA (***Raguso et al., 2003***), onto the Y-maze opening.

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Additional information

Competing interests

ITB: Senior editor, *eLife*. BSH: Vice President of the Max Planck Society, one of the three founding funders of eLife, and a member of eLife's Board of Directors. The other authors declare that no competing interests exist.

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Supplementary materials

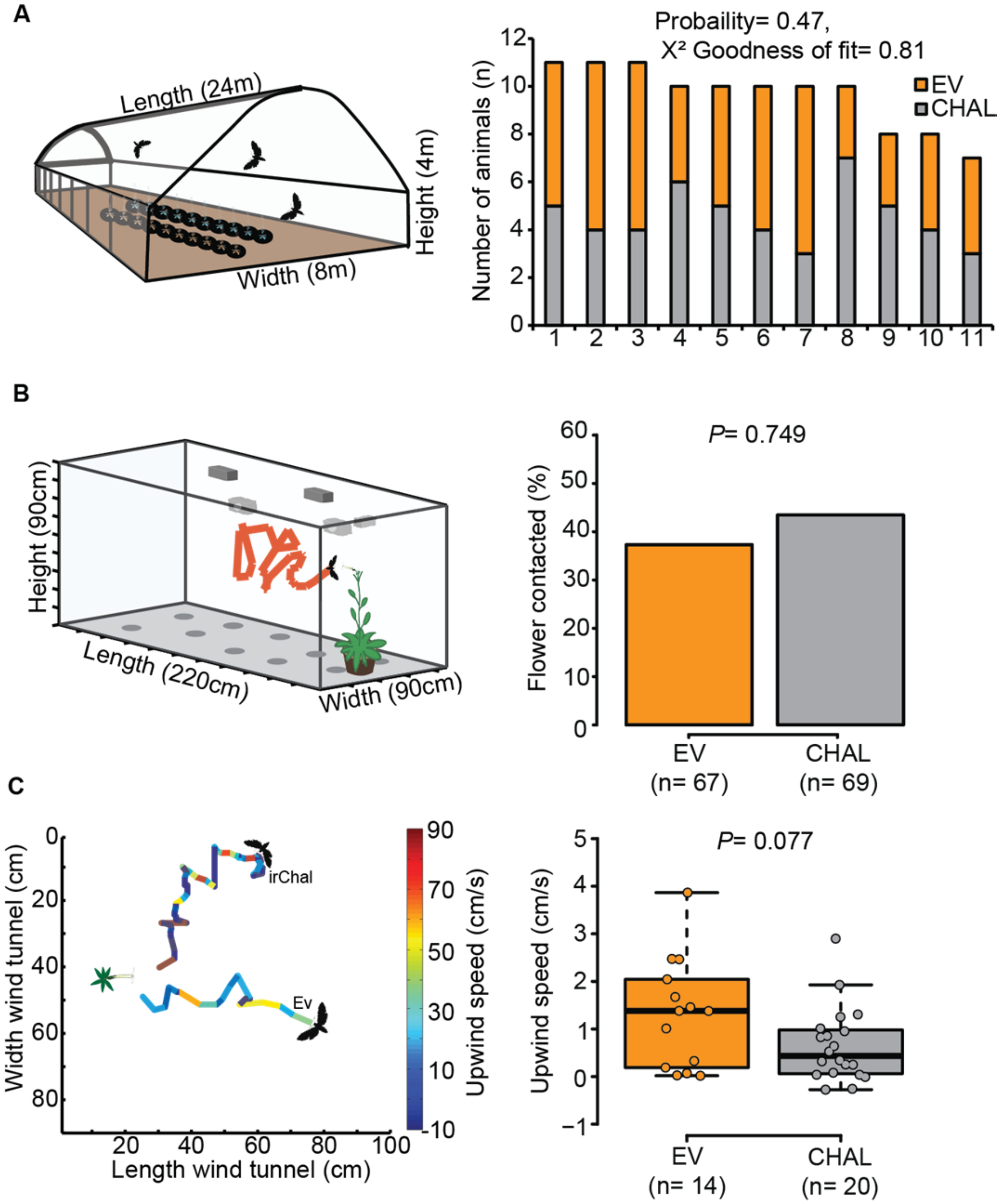

Figure1 supplement 1. Free flight response of *M. sexta* towards flowers emitting and not emitting BA. **(A)** In a dual- choice assay in a free flight tent, moths visited emitting and non-emitting flowers in a random sequence. **(B)** Moths visited a similar percentage of emitting and non-emitting flowers in a no-choice wind tunnel test. (Fisher's exact test, *P*= 0.7493)). **(c)** 3dimensional video tracking analyses revealed a tendency for a higher upwind speed towards EV flowers emitting BA (Wilcoxon rank sum test, *P*= 0.077).

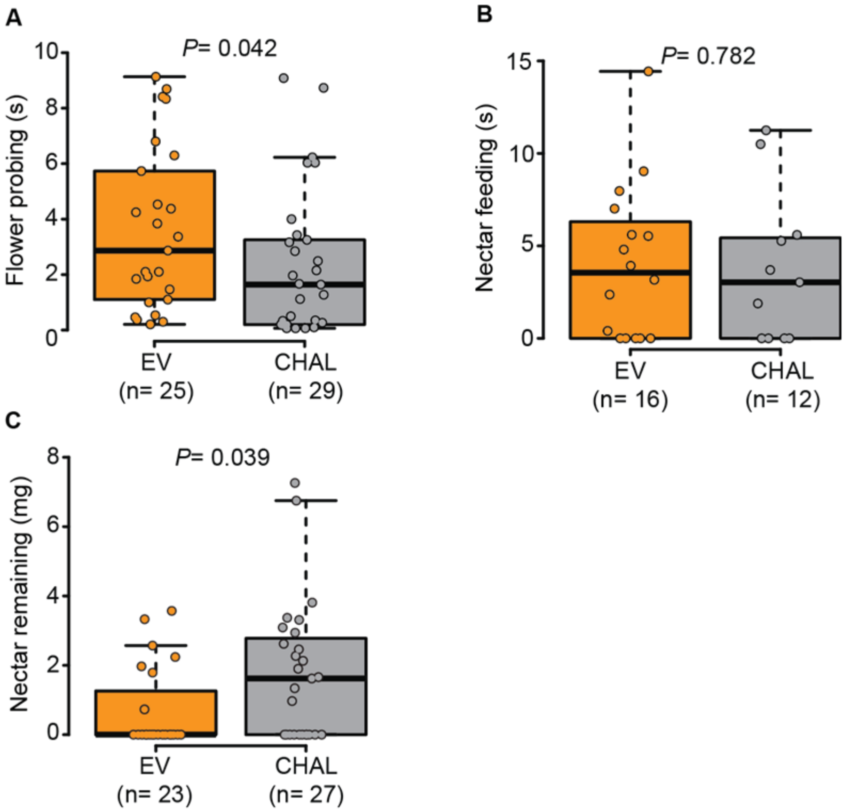

Figure2 supplement 1. Behaviour of *M. sexta* while foraging on EV and CHAL. (**A**) Moth spent significantly more time probing EV flowers in comparison to CHAL flowers, (**B**) but did not differ in their time feeding from the different flowers. (**C**) Moth removed significantly less nectar from CHAL plants in a no-choice wind tunnel assay. Wilcoxon signed rank test was used in all cases.

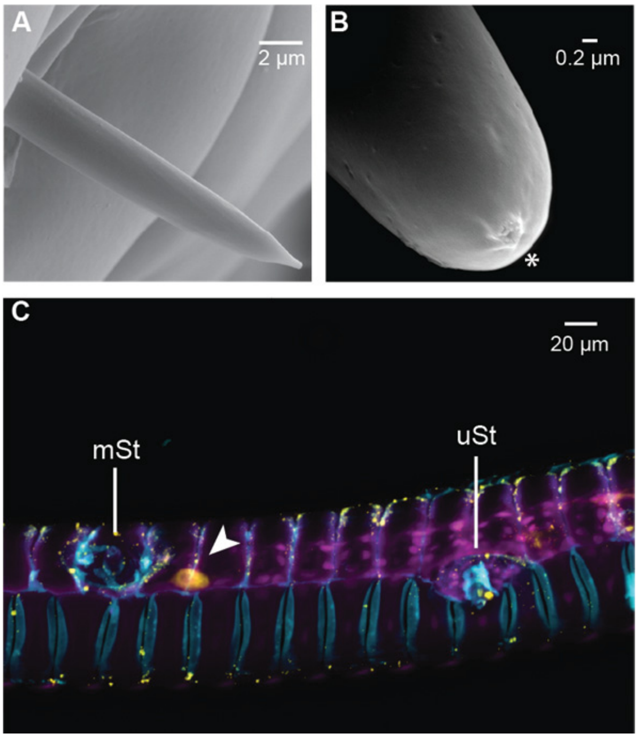

Figure3 supplement 1. Different sensillum types were found in tip region of the *M. sexta* proboscis, but only multiporous sensilla styloconica (mSt) were determined to be Orco positive. (**A**) Aporouse sensillum chaetica (**B**) tip of a uniporous sensillum basiconicum (uBa, Fig. $3b_5$) asterisk indicates tip pore. (**C**) Proboscis section showing the first multiporous sensillum stylochonicum (mSt) and a single Orco positive cell body in close proximity (arrowhead) as well as a uniporous senillum stylochinicum (uSt) without Orco-positive staining.

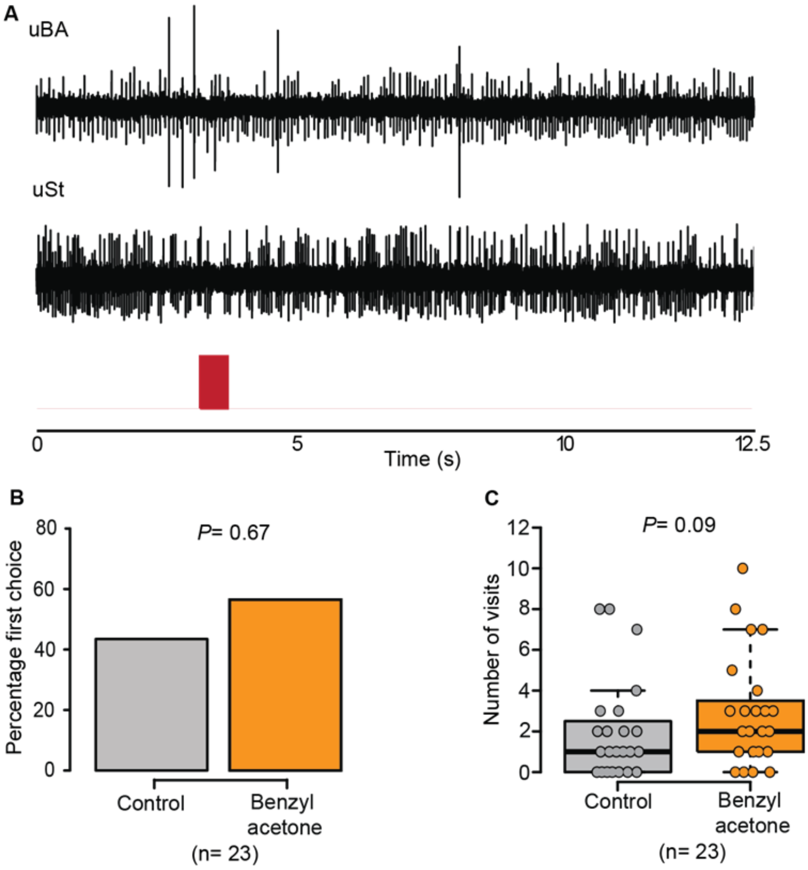

Figure4 supplement 1. BA does not influence all types of sensilla on the proboscis of *M. sexta* and does also not enable the moth to navigate actively towards BA. (**A**) Example traces of a uniporous sensillum basiconicum (uBa) and a uniporous sensillum stylochonicum (uSt) stimulated with the headspace from a 0.1 mM BA suspension, showing no response towards the stimulus. (**B**) Moths choose equally between Y-tube arms containing BA-scented air or solvent control at the first approach (Exact binominal test). (**C**) Moths entered the arm containing the solvent control as often as they entered the arm containing BA-scented air (Wilcoxon signed rank test).

Video 1

M. sexta foraging on EV flowers emitting BA in a free-flight tent.

Video 2

M. sexta attempting to forage on Chal flowers not emitting BA in a free-flight tent.

Video 3

Y-maze of the proboscis choice assay, BA-scented air is provided at the right arm and the solvent control in the left arm. Odours were removed by applying a vacuum at the entrance of the Y-maze.

Figure 4 source data 1

Odorants tested for responses on the multiporouse sensillum styloconica. Chemicals were diluted in hexane (10-2 v/v) and tested on 6 different animals. Odorants are sorted by response magnitude.

Chemical	delta spikes/sec	SEM	CAS number	Source	Purity
benzyl acetone	62.58	8.91	2550-26-7	Aldrich	98.00%
benzyl acetate	47.5	12.42	140-11-4	Aldrich	99.00%
cis-jasmone	37.17	4.44	488-10-8	FLUKA	99.00%
nerol	28	2.68	106-25-2	Aldrich	97.00%
(s)-verbenol	27.92	6.23	19890-02-9	Aldrich	95.00%
linalool	22.5	5.33	78-70-6	Aldrich	97.00%
L(-)-nicotine	21.25	4.87	54-11-5	Riedel-de Haën	98.50%
citral	21.25	8.85	5392-40-5	Aldrich	95.00%
geraniol	21.25	1.48	106-24-1	FLUKA	96.00%
gamma-hexalactone	18.67	4.94	695-06-7	Aldrich	98.00%
methyl salicylate	18	4.74	119-36-8	Sigma	99.00%
phenylacetaldehyde	15.58	2.6	122-78-1	Aldrich	98.00%
eugenol	13.58	1.95	97-53-0	FLUKA	99.00%
benzyl alcohol	13.42	3.51	100-51-6	Aldrich	99.80%
S-(-)-limonene	12.92	1.81	5989-54-8	Aldrich	96.00%
farnesene	9.58	1.52	502-61-4	Aldrich	99.00%
hexanoic acid	8.67	0.86	142-62-1	Aldrich	99.50%
trans-2-hexenal	7.75	1.31	6728-26-3	Aldrich	98.00%
benzaldehyde	7.33	1.54	100-52-7	Sigma	99.00%
cis-3-hexenyl acetate	7.25	1.86	3681-71-8	Aldrich	98.00%
6-methyl-5-hepten-2-one	6.83	1.63	110-93-0	Aldrich	99.00%
beta-pinene	6.58	1.71	18172-67-3	Aldrich	99.00%
trans-2-hexen-1-ol	6.17	1.58	928-95-0	FLUKA	95.00%
R-(+)-limonene	5.75	0.65	5989-27-8	Aldrich	96.00%
alpha-humulene	5.42	1.55	6753-98-6	Aldrich	96.00%
trans-2-hexenyl acetate	5.25	1.38	2497-18-9	Aldrich	98.00%
cis-3-hexenal	5.17	0.35	6789-80-6	Aldrich	50.00%
beta-myrcene	3.92	1.08	123-35-3	FLUKA	95.00%
1,8-cineole	3.42	0.8	470-82-6	FLUKA	99.70%
cis-3-hexen-1-ol	3.17	0.86	928-96-1	Aldrich	98.00%
geranyl acetate	2.42	0.96	105-87-3	Sigma	98.00%
2-methylbutyraldoxime	2	0.66	49805-56-3	Sandra Irmisch (MPI-ICE)	99.00%
farnesol	1.75	0.88	4602-84-0	Aldrich	95.00%
beta-humulene	1.42	0.82	116-04-1	Aldrich	90.00%
dimethyl-disulfied	1.33	0.52	624-92-0	Aldrich	99.00%
acetic acid	1.25	0.76	64-19-7	Aldrich	99.90%
3-methylbutyraldoxime	1.17	0.38	96837-31-9	Sandra Irmisch (MPI-ICE)	99.00%

trans-alpha-bergamotene	0.58	0.57	18252-46-5	Franzisca Beran (MPI-ICE)	38.40%
beta-caryophyllene	0.5	0.41	87-44-5	FLUKA	98.50%
pyrrolidine	0.42	0.87	123-75-1	Aldrich	99.00%
iso-eugenol	-0.08	0.32	97-54-1	Aldrich	99.00%
geranyl acetone	-0.17	0.38	105-87-3	Aldrich	97.00%

Chapter 4

"When analytic thought, the knife, is applied to experience, something is always killed in the process."

Robert M. Pirsig, Zen and the Art of Motorcycle Maintenance:
An Inquiry Into Values, 1974

Title:

Flower orientation in *Nicotiana attenuata* is shaped by biomechanical but not sensory constraints of its hawkmoth pollinator

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Abstract:

Flower signalling and orientation are key characteristics which determine a flower's pollinator guild. The flowers of the wild tobacco *Nicotiana attenuata*, move their corolla upwards towards sunset and downwards after sunrise. We calculated the costs for this flower movement and tested its effect on a major pollinator, the hawkmoth *Manduca sexta.* We found that although flower orientation influenced the approach direction of the moth, it did not alter the overall attractiveness of the flower. Moreover moths invested the same amount of time into handling flowers at different orientations. Flower orientation did not influence the emission and distribution of floral volatiles which was concurrent with the behavioural observations. Hawkmoths were able to enter vertically and upward oriented flowers, but unable to enter downward facing flowers. This strongly constrained nectar and pollen uptake by the moth. Hence flower orientation determined not only the hawkmoth's foraging success but also the plant's fitness. Our study demonstrates how flower orientation might alter the co-evolution of plants and pollinators, leading to potential isolation barriers between flower populations and stresses the importance of the match between flower signals and the flower's accessibility to the sensory and biomechanical capabilities of the pollinator.

Keywords: Flower orientation, pollination, sensory ecology, biomechanics, *Manduca*, *Nicotiana*

1. Introduction

Detecting and subsequently being able to handle a certain flower are two key steps in the interaction between pollinators and plants which strongly influence the specificity of their relationship (Harder, 1985; Riffell et al., 2014). However, the detectability and accessibility of a flower for a certain pollinator is not always constant as many flowers actively open and close or move vertically during the course of the day (Yon et al., 2016). It has been argued that flowers perform these energetically costly movements to attract pollinators, on the one hand, and to protect nectar and pollen on the other. However in spite of its potential importance for plant-pollinator interactions, experimental evidence for the effect of flower movement on the pollinator behaviour remains limited (Fenster et al., 2009).

To the plant, animal pollinated flowers are costly organs that function to advertise and distribute its pollen to ensure outcrossing. Hence natural selection has likely optimized the efficiency of a flower's function in the context of the requirements of the targeted pollinator, so that visitation is maximized, with little energy is wasted on signals or morphological features that are not perceived by or may even hinder a certain pollinator. Rich odour bouquets for example might do little to increase hummingbird visitation, while not investing into a long nectar spur could decrease pollen transfer by hawkmoth (Campos et al., 2015; Fulton and Hodges, 1999). Additionally, flower characteristics might also differ in their cost/benefit to flower function *i.e.* altering the corolla size might be more costly and thus less efficient than changing the corolla orientation, although both can increase pollination by syrphid flies (Fenster et al., 2004; Ushimaru and Hyodo, 2005). Ultimately it might be expected that flowers invest in traits that increase pollination in a most cost- effective manner.

The orientation at which a flower faces the approaching pollinator is an important trait which alters the way a pollinator detects and perceives a flower. Some nectar-feeding bats, for example, locate flowers by the acoustic reflection of a specifically shaped corolla pedal. The strength of the echo-reflection strongly depends on the orientation of the flower pedal, making flowers with downwards facing corollas nearly invisible to pollinating bats (Helversen and Helversen, 1999). Similarly, flower movement might influence the detectability of a flower to a certain pollinator, by altering the visual display size of a flower, and could result in an ethological isolation between plant populations, which move their flowers to different degrees (Fulton and Hodges, 1999). In contrast to sight, the olfactory signal from a flower does not travel linearly, instead directly after their release by the flower, the floral volatise are altered by air movement, which is, in part, determined by the vortex created by the flower in windy environments (Ortega-Jimenez et al., 2013; Riffell et al., 2014). Hence, corolla orientation might have a different effect on the volatile signal than on the visual display of a flower and thus also on pollinators which rely strongly on olfaction in their pollination behaviour. However, these considerations remain unexplored under controlled conditions.

In addition to the flower detectability, flower orientation alters the efficiency by which a pollinator can access the nectar and pollen. Pollen collecting bumblebees for example might profit from flowers oriented downwards, as pollen falls more easily onto their body (Heinrich, 1979b). Hence, less pollen might be collected by bees visiting upwards-oriented flowers, forming a potential mechanical isolation barrier between plants with flowers oriented at different angles. Conversely, syrphid flies and nectar collecting bumblebees land less frequently on flowers with downward orientations, although they approached flowers of all orientations equally often (Ushimaru and Hyodo, 2005). The interplay between these ethological and mechanical isolation barriers is not well understood as it remains often unclear to what extent pollinators perceive and respond to the accessibility of a flower (Campbell et al., 2016).

Nicotiana attenuata is a native plant of the Great Basin Desert (USA) where it is pollinated by *Manduca sexta* among other pollinators (figure 1*a*) (Kessler et al., 2008). *N. attenuata* displays a strong circadian rhythm, in which the flowers move to a horizontal or slightly upward position during the night and to a downward position during the day. Parallel to this, floral volatile emissions are most pronounced during the first half of the night and largely absent during daytime (Yon et al., 2016). These volatiles emissions have been shown to be of particular importance for the interaction with *M. sexta*, which detects these floral emissions both with antenna (Haverkamp et al., 2016b; Riffell et al., 2013b) as well as with specific sensilla on its proboscis (Haverkamp et al., 2016b). However, although these effects of floral volatile emissions have been intensively studied, other floral characteristics such as flower orientation might also have a strong influence on the interaction between *N. attenuata* and *M. sexta*.

Here we aimed to test the effect of floral orientation on the mutualistic interaction of *N. attenuata* with *M. sexta*, by artificially altering the angular position of the flower in wind tunnel assays. In a first step, the effect of this alteration in the flower orientation on the behaviour of the moth was investigated. The results indicated that the flower angle did not influence the detectability of the flower by the moth, likely because the volatile distribution had not been affected by the flower angle. Instead we found that moths were unable to handle flowers with downward orientations. We then investigated the effect of the flower orientation on pollen uptake and delivery as well as on the nectar gained by the moth and found that the inability of moths to handle downward- oriented flowers strongly reduced pollination of the flowers as well as foraging success of the moth. The energetic cost of the flower movement was estimated to consume nearly 20 % as much energy as is contained in the nectar, an investment likely repaid by the enhanced protection of the reproductive tissue and enhanced pollination services. Taken together, these results highlight the high degree of coordination required of both plants and pollinators to ensure successful pollination and foraging.

2. Material and Methods

(a) *Manduca sexta* moth

Moths were reared at the Max Planck Institute for Chemical Ecology (MPICE), Jena, Germany as described previously (Koenig et al., 2015b), in short: eggs were obtained from female moths ovipositing on *N. attenuata* plants. After hatching, the caterpillars were transferred to a growth chamber with 27° C, 70 % relative humidity (RH) and a dark:light cycle of 16:8h and fed an artificial diet (Koenig et al., 2015b). For pupation, caterpillars at the last instar were placed into wooden blocks and kept under the same climatic conditions until one week before enclosure. Pupae were sexed and male and female animals were transferred to separated flight cages with a light regime of 15.5 h daylight, 7.5 h moonlight and 0.5 h transition phases. Climatic conditions were set to 25° C and 70 % RH during the daylight phase and to 20° C and 60 % RH during the dark phase. For all experiments, we only used male moths 72 - 76 h after enclosure.

(b) *N. attenuata* plants

All plants were cultivated at the MPICE, Jena, Germany and originated from wild type *N. attenuata* plants which were collected in a native population at the DI Ranch (Santa Clara, UT, USA). Seeds were sterilized and germinated on Petri dishes with Gamborg's B5 media as described in Krügel *et al*. (Krügel et al., 2002). Petri dishes with 30 seeds were maintained under LD (16 h light and 8 h dark) conditions in a growth chamber (Percival, Perry, Iowa, USA) for 10 days, and seedlings were transferred to small pots (TEKU JP 3050 104 pots, Pöppelmann, Germany) with Klasmann plug soil (Klasmann-Deilmann, Germany) in the glasshouse. After 10 days, plants were transferred to 1 L pots and moved to a York Chamber (Johnston Controls, USA) with the same light regime and climatic conditions as the moth flight cages. In all cases flowers 1 - 3 h after anthesis were used. Flowers were tethered at 45°, 0° or -45° angles using a fine steel wire, which was attached behind the sepal in such a way to maintain the angle, but sufficiently flexible to be moved by the moth.

(c) Wind tunnel assays

All behavioural assays were performed in the wind tunnel of the MPICE. The wind tunnel (220 cm × 90 cm × 90 cm) was run at a laminar flow of charcoal filtered air at a wind speed of 0.37 m/s and 25° C and 70 % R H. Moths and plants were placed into a separated pre-incubation chamber, which had been set to the same conditions as the wind tunnel, at least 1 h before the experiment. Plants were placed into the tunnel directly before the experiment in such a way that the flower would be at a position of 25 cm × 45 cm × 70 cm at the front end of the wind tunnel. The moths were kept individually in small mesh cages (15 cm × Ø13 cm) and were introduced onto a platform at the rear end of the wind tunnel (200 cm × 45 cm ×

40 cm). The animals were given 5 min to initiate wing fanning. Moths which did not start fanning during this time were excluded from the experiment. After take-off, the moths were allowed to fly freely in the tunnel for 4 min during which we observed the foraging behaviour via a video camera (Logitech C615, USA, infrared filter removed) recording at 30 Hz and a resolution of 800 × 600 pixels situated at the back of the wind tunnel. Simultaneously, we recorded the flight tracks of the hawkmoths as described in Haverkamp *et al.* (2016) (Haverkamp et al., 2016). To do so we recorded the flights of the moths via four video cameras and reconstructed the position of the animals at a rate of 10 Hz using a background subtraction method implemented in C. Further analyses were performed using Matlab (Mathworks, USA) and R.

(d) Pollen uptake and delivery

To analyse the effect of flower orientation on male fitness, we tested the amount of pollen, which a moth would take up during their 4 min foraging trial in the wind tunnel from a flower fixed at a certain angle. For this, the moth was captured directly after its flight and the proboscis was washed three times with 1 mL of a 1 % Tween solution. We added 10 µL of a 0.5 % safranin (Sigma Aldrich, Germany) to stain the sporopollenin. The samples were then vortexed and centrifuged for 2 min at 10000 rpm and the supernatant was discarded. We then added 100 µL distilled water, vortexed again and pipetted 10µL of each sample into a four-field Neubauer-counting chamber to determine the number of pollen grains. Each sample was counted two times independently and the mean value was used for statistical comparisons.

To determine the influence of flower orientation on the female fitness of the plant, we analysed the number of pollen grains, which were delivered by a moth to the stigma of emasculated plants. For this, we removed the anthers of still closed flowers to avoid self-pollination by carefully cutting the corolla tube at the side and removing the anther heads with fine forceps (Kessler et al., 2008). Plants were then treated as described above and finally introduced individually into the wind tunnel. Before the start of the experiment, the proboscis of a moth was dusted with pollen collected the previous day, using a fine brush. On average, this method loaded 548.75 (n= 41, SEM= 88.85) pollen grains to a single proboscis.

(e) Pollen counts on stigma

After successful moth-flower interactions, stigmas were fixed and stored in 0.2 mL Eppendorf tubes using a FFA solution (formalin 1; 80% alcohol, 8; acetic acid, 1; by volume). Subsequently stigmas were centrifuged to remove FAA solution, rinsed with distilled water and incubated in sodium hydroxide (8 N) for maximum 4 h to clear tissues. Stigmas were centrifuged to remove previous solution and rinsed again with distilled water. Stigmas were placed in a staining solution with 0.1% aniline blue dye (fluorescence) and 0.1% safranin (bright field). Samples were removed from the staining solution and squeezed with the stigmas facing upwards in a microscopy glass slide. Pollen grains in the stigma were then counted under a fluorescence microscope.

(f) Flower scent measurements

Flower scent volatiles were trapped using PDMS, for a 30 min collection time at 2.5, 5 and 10 cm distance from the corolla limb under same wind tunnel conditions as in section (c). The samples were processed using a TDU-GC-MS as described in Kallenbach *et al.* (2014) (Kallenbach et al., 2014) . 11.8 pg bromohexane was added as an internal standard for relative quantification of the major floral volatile, benzyl acetone.

(g) Flower movement energy estimation

The model for the flower movement energy was calculated by considering the mass (mg) of the flower, rotational angle estimated to the tip of the pedicel, and the work as energy (J) needed to achieve it. In our model the pedicel movement also implies an active elongation of the pedicel (Cortés Llorca, 2014) not just a change in turgor pressure . Given that the mass point is at tip of flower, the inertia effect is considered as for a solid bar with rotation at one single end. The rotational kinetic energy was calculated with the following formula:

$$E(J) = \frac{\frac{(m * l^2)}{3} * \omega\hat{}2}{2}$$

Where E: work energy expressed in Joules, m: mass of the flower, l: length of the flower, and ω: angular velocity.

The position coordinates of the corolla tip and the calculations were done using the software Tracker v. 4.92 (Douglas Brown, http://physlets.org/tracker/). A total of three replicates, pedicel video sequences, were used for the calculation.

3. Results and Discussion

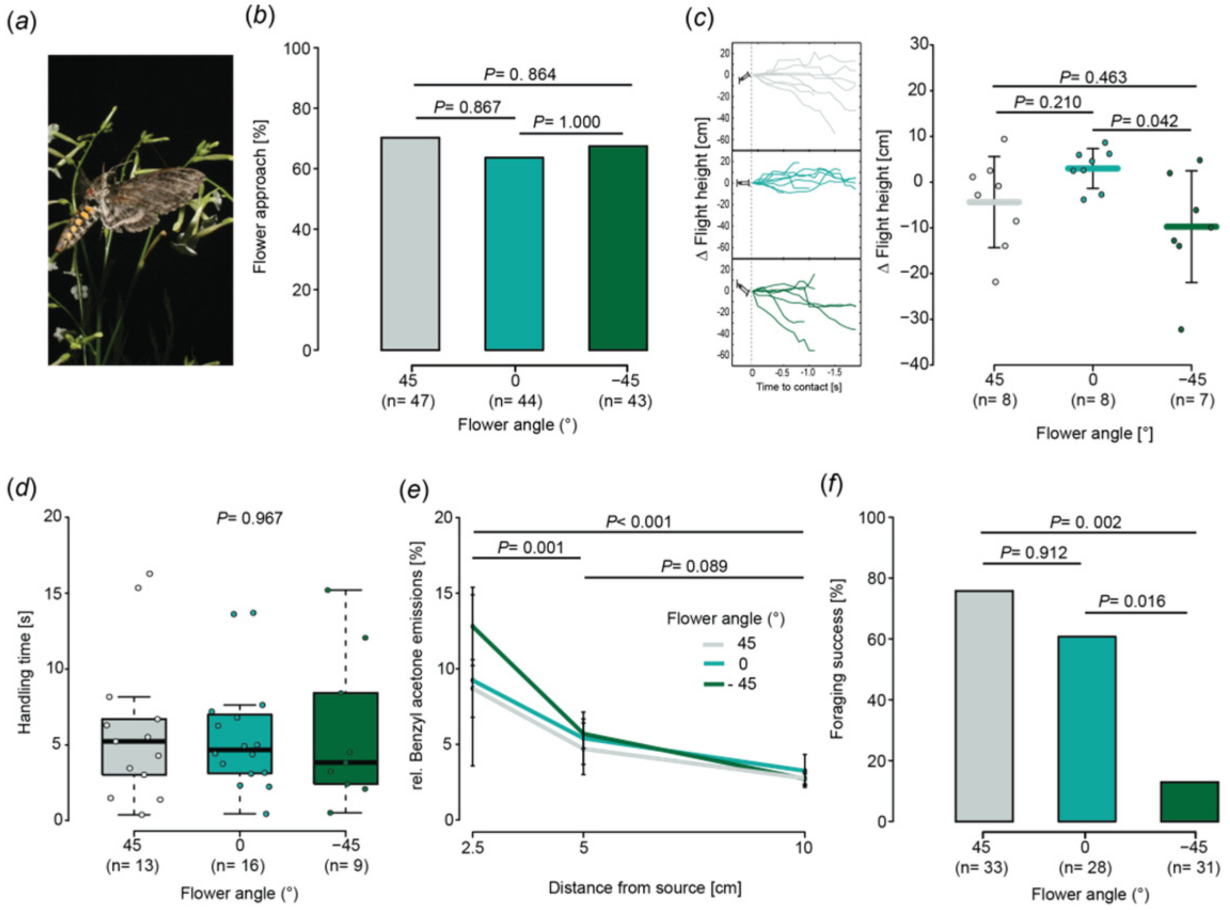

Figure 1. Hawkmoth approached flowers at all orientations, but had higher success rates on upwards and horizontally facing flowers. (*a*) *Manduca sexta* attempting to forage on a downwards oriented flower. (*b*) Moth visited the same number of flowers at an angle of +45°, 0° and -45°. (*c*) Left panels: Lines show the flight altitude during the last 2 seconds before flower encounter. Right panels: Mean flight altitude was significantly higher when the moths approached flowers at an angle of 0° than when they approached flowers at an angle of -45°. Coloured lines show mean values, error bars indicate standard variation. (*d*) Handling times did not differ among flowers at different angles. (*e*) Amount of benzyl acetone collected relative to the internal standard (bromohexane) at the different flower orientations and at different distances from the corolla. The amount of floral volatiles was strongly dependent on the distances from the corolla, but not on the flower orientation (n= 6-8 for each data point). Error bars indicate SEM. (*f*) Moths were significantly more successful in entering the corolla tube at +45° and 0° than at -45°. Holm corrected Fischer's exact test in (*b*) and (*f*), ANOVA followed by a Holm corrected Welch t-test in (*c*) and (*e*) and Kruskal-Wallis test followed by Holm corrected Wilcoxon- rank sum test in (*d*).

(a) Hawkmoth behaviour and floral volatile distribution

To investigate the mechanism by which the moths' foraging behaviour influences plant fitness we closely observed the interaction of *N. attenuata* flowers and their pollinators in a wind tunnel. We found similar proportions of hawkmoths approaching the flowers at all three different angles, indicating that flowers at all three orientations were equally detectable to *M. sexta* (figure 1*b*). However, we found that the moths were generally flying at a higher altitude, slightly above the flower when the corolla was oriented upwards (figure 1*c*), potentially because the upward oriented flower offered an additional visual landmark, which might have influenced flight altitude (Frye et al., 2003). Upon arrival, moths invested similar amounts of time into handling flowers at the different orientations (figure 1*d*), suggesting that the animals expected similar foraging gains from flowers at all three angles. These findings agree with other studies, which found that *M. sexta* allocates its foraging time and energy resources mainly based on the volatiles emitted by a flower (Haverkamp et al., 2016b, 2016c; D. Kessler et al., 2015). We found that the amount of odour present in front of a corolla was not dependent of the flower angle but only on the distance from the flower (figure 1*e*), which is likely due to the turbulent air vortices that usually occur around flowers within an air stream (Ortega-Jimenez et al., 2013). In contrast to our findings, the hawkmoth *Hyles lineata* preferred upward facing flowers of *Aquilegia pubescens* when these were directly compared with downward facing flowers in a two-choice assay (Fulton and Hodges, 1999). In part these differences might be explained by the difference in the experimental procedures, but also suggests that the crepuscular *H. lineata* might rely more strongly on visual cues (D. Kessler et al., 2015). Similarly, different species of African hawkmoths preferred to first probe upward oriented flowers of the fly-pollinated *Zaluzianskya microsiphon*, which does not emit any floral scent attractive to hawkmoths (Campbell et al., 2016). However, even though in our study, *M. sexta* allocated similar amounts of time into flowers at all three orientations, they were less successful in inserting their proboscis into the corolla tube of downward facing flowers than with upward or horizontally oriented flowers (figure 1*f*). This resulted in a significantly lower nectar gain for moths foraging on downward-facing flowers when compared to upward-facing flowers (figure 2*d*). *M. sexta* usually maintains a body angle 34.3° ± 3.3° while hovering in front of a flower (Hedrick and Daniel, 2006). Moths might be reluctant to take a steeper body angle as this will also require a steeper wing angle which will reduce lift forces and might make hovering flight too unstable for efficient nectaring (Willmott and Ellington, 1997). Although, some Lepidoptera appear to be able to lift their proboscis tip above their body axis (Krenn, 2010), we did not observe this for *M. sexta* which limits the maximum downward orientation of a flower on which *M. sexta* can forage to the body angle of the moth. For moths with shorter proboscis, these limitation could be bypassed by increased body or proboscis manoeuvrability (Farina et al., 1994), or by either being capable of landing in flowers that can support their weight (Ushimaru and Nakata, 2002). Other studies, which investigated the influence of more rapid flower movement induced by wind found that following a moving flower along a looming arc is more challenging for *M. sexta* than along a horizontal arc (Jordanna D H Sprayberry and Suver, 2011). Interestingly, parallel measurements of these flower movements found that flowers more often move along a horizontal arc and at a frequency, which can be tracked well by the moth. Hence, the flight abilities of *M. sexta* might indeed be most adapted to those flower movements that are most likely to occur under natural conditions (Sponberg et al., 2015; Jordanna D H Sprayberry and Suver, 2011).

Pollinators are always faced with the dilemma that they are unable to directly perceive the energy which they might be able to gain on a certain flower. Due to this constraint, pollinators rely on floral advertisements to make their foraging decision. The hawkmoth *M. sexta* relies extensively on floral volatiles when foraging. At the same time the energy gained during nectaring strongly depends on the morphological features of the flowers they visit (Haverkamp et al., 2016b). Hence, a strong correlation of volatile emissions and floral characteristics, such as flower orientation is of crucial importance for the interaction between plant and pollinator. Notably in *N. attenuata*, flower orientation and floral volatile emissions are under the influence of the same circadian genes, which ensures the coordination of angle and odour under natural conditions (Yon et al., 2016).

(*b*) Pollination service and foraging success

Depending on their behaviour and body shape, pollinators can influence the male and the female fitness of a plant to different degrees. In *N. attenuata* the stigma is only slightly shorter than the corolla tube of the flower and the anthers even protrude over the corolla opening. Hence, even an unsuccessful visit for a moth might lead to a successful pollen delivery or removal for the plant. To test this hypothesis, we washed the proboscis of each moth that had contacted a single flower and quantified the number of pollen grains that had been taken-up by the moth during this interaction. These results indicated that the moths had not taken up any pollen when they handled flowers with downward orientations whereas moths foraging on horizontal or upwards oriented flowers received between 16250 and 21250 pollen grains per trial (figure 2*c*). Additionally, we dusted the proboscis of the moths with pollen of *N. attenuata* using a fine brush and released them in the wind tunnel with an emasculated *N. attenuata* flower fixed at particular orientations. When we analysed the number of pollen grains on the stigma (figure 2*a*) we found a significantly higher number of pollen grains on the stigma of upward and horizontally oriented flowers in comparison to downward facing ones (figure 2*b*). Combined, our results indicate that both male and female fitness of the plant are similarly influenced by the flower angle which should place this trait under a special selection pressure similar to what had previously been reported for floral volatile production (Kessler et al., 2008). Parallel to the pollen delivery and take-up from the flower, we also analysed the amount of nectar, which a hawkmoth could remove from flowers at different angles. We found that in case of an upward and horizontal flower orientation, moths were able to empty the flower in nearly all cases, whereas significantly more nectar remained when the moth had to handle downward oriented flowers (figure 2*d*). Hence, hawkmoths and flowers appear to be similarly penalized by flowers oriented at angles unsuitable for *M. sexta*.

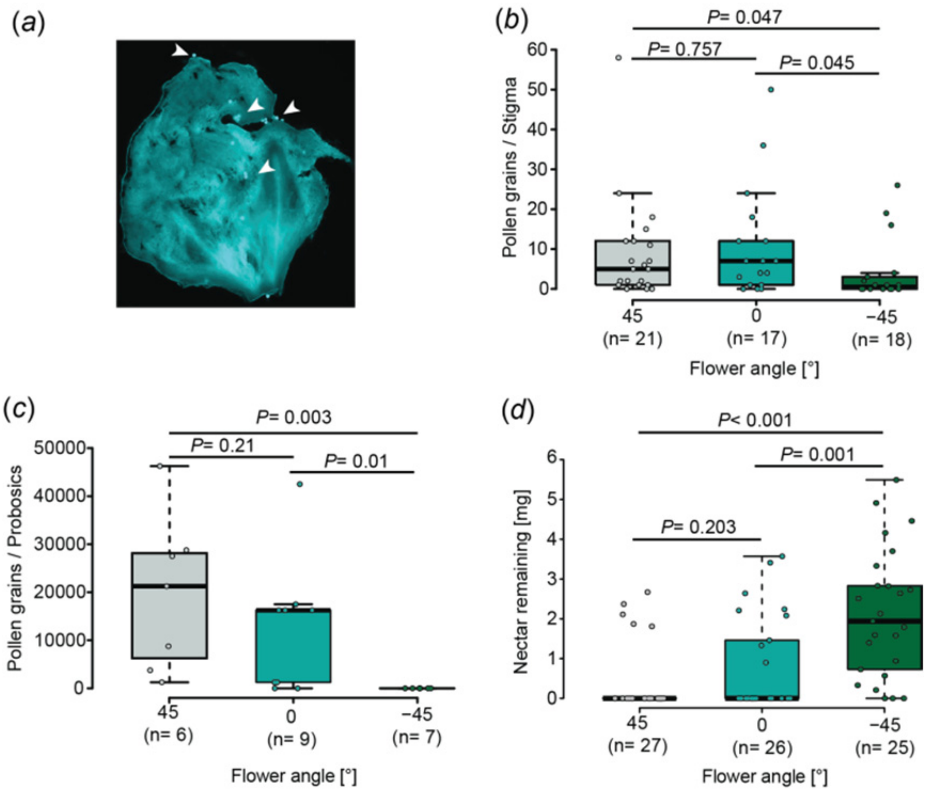

Figure 2. Flower orientation influenced both pollen uptake and pollen delivery. (*a*) Pollen grains (arrows) on the stigma of *N. attenuata* after pollination by *M. sexta.* (*b*) Moths with similar pollen loads on their proboscis delivered significantly more pollen to stigmas of emasculated flowers fixed at 45° or 0° angles than to flowers at a -45° angle. (*c*) More pollen was taken up by moths foraging on flowers at +45° and 0° angles. (*d*) Less nectar remained in flowers at an angle of 45° than at any of the lower angle positions, indicating a higher foraging success of the moths at this angle. Kruskal-Wallis test followed by Holm corrected Wilcoxon- rank sum test in (*b*), (*c*) and (*d*).

(c) Energetic costs of flower movement

Using the specific weight of a flower and the angular distance displacement, we calculated the amount of energy required for the flower movement. Similar to a previous study, freely moving flowers altered the angle between -90° during the day to around +45° degree at night (Yon et al., 2016). This movement is achieved through asymmetric cell grow on either upper/lower flank of the pedicel, making both the upwards and the downwards movement of the flower an active process (Atamian et al., 2016; Cortés Llorca, 2014). On average the flower movement required 1.69 J in total for the upward and downward movement (figure 3). The nectar of *N. attenuata* contains on average 8.59 J, meaning that the movement of the flower requires nearly 20 % as much energy as the plant normally invests into floral nectar (Haverkamp et al., 2016b). This investment resulted into 61.47 % more moths succeeding in entering the corolla tube (figure 1*f*), significantly increasing pollen uptake und delivery for the plant (figure 2*b*, *c*).

Therefore, moving the flower to an upward orientation appears to be a viable investment for the plant, especially as nectar on its own increases pollination success by *M. sexta* only by 44.6 % (D. Kessler et al., 2015). However, our estimations also suggest that the costs which might arise through nectar thieves, desiccation or other factors when the flower is not moved to a downward position excide the costs of the flower movement.

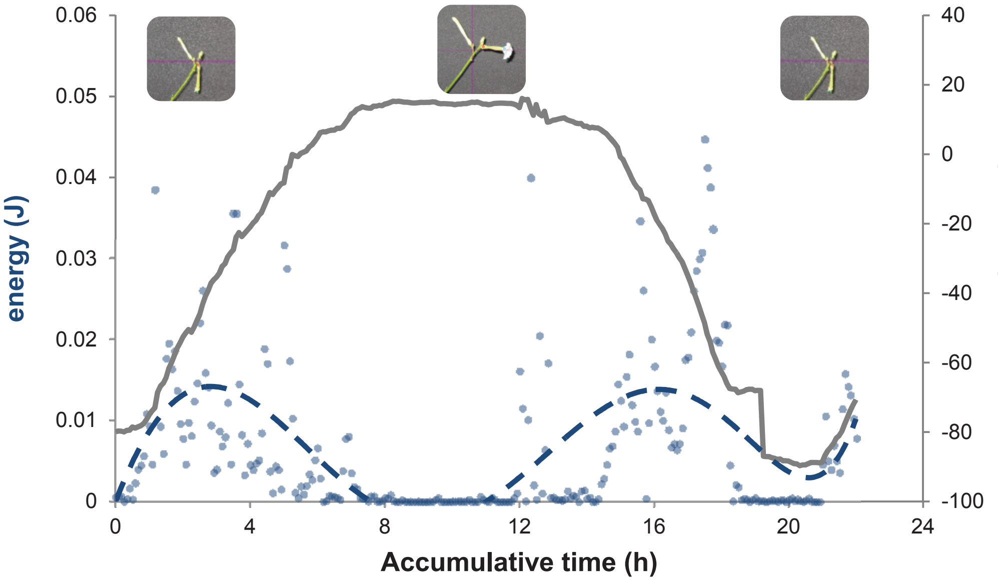

Figure 3. Both the upward and downward movement of a flower consumes energy. Blue line indicates mean calculated movement energy (J) of three flowers. Grey line indicates mean flower angle.

4. Conclusions

Within the mutualistic interaction of *N. attenuata* and *M. sexta* an upward or horizontal orientation of the flower is crucial to ensure the pollination of the flower as well as successful foraging by the hawkmoth. The importance of the flower angle was, however, not due to an altered detectability of the flower. *M. sexta* evaluates flowers mainly by their floral volatiles, which were not affected in their spatial distribution by the flower angle. Instead, moths were unable to maintain a hovering position sufficiently steep to reach into the corolla tubes of flowers with downward orientations and were thus not able to collect nectar or to deliver any pollination services to the plant. In addition to the demonstrated importance of a positive angle for pollination, a downward floral orientation might offer additional benefits in environments with high abiotic and biotic stresses as it can help to prevent the loss or thievery of pollen and nectar as well as damage to reproductive organs by unwanted visitors or excessive solar radiation (Aizen, 2003; Kessler et al., 2013). The latter is particularly relevant in the desert environments of *N. attenuata* where a floral display could easily loose its attractiveness by desiccation during day-time.

Although, these benefits of downward orientation are difficult to estimate, they likely out-weigh the costs of flower movement, which represent about 20 % of the energy invested in nectar. Pollinators are unable to directly perceive the energy which might be gained from a given flower and therefore have to rely on the floral signals. These results highlight the importance of synchronizing floral signals with accessibility for the stability of plant-pollinator interactions and point to mechanisms, which flowers might have evolved to maintain floral signals for suitable pollinators while minimizing both biotic and abiotic stresses.

Data accessibility. Haverkamp, A., Hansson, B. S., Baldwin I. T., Knaden M. and Yon F. (2016) Data from: Flower orientation in *Nicotiana attenuata* is shaped by biomechanical but not sensory constraints of its hawkmoth pollinator. Dryad Digital Repository. doi:10.5061/dryad.h6g2s

Author's contributions. All authors contributed to the experimental design of the study. A.H and F.Y. performed all experiments and analysed their results. F.Y. performed energy calculations of the flower movement. A.H. wrote the first draft of the manuscript and all authors contributed to the revision.

Competing interest. The authors declare no competing interests.

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Discussion

“We hope that when the insects take over the world,
they will remember with gratitude how we took them along on all our picnics.”
William E. Vaughan

Throughout this thesis we have aimed to link the foraging behaviour of *M. sexta* to its olfactory preference. From the research conducted as part of this thesis it could be shown that:

> *M. sexta* prefers the floral volatiles of those flowers, which will yield the highest energy balance for the moth during foraging, indicating that the olfactory system has partly been shaped by the pressure to optimize foraging efficiency (chapter 1).
>
> Furthermore, we found that the olfactory preference towards different host plants during the moth’s search for oviposition sites is dependent on the composition of the odour plume rather than on the plume concentration. A principle which is also likely to apply to floral odour plumes (chapter 2).
>
> The precise detection of the different odours emitted by a flower holds different challenges over a distance of several meters than it does on the scale of centimetres. We therefore investigated how *M. sexta* might evaluate odours sources at a close range and found that the moth uses additional olfactory receptors on its proboscis for this task (chapter 3).
>
> Finally, we show that the vertical orientation of a flower does not alter the distribution of the floral odour plume and in such cases *M. sexta* also allocated the same amount of hovering time to flowers at different orientations, even though the moths were not able to forage on downwards orientated flowers. This suggested that the moth invests its foraging energy, mainly based on the olfactory input from its antenna and proboscis (chapter 4).

Combined, the results of this thesis highlight the importance of olfactory information for the decision making of *M. sexta* during foraging and emphasises the tight co-evolution between the sender of these information - the flower - and the olfactory system of the moth as the receiver of these volatile messages.

I. Odour guided foraging in hawkmoths is shaped by energetic trade-offs

Energy is the fundamental currency of all interactions within an ecosystem and during foraging animals act as “economic agents” trying to maximise their energy gain while minimising their costs (Heinrich, 1979a). In order to make these energy-economic decisions animals have to rely on their sensory systems to collect all available and relevant information. However, sensory systems themselves can create considerable metabolic costs, both directly through the energetic needs of the neurons, as well as indirectly by creating an ineffective decision making process. These constraints force the animal again, to balance its investment into the sensory system against the foraging advantages it gains through a better but metabolically more expensive sensory system (Laughlin, 2001). Moreover, these energetic constraints do not only influence the sensory system of the animal, but do also shape the interaction with the sender of this information, such as a flower. Hence, using energy as a common currency might also provide interesting insight into the evolutionary forces, which shape the interaction between different species (Pyke, 1978).

Nectar collecting animals such as honeybees, hummingbirds, but also bats and hawkmoths, have captured human fascination due to their restless activity by which they visit flower after flower (Heinrich, 1979a). These obvious efforts led naturalists to wonder about the balance of the energy gained though the floral nectar and the energy spent by the animal during their flower visits. An important aspect which influences this energy balance is the efficiency by which the animal can locate and evaluate a certain flower resource. Therefore, the sensory system of an animal plays a crucial role in shaping the outcome of the animal’s foraging efforts on different flowers and thus also for its evolutionary fitness. This connection between energy gained during foraging and biological fitness is particularly strict in the different species of hawkmoths which only feed on floral nectar. In these species the carbohydrates derived from the nectar are the most limiting factor for egg production in females, even more than essential amino acids, which are commonly taken up in sufficient amounts during the larval stage of the moth (O’Brien et al., 2002). Due to this we would expect a strong evolutionary pressure on factors, such as the sensory systems, which directly influence foraging efficiency in the hawkmoth.

How to select the right amount of information: As we have seen throughout the different chapters of this thesis, the olfactory system is crucial for hawkmoths both to find nectar resources as well as to estimate the quality of a certain flower (Haverkamp et al., 2016a, 2016b; Riffell et al., 2013a). The two antenna of *M. sexta* combined contain a little less than half a million receptor neurons (Homberg et al., 1988). At rest these neurons probably introduce considerable metabolic cost, similar as the visual system in the fly, which has been calculated to make up about 13 % of a fly's resting metabolic rate (Laughlin, 2001). In contrast, as soon as the fly takes off the metabolic rate increases more than 30 times and the direct energy costs of the visual system become almost negligible. In this situation, the weight and the aerodynamic drag of the sensory system might then become more important factors (Niven and Laughlin, 2008). However, as every second of flight is energetically highly demanding the most severe caloric costs associated with the sensory systems most likely do not arise from the direct costs of the neurons, but from inefficient or faulty decision making. In chapter 1 for example we have seen that *M. sexta* gains about 28.27 J per visit to an *N. alata* flower, but only 8.07 J per visit to a flower of *N. sylvestris* (Haverkamp et al., 2016a). Hence, the energetic opportunity costs [2] for not having the receptors to discriminate between the two flowers would come to 10.1 J per flower handling, assuming that the moth then takes a random decision. Additional cost would of course also arise if the moth had to take the decision at a certain distance from the plant, adding traveling costs of 0.13 J per second flight (Willmott and Ellington, 1997). Notably though, hovering costs are likely to have a far greater impact on the foraging balance of animals which collect nectar in hovering flight than, the traveling costs between flowers (Casey, 1976; Voigt and Winter, 1999; Winter and von Helversen, 2001). Hence, it is conceivable that *M. sexta* has developed the olfactory receptors on its proboscis, which have been shown in chapter 3 of this thesis, as a final control sensor, before engaging into costly hovering manoeuvres (Haverkamp et al., 2016b). Additionally, having a sensory system, which is able to precisely recognise the chemical profile of a certain flower, will not only aid in finding this flower, but will also, help to accurately remember this flower, either positively or negatively, after its nectar content has been evaluated (Wright and Schiestl, 2009).

In contrast, having too many receptors may not only create more direct metabolic costs but might also introduce information noise by adding irrelevant information and thereby extending decision time or even causing incorrect decisions (Warrant, 2016; Wehner, 1987). In chapter 1 it was shown that the olfactory system of *M. sexta* did not or only weakly responds to acyclic sesquiterpenes such as farnesal or trans-farnesol (Haverkamp et al., 2016a). As these are very ubiquitous plant compounds, which occur in many different contexts, the detection of these compounds would probably not aid the search of the hawkmoth for a certain flower. On the contrary such a receptor might even create an additional burden on the olfactory system as

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[2] Opportunity costs are here taken as the hypothetical loss from having made one decision instead of another (Sterling and Laughlin, 2015).

these signals would have to be filtered out at a later stage. Hence, it seems reasonable that such a specific farnesal detector did not develop in the moths through the cause of evolution or has been lost at a later stage. Interestingly, one case were such a receptor loss has been suggested is the butterfly family of the Nymphalidae. These day active butterflies normally forage on flower nectar and possess four different types of colour receptors in their eyes; however those family members, which have diverged from nectar foraging to feeding on tree saps, rotten fruits and dung have lost their receptor for red light (wavelength sensitivity > 565 nm), apparently because it did not give beneficial information during nectar foraging (Briscoe and Chittka, 2001).

In addition to not providing novel information, sensors might also provide faulty or unreliable information if their input cannot be evaluated in the right context. The results shown in chapter 3 and 4 indicate for example that *M. sexta* fully relies on those olfactory receptors, which are detecting the main flower volatile of *N. attenuata*, when deciding whether a certain flower is suitable or not (Haverkamp et al., 2016b; D. Kessler et al., 2015, chapter 4). In the presence of this odour, the moth always allocated the same amount of hovering time to each *N. attenuata* flower, which under normal conditions ensures a high foraging success (Haverkamp et al., 2016b). However, in certain *N. attenuata* populations the coordination of flower movement and volatile emissions is impaired (chapter 4, Yon et al., 2015), causing the flower to be in an angular position which cannot be accessed by *M. sexta*, even though they are attracted by the floral volatiles. The hawkmoths foraging in these populations might either learn that the signal from the receptor detecting the *N. attenuata* odour is unreliable or, on an evolutionary time scale, might lose such a receptor altogether.

How to optimize an olfactory system: Taken together, we have argued that a foraging hawkmoth should possess enough receptors to reliably detect the most suitable nectar source, while not having too many unnecessary receptors, which might create unwanted information noise. But can we also predict how much *M. sexta* should invest into the sensitivity of its olfactory system in order to forage efficiently?

For some olfactory receptors such an energetic cost / benefit analyses is probably not possible as the survival and reproduction of the animal depends on them to such an extent that no compromise can be made with regards to their sensitivity. These channels include for example the pheromone system or channels which detect fatally toxic compounds (Hansson, 1995; Stensmyr et al., 2012). Often these channels consist of so called ‘labelled lines’ through which highly specialized receptors send their information almost directly towards higher processing centres in the brain (Grabe et al., 2016).

In contrast to these specialised connections, the more broadly tuned channels which are involved in the detection of food should be under a high optimisation pressure with regards to the number and size of neurons detecting and processing these food related odours. An increased number and size of neurons both influence the sensitivity of the olfactory system, in the way that they increase the likelihood that a certain odour molecule is detected (Dekker et al., 2006) and that the animals are less vulnerable to signalling noise (Niven and Laughlin, 2008). However, having more and larger neurons also increase the energetic cost of the sensory system and we might therefore expect that an animal only invests into a greater olfactory sensitivity when necessary. If for example foraging costs are relatively high, the animal, in evolutionary terms, should invest strongly into its olfactory sensitivity to reduce decision making time and to minimize the change of energetically costly errors. If in contrast, foraging costs are low, the animal might be better off to save cost on maintaining sophisticated sensory systems and to rather adopt a ‘trial- and error’ strategy (Chittka et al., 2009). Heinrich and Raven (1973) for example estimated that a 0.1 g bumblebee which walks over a florescence to collect nectar spends about a 140 times less energy than a 3 g hawkmoth, which collects nectar while hovering. Based on such extreme differences in foraging costs, one would predict hovering flower visitors to invest highly into both speed and precision of their sensory systems to facilitate their decision making. Such investments have indeed been shown for the insect eye, were it was found that day-active hawkmoth, which select flowers during costly hovering flights, have particularly well adapted visual systems (O’Carroll et al., 1996; Warrant, 2001).

Hence, animals should use less energy to increase both speed and accuracy of their olfactory channels, whenever their decision costs are low and invest strongly when the costs of a decision are high (Chittka et al., 2009). Such an argument might be further supported by observations in bumblebees, which have been shown to become the more selective in their flower choice, i.e. investing more into their decision making, the higher their flight cost become (Heinrich, 1979a). It might therefore be an interesting hypothesis for further experiments to determine whether animals with higher decision making costs, invest more into the sensitivity of their olfactory system than animals in which decision costs are low.

Figure 5 Important competitors of *Manduca sexta* for floral resources: The hawkmoths *Hyles lineata* (a) and *Manduca quinquemaculata* (b) and the hummingbird *Archilochus alexandri* (c). Pictures by Danny Kessler.

The ecological context shapes the receptor repertoire: In most ecosystems, pollinators are also competing strongly for flower resources (Goulson, 2003). Hence, to fully understand the preferences of a given pollinator towards certain flower resources it might in some cases be crucial to also take the preferences and adaptions of competing pollinators into account. In the Great Basin Desert in Utah, USA, *M. sexta* shares its habitat and its flower resources mainly with the hawkmoth *Hyles lineata* and *Manduca quinquemaculata*, as well as with the hummingbird *Archilochus alexandri* (Alarcón et al., 2008; D. Kessler et al., 2015) (Figure 5). Potentially all of these pollinators can feed on an overlapping range of flowers, although most probably with different efficiencies. The flowers of *N. attenuata* for example have a mean length of 2.7 cm and visits by all four pollinators have been recorded (D. Kessler et al., 2015). In spite of this we have seen in chapter 1 that the energy gain by foraging on this flower might even be negative for *M. sexta* due to the mismatch of proboscis and flower length (Haverkamp et al., 2016a). In contrast to this, the 3.2 to 4.6 cm long proboscis of *H. lineata* matches the *N. attenuata* flower much better and we would therefore expect a competitive advantage of *H. lineata* over *M. sexta* while foraging on these flowers. Interestingly the visitation rate of the short-tongued *H. lineata* over *M. sexta* on *N. attenuata* flowers is also about 50:1 (D. Kessler personal communication, Sept. 2nd, 2016), even after correcting for potential differences in population size according to Alarcón et al. (2008). In contrast to this, the deep corolla tubes of *D. wrigthii* provide an exclusive nectar source for long-tonged hawkmoths in the Great Basin Desert and pollen counts on the proboscis of the three hawkmoth species mainly present in this habitat also indicate that this flower is indeed largely visited by *M. sexta* and *M. quinquemaculata* (Alarcón et al., 2008). These observations support the general hypothesis that hawkmoths should specialise according to their proboscis length; however is this specialisation also reflected in their olfactory preference?

Unfortunately no direct comparison of the olfactory systems in these hawkmoth species has been conducted so far, but both *M. sexta* and *H. lineata* have been shown to mainly respond to oxygenated aromatic and terpenoid compounds typical for hawkmoth pollinated flowers (Fraser

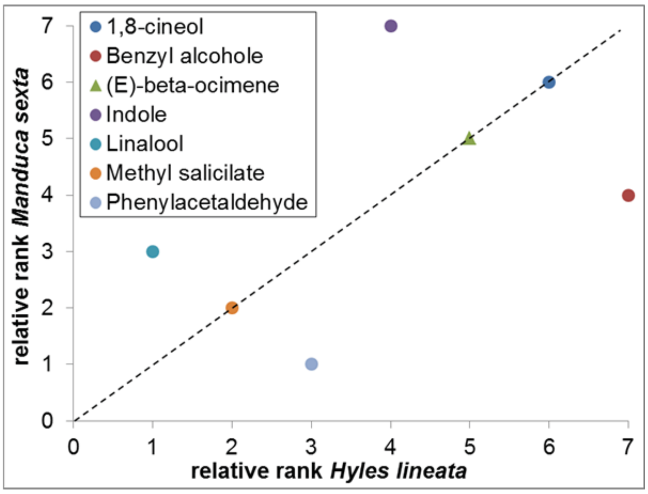

Figure 6 relative ranks of antenna responses in *Manduca sexta* and *Hyles lineata* towards seven common flower compounds. Based on data of Fraser *et al.* (2003) for *M. sexta* and Raguso *et al.* (1996) for *H. lineata*.

et al., 2003; Haverkamp et al., 2016b; Raguso et al., 1996; Riffell et al., 2013a). Nevertheless, some differences in the relative importance of certain compounds become apparent when we compare the results of Fraser *et al.* (2003) for *M. sexta* and Raguso *et al.* (1996) for *H. lineata*, which both performed electroantennogramm (EAG) recordings using a partly overlapping set of compounds (Figure 6). Phenylacetaldehyde for example elicited the highest relative response in *M. sexta* whereas linalool elicited the highest relative response in *H. lineata*. To fully elucidate the importance of olfactory preferences in the interaction between *M. sexta* and *H. lineata* a single consistent study would be required. However, the comparison shown here still suggests that even though both moth species detect a similar set of compounds, the relative differences in the response could still contribute to segregate these two species into different foraging niches.

The costs of being a generalist: Additionally, animals might invest into new olfactory channels, during their evolution, in order to acquire additional resources and to thereby broaden their diets. Hence, we would expect to find a higher investment into the nervous system in pollinators that visit a broad range of flowers than in those that are more specialised (Bernays, 2001). *Apis mellifera* for example, which is sometimes considered a ‘super-generalist’ (Waser et al., 1996), has about 163 Or genes whereas *M. sexta,* which is specialised mostly on sphingophilouse flowers has only about 70 Or genes, indicating that the bee might be able to perceive a larger odour panel than the moth (Koenig et al., 2015a; Riffell et al., 2013a; Robertson and Wanner, 2006). Moreover in spite of being about ten times smaller than *M. sexta*, the honeybee maintains an olfactory centre and a brain only about three times smaller than the one of *M. sexta*. Theoretically, this would imply that for a bee of the same size as *M. sexta* the brain and the olfactory centres would roughly consume three times as much energy as in *M. sexta*. This increment could partly be viewed as the costs *A. mellifera* has to pay for its low degree of specialisation. Importantly, *M. sexta* will only save energy in the case where the travel costs, between the flowers it has specialised on, do not excide the energy saved by the reduced neuronal tissue (Lewis, 1986). Interestingly though, *M. sexta* is well adapted for travelling large distances due to its relatively long and slender wings (Willmott and Ellington, 1997). These specific morphological characteristics reduces travelling costs in *M. sexta*, but also decreases hovering abilities and probably support a specialisation on certain high rewarding, but sparsely distributed flower types (Haverkamp et al., 2016a; Winter and von Helversen, 2001). Nevertheless, *M. sexta* has also been observed to opportunistically switch to other flowers in the case where the flower species they innately prefer fall below a certain density, but switches back to their innate choice as soon as their preferred flowers become again sufficiently abundant (Riffell et al., 2008b).

II. Odour guided searching

Olfactory signals can be distributed by the wind over large distances, giving them the capacity to guide pollinators over 15 m or more to a flower (Raguso and Willis, 2005). At the same time flower odours also act as local cues eliciting the probing response of the moth at a close range (Haverkamp et al., 2016b; Raguso and Willis, 2005). However, over this wide spatial scale, olfactory signals vary greatly in their physical representation. At a distance of several meters from the source, insects encounter odour plumes as brief pulses with a certain frequency whereas at a range of centimetres the insect will be confronted with a continuous scent cloud, which provides a chemical gradient towards the source of the odour (Cardé and Willis, 2008). These two very different forms of odour plume representation are probably difficult to detect accurately with a single olfactory organ and many insects therefore carry olfactory receptors not only on their antennae but also on different other body parts such as their labial palps (Dweck et al., 2016; Guerenstein et al., 2004a), their abdomen (Klinner et al., 2016) or, as we have demonstrated in chapter 3 of this thesis, on the proboscis (Haverkamp et al., 2016b).

Odour detection at a distance: The antenna is the primary olfactory structure of most insects and it is the main organ involved in odour-guided orientation, enabling flying insects to track odour sources over large distances (Vickers, 2006). Such odour-plume tracking, as we have shown in chapter 1 of this thesis, depends on accurate measurement of the wind direction by the animal (Cardé and Willis, 2008). It is often assumed that insects estimate the wind direction by visually measuring their drift during flight. Although this concept appears intuitive, it has so far not been demonstrated experimentally. Moreover, many hawkmoth species operate under extremely dim light conditions and have evolved the ability to slow down their visual processing in order to increase their spatial accuracy (Sponberg et al., 2015; Stöckl et al., 2016b). This on the one hand could increase the ability of the moth to estimate drift at low wind speeds but could potentially limit their performance under more turbulent conditions. Alternatively, insects often use mechano-sensory information whenever visual information is not available at the required temporal resolution (Daniel et al., 2012). Flying insects are able to measure wind forces as well as turbulences using their antenna (Burkhardt and Schneider, 1957; Sane et al., 2007). However, how this information might be employed during odour tracking in turbulent environments has yet to be explored. In this context, it might also be of interest to determine how mechano-sensory neurons from the Johnston-organ, which detects wind and Coriolis forces at the base of the antenna, interacts with olfactory neurons in the central nervous system (Dieudonné et al., 2014). In addition to turbulences induced by wind forces, hawkmoths themselves might produce fluctuations in the wind speed through their wing movement, which might also alter the odour perception of the animal (Tripathy et al., 2010). Interestingly, recent, anatomical studies have identified neurons innervating both the flight muscles and the antenna lobe of *M. sexta* and could thus potentially help to adjust the olfactory system to the wind turbulences induced by the animals own movements (Bradley et al., 2016). Taken together, this data suggests that hawkmoth might possess a far more complete picture of their own movement within their turbulent environment than it is assumed so far and further investigations might even find a similar use of internal models and planning during odour guided navigation as it has recently been shown in dragonflies during visual prey pursuit (Mischiati et al., 2014).

In addition to the antenna´s function during odour-guided orientation, the olfactory receptors on the antenna probably also contribute to the evaluation of the olfactory stimulus. In the 1st chapter of this thesis we demonstrated that *M. sexta* responded to the first encounter of the *N. alata* odour blend with a significant increase in upwind flight within less than 500 ms, whereas other flowers odour blends did not trigger any significant change in the upwind movement after one encounter (Haverkamp et al., 2016a). However, it is not clear whether this increase in upwind speed for *N. alata* was mainly due to an enhanced detectability of the odour blend or whether the compounds within this blend carry a greater attractive value for the moth than other floral odours. When we tested the physiological response of the hawkmoth antenna towards odour blends of *N. alata* and *N. sylvestris* we found, that although the odours from *N. alata* had the tendency to elicited a higher mean response, the responses towards both blends were in a similar range (mean *N. alata*: 7.25 µV ± STD 3.85; mean *N. sylvestris*: 5.64 µV ± STD 1.17; n= 3 for both flowers). This indicates that the stronger upwind response of *M. sexta* in case of *N. alata* was not only due to a higher number or greater sensitivity of the antennal receptors, but also due to some differences in the processing of these odours in the central nervous system of the moth. Similarly, the results shown in chapter 2 demonstrated that moths did not discriminate when they were given the choice between the headspace of one host plant against the same headspace five times diluted by clean air. In contrast, when the blend composition was altered by mixing it with the headspace of another attractive host plant, the moth was not attracted any more to the odour source (Spaethe et al., 2013a). Hence comparable to what we have seen for the floral odour blends, the moth's response was less dependent on the strength by which the OSNs on the antenna were activated, but rather on further processing steps in the brain. Potentially these differences in processing could have been established through uneven convergence ratios between the OSNs and projection neurons in the antennal lobes as it has been shown in *D. melanogaster* (Grabe et al., 2016) or through differential processing in the antennal lobe by local interneurons, which would then create a blend specific 'odour image' (Kuebler et al., 2011).

Close range odour evaluation: Within a few meters from the flower the floral odour plumes can become quickly intermixed with background odours, which can make it challenging to the moth to establish the identity and quality of an odour source at a distance as these background odours might interfere with the odour blend processing (Riffell et al., 2014). The final decision by the moth whether or not to engage with a certain flower might thus only be taken in a close vicinity of less than 20 cm from the flower. However, as the hovering flight is energetically particularly expensive, hawkmoths throughout their evolution appear to have invested heavily into their sensory equipment, which might enable them to quickly and precisely evaluate a certain flower. At a close range for example hawkmoths have been shown to use humidity sensors like those found on their antenna (Lee and Strausfeld, 1990) to detect elevated evaporation levels from freshly opened and potentially nectar filled flowers (von Arx et al., 2012). Furthermore, the floral nectar has been found to emit a surprising diversity of volatile compounds, which are often overlooked in the chemical analyses of the total floral headspace due to their low concentration. They do however accumulate strongly in the direct surrounding of the flower and in its corolla tube (Kessler and Baldwin, 2007). Yet, due to their long proboscis hawkmoth are usually more than one body length removed from the flower when evaluating the plant, which might have promoted the development of additional olfactory sensilla especially on the moth proboscis (Haverkamp et al., 2016b). In chapter 3 of this thesis I could show that these olfactory sensilla are able to detect and mediate attraction to the main compound in the *N. attenuata* flower headspace.

Interestingly though, when we tested the response of the moth's proboscis in a two-choice assay the hawkmoth was not able to actively navigate with its proboscis towards the odour. Instead it probed both sides of the assay at random and then remained at the side with the floral odour. This lag of navigation behaviour might potentially also be due to the limited space which our assay offered to the proboscis and a more rigorous testing might be required to fully exclude any odour guided navigation based on the proboscis input. However, our current data rather supports the idea that the olfactory input from the proboscis mainly serves to determine the quality of a flower and to maintain the motivation of the moth during hovering. Hence, it might be conceivable that the axons of these sensory neurons do not or not only project towards the antennal lobe but do instead innervate similar areas in the suboesphagal ganglion as the sugar sensitive neurons and that the olfactory input from the proboscis might therefore also been perceived as a direct reward (Riabinina et al., 2016; Spingler, 2016). In the honeybee sugar sensitive neurons synapse onto large octopaminergic neurons which then help to connect this positive sugar signal to higher brain centres such as the mushroom bodies (Hammer, 1993). Similar octopamineric neurons and effects of this biogenic amine have also been found in *M. sexta* (Dacks et al., 2005; Riffell et al., 2013a), supporting the hypothesis that the activation of these neurons by the OSNs on the moth proboscis leads to an enhanced foraging motivation of *M. sexta* (Haverkamp et al., 2016b).

In summary, it could be hypothesised that the olfactory neurons on the proboscis have developed due to the need to carefully evaluate a certain flower at a close range before allocating expensive hovering time to that particular flower. These neurons might therefore also give a direct positive signal to the brain comparable to the sugar sensitive neurons on the proboscis.

III. Pollinator- plant interactions

The co-evolution of plants and pollinators has often been described as rather one sided. Long-tongued hawkmoths for example appear to exert a selection pressure on flowers to evolve ever longer corolla tubes, while hawkmoths with a long-proboscis have been found to forage on flowers with both short and long corolla tubes (Johnson et al., 2016). However, as we have shown in chapter 1, the long-tongued hawkmoth *M. sexta* readily forages on many different flowers, but only those which match the length of the moth proboscis were ultimately energetically profitable (Haverkamp et al., 2016a). These results are also consistent with the findings in other hawkmoth species in the wild that although these moths can potentially feed on a variety of plants, they usually concentrate their foraging efforts on flowers matching their proboscis length (Sazatornil et al., 2016). Moreover, although the olfactory system of *M. sexta* is able to detect a large variety of floral compounds, it is most adapted to detect those compounds emitted by flowers that match the moth's proboscis (Haverkamp et al., 2016a; Riffell et al., 2013a). Hence, flowers might place a greater pressure on the evolution of certain pollinators than it might by assumed based on observations of the pollen load carried by a certain species in a particular habitat (Johnson et al., 2016).

It is all in the genes: *M. sexta* is a major pollinator of different *Nicotiana* and *Petunia* species, i.e. two genera, which have been intensively used in genetic studies over the last decades, revealing many of the genes important for flower development and floral volatile production (Kessler et al., 2013, 2008a; Klahre et al., 2011; Yon et al., 2015). Recently also genetic data for *M. sexta*, both on a genomic and on a transcriptional level (Kanost et al., 2016; Koenig et al., 2015a), has become available, which might greatly facilitate the study of plant-pollinator co-evolution. In chapter 1 of this thesis we have seen that the olfactory system of *M. sexta* is mostly tuned towards the volatiles emitted by *N. alata* (Haverkamp et al., 2016a). Interestingly, *N. alata* shares most of its floral volatiles with other plants for which *M. sexta* is also an important pollinator such as *D. wrightii* or *Petunia axilliaris.* In *P. axilliaris* the enzymatic cascade for the production of the individual flower volatiles is largely known (Kessler et al., 2013) and it has been shown, that changes even in a single gene can lead to a variety of compounds attractive to *M. sexta* (Klahre et al., 2011). Conversely, two lineage specific duplication events have been found in the olfactory gene set of *M. sexta* (Koenig et al., 2015a). One cluster of six genes (MsexOR 8, 10, 33, 36, 80 and 85) most closely resembles the *Bombyx mori* receptor BmOr24, a very broadly tuned receptor also expressed in the *B. mori* caterpillar (Tanaka et al., 2009), whereas the other cluster of three genes (MsexOR 9, 26 and 65), resembles a *B. mori* receptor gene for which the function is unknown, but is most closely related to another *B. mori* receptor involved in the detection of linalool. It would be interesting to see if the development of any of these *M. sexta* receptor gene clusters correlates to the origin of those genes, which control the emission of floral volatiles and thereby linking the evolution of flower odour production to the evolution of floral odour detection in *M. sexta*.

Pollinator-herbivore interactions: Contrasting to what has been predicted for plant-pollinator interactions where a strong selection pressure of the insect onto the plant has been hypothesised, many studies on plant-herbivore interactions have assumed quite the opposite: here it has been predicted that plants create a strong selection pressure on the insect herbivore, whereas the herbivore does not impose a strong selection pressure on the plant (Schoonhoven et al., 2005). In this context it is interesting that several volatiles released by *P. axilliaris* are not only attractive to *M. sexta*, as one of their main pollinators, but are also repellent to several generalist flower-feeders (Kessler et al., 2013). Moreover, the phytohormone jasmonoyl-L-isoleucine (JA-Ile) one of the master regulators of plant defence is also responsible for the control of flower opening and the emission of floral volatiles (Stitz et al., 2014), suggesting that the evolution of floral volatiles has not only been shaped by the mutualistic interaction of a plant with its pollinators but also by the plants interactions with its herbivorous antagonists.

Figure 7 *Hyles lineata* caterpillar feeding on a flower of *Mirabilis spp*. a plant mainly pollinated by the adult hawkmoth. Picture by D. Kessler

In cases of *M. sexta* and other hawkmoth where the adult moth is an important pollinator of many solanaceous plants, the caterpillars are also major herbivores on these plants (Figure 7). *M. sexta* caterpillars are specially adapted to deal with high nicotine levels in their plant food (Self et al., 1964), being about 5000 times more tolerant to this toxin than mammals such as mice (Kanost et al., 2016). However, nicotine cannot only be found in plant leaves, but is also present in the nectar of different *Nicotiana* plants (Kaczorowski et al., 2014; Kessler and Baldwin, 2007). Currently, the role of the nectar nicotine in the interaction of adult *M. sexta* and different *Nicotiana* plants is still largely unknown and although *M. sexta* is to some extent deterred by nicotine, it seems to be less sensitive than other competing nectar feeders, such as hummingbirds (Kessler and Baldwin, 2007). *M. sexta* caterpillars excrete nicotine through their Malpighian tubules (Maddrell and Gardiner, 1976), and actively pump nicotine away from their central nervous system through ABC-transporter in their blood-brain barrier (Murray et al., 1994). Adult moths however, are unable to eliminate nicotine from their haemolymph through their Malpighian tubules (Maddrell and Gardiner, 1976) and it is currently unknown whether the adults possess the same transporter proteins in their neurolemma as the caterpillars. If they would, this could suggests that although the adult hawkmoths are not as resistant to high nicotine levels as the caterpillar, they might still be more tolerant than other pollinators and nicotine could thus act as a specific filter against all but hawkmoth pollinators (Raguso, 2004). Furthermore, although nicotine is a strong neurotoxin and has also been considered as a repellent bitter compound (Tiedeken et al., 2014), it has also been argued that low concentrations might enhance memory formation in insects by acting as an antagonist of the acetylcholine receptor in the insect brain (S. C. Kessler et al., 2015), similarly to what has been shown for caffeine, another alkaloid, sometimes found in floral nectar (Wright et al., 2013). Hence, nectar nicotine could act to promote *M. sexta* pollination in *Nicotiana* flowers by repelling or intoxicating unwanted flower visitors on the one hand and by strengthening the memory formation of *M. sexta* for *Nicotiana* flowers on the other hand (Stevenson et al., 2016). In chapter 3 of this thesis we have identified an olfactory sensillum on the proboscis of *M. sexta*, which is able to detect volatile nicotine (Haverkamp et al., 2016b). This finding might enable further research on how nicotine is detected by *M. sexta* and on how these information are processed in the brain of the hawkmoth.

IV. Future prospects for olfactory research in hawkmoth

Within this thesis a great variety of methods and techniques was used to study the chemosensory ecology of *M. sexta* during foraging. Our approach has ranged from gene analyses over physiological measurements to behavioural assays in a wind tunnel and in a semi-natural free flight tent. However, one level we still have to investigate is the behaviour of the hawkmoths in their natural environment and many general aspects concerning the natural history of *M. sexta* remained still unanswered. For example, it is not yet known how far floral volatiles act to attract hawkmoth pollinators and how faithful the moths are in visiting only a certain type of flower species. Other open questions concern the background volatiles against which the moths have to identify the floral headspace: Do moths have greater difficulties in finding a certain flower in a meadow of other flowers than in finding the same flower in a desert environment? These questions have so far been very difficult to answer due to the large foraging range of the hawkmoth and due to their night-time activity. Radar based tracking has so far been used to investigate the movement strategies of nectar foraging honeybees and butterflies (Cant et al., 2005; Reynolds et al., 2009). However even though this method provides interesting insights into the behaviour of pollinating insects, its application has been limited by the costs and technical difficulties of the radar equipment to such an extent that it has so far only been used at two sites in northern Europe. Alternatively, new developments in the design of light weight radio transmitters have also made these devices applicable for the tracking of insect movements throughout remote landscapes (Kissling et al., 2014). Hence, this technique might in the future provide a viable method to investigate the behaviour of *M. sexta* in its natural habitat in North and South America.

Hawkmoths are a very diverse group of moths and their phylogeny is among the most intensively studied in all insects (Kitching and Cadiou, 2000). However, this wealth of information has so far been rarely utilised in studies on the evolution of the hawkmoth olfactory system (Bisch-Knaden et al., 2012), even though similar approaches using different species of *Drosophila* have already proven to be extremely fruitful (Ebrahim et al., 2015; Keesey et al., 2016; Stensmyr et al., 2012). In this context a comparison of the closely related *M. sexta*, *M. rustica* and *M. quinquemaculata* might give exciting insights, as these species are morphologically very similar and are potentially able to utilise the same nectar resources. Nevertheless, all three species can coexist in the same habitat, which might be due to a niche segregation based on olfactory preferences, similar to what has been suggested in the previous chapter for *M. sexta* and *H. lineata*.

On another level we have found that the moth proboscis is able to detect not only nectar sugars but also a great variety of volatile compounds (Haverkamp et al., 2016b), however, it remains unclear how this information is processed in the brain of the moth. Commonly it has been assumed that all olfactory information is processed in the antennal lobes of the insect brain, but recent work in the mosquito *Anopheles gambiae* suggests that glomerular structures, like those found in the antennal lobe, also exist in the suboesophagal ganglion, a part of the brain which normally receives mainly gustatory input (Riabinina et al., 2016). Hence, it will be interesting to see whether the olfactory neurons in the proboscis of *M. sexta* are processed in a similar way and how these neurons are then integrated with information from the sugar and bitter sensitive gustatory neurons, as well as with input from hydro- and CO_2 detecting receptors and how all these information then lead to a specific foraging decision in the hawkmoth.

Foraging efficiency is one of the main objectives for the sensory system of an animal. Ecological theories have identified three major steps, which influence the foraging efficiency of an animal within a certain habitat: search time, handling or capture time and “give up time” (Charnov, 1976). In this thesis we identified certain factors, which influence all three of these steps, but our picture still remains more than incomplete. Tracking foraging flights of hawkmoth in their natural environment and increasing our understanding how flowers are evaluated by the nervous system of the hawkmoth might help us to generate a more complete picture of the foraging behaviour of the moth and thus also of the forces which have shaped the evolution of the nervous system not only in hawkmoths.

Summary

"Time is Honey"
Bernd Heinrich

While foraging, all animals need to balance their energetic cost and gains in order to accumulate resources efficiently. The sensory systems provide the information, which form the bases for these energy-economic decisions made during foraging and thus, linking the sensory input directly to the evolutionary fitness of the animal. In night-active hawkmoth species, this is particularly true for the olfactory system which detects the floral volatiles emitted by those plants usually visited by the moth. The aim of this dissertation was to examine the link between the olfactory system and the foraging decisions of the hawkmoth *M. sexta* and to thereby gain further insights into the ecological pressures which might have directed the evolution of the olfactory system in hawkmoths and their coevolution with the flowers they visit.

Darwin was among the first to predict that a hawkmoth foraging efficiently would be greater on flowers that match the length of its proboscis. We tested this hypothesis in *M. sexta* foraging on different *Nicotiana* species. For this a respiration chamber was developed which allowed the hawkmoth to freely forage on a certain flower while its CO_2 emissions, as a proxy for its energy usage, was measured in near real-time. The results obtained through this analysis supported Darwin's initial hypothesis, showing that the moths did indeed forage most optimally, from an energetic point of view, on the flowers matching their proboscis in length. Moreover, by tracking the moth flight in a wind tunnel and by correlating this flight data to an odour plume model it could be shown that the moth could already detect the most energetically profitable flower at their first encounter with the odour plume. The data presented in the chapter 1 therefore strongly suggest that the olfactory system of the *M. sexta* has indeed adapted to detect those flowers which provide the best energy balance.

But how did the moth then recognise the most suitable flower? In chapter 2 we tested the response of a *M. sexta* females during the search for an oviposition side. The moths did prefer the two tested host plants over a clean air control at different concentrations, but lost their preference when the composition of the two plant odour blends were altered by mixing them together to a single artificial bouquet. These findings suggest that the chemical composition of a certain odour blend is of higher importance for the moth than the strength of the olfactory signal, a conclusion which is also likely to apply to the recognition of a certain flower during foraging.

However, floral volatiles are most reliable in the close vicinity of the flower corolla, as in natural environments flower odours are rapidly intermixed with volatile compounds from the surrounding. Due to this, close range detectors might be of particular importance for hawkmoth engaged in costly hovering manoeuvres. In chapter 3 of this dissertation we compared the foraging behaviour of *M. sexta* on flowers of *N. attenuata* with the moth behaviour on flowers, in which the floral volatiles had been genetically silenced. We found that although the moths approached the two flower types equally, both in a wind tunnel assay, as well as in a semi-natural flight tent environment, they invested significantly less handling time into the scentless flowers. Following these results additional mechanisms for a close range volatile detection were hypothesised and we could demonstrate the presence of olfactory neurons on the tip of the moth proboscis by electrophysiological, morphological and molecular methods. Moreover, when the response of the proboscis alone towards the flower volatiles of *N. attenuata* was tested in a specially designed bio-assay, it was found that the detection of these volatiles by the proboscis greatly enhanced the hovering time of the moth in front of a scented flower. The combination of these results suggested that *M. sexta* does indeed use the olfactory receptors on the tip of its proboscis to assign hovering time and thus energy to a certain flower.

Hawkmoth and the flowers they pollinate are thought to have coevolved in many different traits simultaneously. Flowers for example, do not only match the proboscis of their main pollinator in length, they furthermore release the volatiles most attractive to the hawkmoth, precisely when the moths are most active. Moreover, flowers do not only time their volatile release to the activity of the moth, but also alter their orientation according to the foraging time of the moth. In the 4th and final chapter of this thesis we examined the influence of such altered flower orientation on the detectability of the flower by the moth and on the moth's foraging efficiency. Strikingly, we found that the moths were unable to forage on flowers with a downwards orientation. Nonetheless, the moth still allocated the same amount of hovering time to these flowers. Notably, the flower orientation did not alter the volatile distribution in the close vicinity of the corolla. This suggested that the moth did not use visual or mechansensory information, which would have both been altered by the flower angle, but instead allocate its hovering time mainly based on the olfactory input from its antenna and proboscis. Taken together these results suggest that the moth and flower have not only co-adapted with regards to the volatiles emitted by the plant and detected by the moth, but also with regards to the orientation of the flower and the flight abilities of the hawkmoth.

This last chapter highlights again one of the main schemes underlying this dissertation: the link between the olfactory system of a hawkmoth and the specific behavioural characteristics of that moth. The hovering flight of a hawkmoth is one of the most costly forms of movement in the animal kingdom, but it also provides the hawkmoths with a unique ability to rapidly visit specifically shaped flowers and thus, largely defines the foraging niche of this diverse group of moths. However, these high energetic costs have also created a strong selection pressure for the sensory system of these moths to gather and use information in an effective way, leading to some of the most remarkable adaptions found in flower visiting insects. Moreover, these energetic pressures which act on the sensory system might then also reinforce the co-adaption between the moths and the flowers they visit. The high foraging costs enhance the need for an olfactory system that is tuned to detecting the most profitable flowers, which in turn might drive an ever stronger relationship between a certain flower and its hawkmoth pollinator. Hence, by studying the foraging strategies of hawkmoth pollinators we might not only gain new insights in to the evolution of the sensory systems, but also on how the information gathered by these systems shape the interaction with other species in their environment and their ecological niches. Understanding the sensory and behavioural ecology of hawkmoth pollinators will then not only help to maintain the ecosystem services on which we so crucially depend, but will also enrich our everyday lives by the small wonders these animals are still to reveal.

Zusammenfassung

„Kurz, man muß die Natur auf der That zu ertappen suchen."
Christian Konrad Sprengler, „Das Entdeckte Geheimnis der Natur", 1793

Auf der Suche nach Nahrung müssen alle Tiere, ständig die Kosten und den Nutzen ihres Verhaltens gegeneinander abwägen, um so die Effizienz ihrer Entscheidungen zu gewährleisten. Die Basis für diese Entscheidungen bilden die Informationen die dem Tiere von seinen Sinnesorganen zur Verfügung gestellt werden. Auf diese Weise wirken sich die Eigenschaften eines Sinnesorgans auch direkt auf die biologische Fitness des Tieres aus. Bei nachaktiven Schwärmern, kommt hierbei dem olfaktorischen System eine besondere Bedeutung zu, da dieses die Duftstoffe der Blüten, die den Schwärmern als Nektarquelle dienen, detektiert und der Motte so ein rasches Auffinden der Blüten ermöglichen. Ziel dieser Dissertation war es, diesen Zusammenhang zwischen dem olfaktorischen System des Tabakschwärmers *M. sexta* und der energieeffizienten Entscheidungsfindung während der Nektarsuche näher zu untersuchen und so ein genaueres Bild der ökologischen Kräfte zu gewinnen, die die Evolution des olfaktorischen Systems und damit auch die Anpassung zwischen Blüten und Bestäubern beeinflussen.

Darwin war einer der Ersten, der vermutete, dass die Nektaraufnahme eines jeden Schwärmers immer bei genau der Blüte am effektivsten sei, bei der die Blütenlänge der Länge des Schwärmerrüssels entspricht. Im ersten Kapitel dieser Arbeit wurde diese Hypothese an Hand der Interaktion zwischen *M. sexta* und sieben *Nikotiana*- Arten mit Blüten unterschiedlicher Länge überprüft. Zu diesem Zweck wurde eine Respirationskammer entwickelt, die es dem Schwärmer erlaubte an jeder Blüte freischwebend nach Nektar zu suchen, während gleichzeitig sein CO_2 Ausstoß, als Maß des Energieverbrauchs, mit hoher zeitlicher Auflösung gemessen werden konnte. Diese Messungen unterstützten Darwin's Ausgangshypothese und zeigten, dass die Schwärmer tatsächlich den größten Netto-Energiegewinn an Blüten erzielten, bei denen ihre Proboscislänge mit der Blütenlänge übereinstimmt. Parallel zu diesen Tests wurde das Flugverhalten der Schwärmer in einem Windkanal aufgezeichnet. Eine anschließende Korrelation der rekonstruierten Flugpfade mit einem eigens erstellten Model der räumlichen Duftverteilung zeigte dann, dass die Blüte, bei der letztendlich der größte Energiegewinn erzielt wurde, bereits beim ersten Zusammentreffen der Motte mit dem Blütenduft ein signifikantes Orientierungsverhalten auslöste. Diese Ergebnisse legen es nahe, dass sich das olfaktorische System des Tabakschwärmers tatsächlich im Laufe seiner Evolution speziell an die Wahrnehmung effizienter Nektarquellen angepasst hat.

Wie aber erkennt der Schwärmer den Duft der passenden Blüte und wie unterscheidet er diesen von Düften anderer Blüten? Bei Versuchen mit weiblichen Tabakschwärmern, die eine geeignete Pflanze für die Eiablage im Windkanal auswählen sollten, zeigte sich, dass diese nicht zwischen dem Duft von zwei gleichen Pflanzen in unterschiedlicher Konzentration unterschieden. Wurden hingegen die Düfte von zwei attraktiven Pflanzen, die jedoch unterschiedlichen Arten angehörten, vermischt, verlor die Mischung schlagartig ihre Attraktivität und wirkte sogar tendenziell abstoßend. Diese Ergebnisse lassen den Schluss zu, dass die Schwärmer das Duftbouquet einer attraktiven Pflanze hauptsächlich an der spezifischen Zusammensetzung der enthaltenden Duftstoffe erkennen und weniger an der Konzentration der gesamten Duftmischung. Diese Mechanismen, die im zweiten Kapitel für die Auswahl grüner Pflanzen während der Eiablage gezeigt wurden, lassen sich so vermutlich auch auf das Erkennen geeigneter Blüten anwenden.

In der natürlichen Umgebung der Schwärmer vermischt sich das Duftbouquet einer Blüte jedoch schnell mit dem Duft andere Blüten, oder grüner Pflanzenteile. Aus diesem Grund sollten die Schwärmer auch kurz vor der Blüte noch in der Lage sein den Duft der Pflanze wahrzunehmen, um so energetisch kostspielige Flugmanöver vor unprofitablen Blüten zu vermeiden. Im dritten Kapitel dieser Dissertation wurde das Verhalten von *M. sexta* beim Besuch der Blüten des wilden Tabaks *N. attenuata*, mit dem Verhalten der Motten beim Besuch von Blüten derselben Tabakart verglichen, beim denen die Emission des wichtigste Blütenduftstoffs genetisch ausgeschaltet wurde. Erstaunlicherweise besuchten Schwärmer, sowohl bei Tests im Windtunnel, als auch unter semi- natürlichen Bedingungen in einem großen Flugzelt, gleich viele Blüten von beiden Pflanzentypen. Im Gegensatz dazu investierten die Motten jedoch signifikant mehr Zeit in die Handhabung der duftenden Pflanzen sobald sie diese erreicht hatten. Aufgrund dieser Ergebnisse begannen wir eine nähere Untersuchung der Mechanismen mit denen *M. sexta* Blütendüfte auf kurze Distanzen wahrnimmt. Wir konnten schließlich durch eine Kombination von molekularen, morphologischen und elektrophysiologischen Methoden die Existenz von bisher unbekannten olfaktorischen Neuronen an der Spitze der Proboscis nachweisen. Zusätzlich konnte bei Verhaltenstests, in denen nur die Proboscis der Motte dem Blütenduft ausgesetzt war, gezeigt werden, dass die Anregung dieser olfaktorischen Neurone eine längere Verweilzeit der Schwärmer an der Duftquelle hervorrief. Zusammengenommen legen diese Ergebnisse nahe, dass *M. sexta* die olfaktorischen Neurone an der Spitze der Probosics benutzt, um eine Blüte auf kurze Entfernung zu bewerten, um dann entsprechend eine gewisse Flugzeit und somit Energie in diese Blüte zu investieren.

Schwärmer und die Blüten, die von ihnen bestäubt werden, haben sich parallel in vielerlei Eigenschaften aneinander angepasst. So entsprechen sich Blüten und Bestäuber beispielsweise nicht nur in der Länge von Proboscis und Korollastiel. Zusätzlich, geben die Blüten ihren Duft auch zu genau der Zeit in der Nacht ab, wenn die Motten ihre größte Aktivitätsphase haben. Die Blüten richten sich jedoch nicht nur bezüglich ihrer Duftemission nach dem Verhalten der Schwärmer, sondern auch in Bezug auf die Ausrichtung ihrer Blüten. In dem vierten Kapitel dieser Arbeit wurden die Auswirkungen dieser Blütenorientierung auf die Wahrnehmung der Blüte durch die Schwärmer und auf die Nektarsammeleffizienz der Motten getestet. Interessanterweise wurde festgestellt, dass die Motten nicht in der Lage waren Nektar, aus abwärts gerichteten Blüten zu saugen. Nichtsdestotrotz investierten die Schwärmer dieselbe Flugzeit in diese abwärts gerichteten Blüten. Bemerkenswert war dabei aber auch, dass sich die Orientierung der Blüte nicht auf die Verteilung des Blütenduftes in der direkten Umgebung der Blüte ausgewirkte. Diese Ergebnisse zeigen, dass sich die Motten bei der Allokation der Flugzeit, weniger an den visuellen und mechanischen Sinneseindrücken, die sich beide durch die Blütenorientierung veränderten, orientierten, sondern hauptsächlich ihr olfaktorisches System benutzten. Zusammenfassend lässt sich daher folgern, dass sich die Schwärmer und die Blüte nicht nur bei den Blütenduftstoffen und deren Wahrnehmung aneinander angepasst haben, sondern, dass sich auch die Orientierung der Blüte an die spezifischen Flugfähigkeiten der Motte angepasst hat.

Mit dieser Aussage gibt das zuletzt genannte Kapitel eines der wesentliche Themen dieser Doktorarbeit wieder: Die Verbindung zwischen der olfaktorischen Sinneswahrnehmung der Schwärmer und deren spezifischen Verhaltenseigenschaften. Der Schwebeflug der Schwärmer ist eine der energieaufwändigsten Fortbewegungsarten im Tierreich. Gleichzeitig bietet diese Fortbewegung aber auch die Möglichkeit in kurzer Zeit viele Blüten zu besuchen und definiert somit zu einem großen Teil die ökologische Nische der Schwärmer während der Nektarsuche. Der große Energieaufwand während der Nahrungssuche erzeugt jedoch auch einen hohen Druck auf das olfaktorische System der Motte, Information auf eine effektive Weise zu sammeln und zu nutzen. Zugleich hat dieser hohe Energieaufwand, aber auch die Anpassungen zwischen Motten und Blüten bestärkt, da diese energetischen Kosten die Schwärmer dazu zwingen, besonders sensitiv auf die spezifischen Blütendüfte der optimalen Nektarpflanzen zu reagieren, was dann wiederum die besondere Anpassungen zwischen Blüten und Schwärmern bestärkt.

Aus diesem Grund können wir aus der Betrachtung des Nektarsuchverhaltens der Schwärmer nicht nur neue Einsichten über die Evolution der Sinnesorgane gewinnen, sondern auch über die Interaktionen verschiedener Arten in einem Ökosystem. Ein tiefergehendes Verständnis der Sinnes- und Verhaltensökolgie blütenbesuchender Schwärmer, wird nicht nur dazu beitragen wichtige Ökosystemleistungen wie die Blütenbestäubung zu verstehen und zu erhalten, sondern kann auch dazu verhelfen unser alltägliches Leben durch die kleinen Wunder die diese Tiere in sich tragen, zu bereichern.

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Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller-University Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate theses.

The thesis has not been previously submitted whether to the Friedrich-Schiller-University, Jena or to any other university.

Jena, November, 22nd …………………………………

Alexander Haverkamp

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“Ugly or beautiful, it is the little creatures that make the world go round.

We should celebrate and appreciate them in all their wonderful diversity.”

Dave Goulson

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Curriculum Vitae

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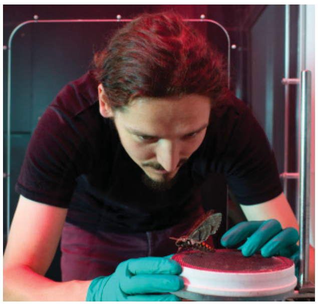

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Dec. 2008	Bachelor of Science in Forest Science at the Technical University of Munich, Germany, with merit
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Skills and Interests

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Student supervision

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Jan. - Jun. 2016	Tamara Springler, Bachelor thesis at Friedrich-Schiller-University: "*Die Nase auf der Zunge: Der Tabakschwärmer* Manduca sexta *erkennt Blütendüfte mit dem Proboscis*"
Jun. - Jul. 2015	Maria Demmig, student intership at the Berufsbildendes Schulzentrum Jena-Göschwitz: „*Die olfaktorische Wahrnehmung von Manduca sexta-Larven. Eine neuroethologische Versuchsreihe.*"
Jan. - Aug. 2015	Julia Bing, Magister thesis at Friedrich-Schiller-University, Jena: *"The better the fit, the bigger the profit? - Quantifying the interaction between the pollinator* Manduca sexta *and* Nicotiana*-flowers"*

Public outreach and commission work

Mar. 2016	Regional Television interview on *Manduca* research
Jul. 2015	Student lecture, together with Shimaa Ebrahim "Der Riechsinn der Insekten: Von der Fliege bis zur Motte und vom Rüssel bis zur Antenne"
Jan. 2015	Student lecture, togehter with Elisa Badeke: "Nachtflug der "Nase" nach: Wie Motten und Schwärmer mit Hilfe von Düften ihr Ziel finden."
Nov. 2013	5th Long Night of the Sciences "Nachtschwärmer im Windkanal"
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Publications

2016 Keesey, I., Koerte, S., Retzke, T., **Haverkamp, A.**, Hansson, B. S., Knaden, M. (2016). Adult frass provides a pheromone signature for Drosophila feeding and aggregation. Journal of Chemical Ecology, 42(8), 739-747. doi:10.1007/s10886-016-0737-4

Haverkamp, A., Yon, F., Keesey, I., Mißbach, C., König, C., Hansson, B. S., Baldwin, I. T., Knaden, M., Kessler, D. (2016). Hawkmoths evaluate scenting flowers with the tip of their proboscis. eLife, 5: e15039. doi:10.7554/eLife.15039.

Haverkamp, A., Bing, J., Badeke, E., Hansson, B. S., Knaden, M. (2016). Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. Nature Communications, 7: 11644. doi:10.1038/ncomms11644

Badeke, E., **Haverkamp, A.**, Hansson, B. S., Sachse, S. (2016). A challenge for a male noctuid moth? Discerning the female sex pheromone against the background of plant volatiles. Frontiers in Physiology, 7: 143. doi:10.3389/fphys.2016.00143.

2014 **Haverkamp, A.**, Smid, H. M. (2014). Octopamine-like immunoreactive neurons in the brain and subesophageal ganglion of the parasitic wasps Nasonia vitripennis and N. giraulti. Cell and Tissue Research, 358(2), 313-329. doi:10.1007/s00441-014-1960-3

2013 Späthe, A., Reinecke, A., **Haverkamp, A.**, Hansson, B. S., Knaden, M. (2013). Host plant odors represent immiscible information entities - blend composition and concentration matter in hawkmoths. PLoS One, 8(10): e77135. doi:10.1371/journal.pone.0077135.

Oral presentation

Sep. 2015 **Haverkamp A.**, Bing.J, Hansson B.S., Knaden M.: Optimal foraging by smell. Talk presented at 14th European Symposium for Insect Taste and Olfaction ESITO, Villasimius, IT

Apr. 2014 **Haverkamp A.**, The Ups and Downs of flower perception: The influence of flower position and volatile emissions on the foraging behavior of Manduca sexta. Talk presented at 13th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE

Jun. 2013 **Haverkamp A.**, A bouquet for a hawkmoth: The influence of flower odor on the foraging behavior of Manduca sexta. Talk presented at ICE Symposium, MPI for Chemical Ecology, Jena, DE

2012 **Haverkamp A.**, Bisch-Knaden S. and Hansson B.S.: Be quick or be hungry: Rapid detection of natural stimuli by the hawkmoth *Manduca.* PhD Symposium Rothamsted

Poster presentation

May 2016	**Haverkamp A**., Yon F., Keesey I., Mißbach C., König C., Hansson B.S., Baldwin I.T., Knaden M., Kessler D. Hawkmoths evaluate scenting flowers with the tip of their proboscis. Poster presented at Linnaeus Conference - IC-E3 Final Symposium, Alnarp, SE
2015	Yon F., **Haverkamp A.**, Kessler D., Knaden M., Hansson B.S., Baldwin I.T.: Floral scents to the touch: Scents inform moth's proboscis for probing and foraging, thus determining the plant fitness. Poster presented at 31st ISCE Meeting, International Society of Chemical Ecology, Stockholm, SE
2015	**Haverkamp A**., Bing J., Hansson B.S., Knaden M.: Smelling a low-cost meal: Hawkmoth use olfactory information to optimize their foraging behavior. Poster presented at 14th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, DE
2014	**Haverkamp A.**, Bing J., Yon F., Badeke E., Knaden M., Hansson B.S.: Odour-guided foraging behaviour of the hawkmoth Manduca sexta. Poster presented at SAB Meeting 2014, MPI for Chemical Ecology, Jena, DE
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2012	**Haverkamp A.** : How to search in a turbulent environment? Suggestions from a hawkmoth flight. Poster presented at Frontiers of Chemical Ecology, Jena, DE
Jun. 2012	**Haverkamp A.,** Bisch-Knaden S., Knaden M., Hansson B.S.: Tracing the neuronal correlate of olfactory flower perception in *Manduca sexta*. Poster presented at XVI International Symposium on Olfaction and Taste (ISOT), Stockholm, SE

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Jan. 2012 - Dec. 2014	Stipend of the International Max Planck Research School (IMPRS)

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