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Thema:

**The better the fit, the bigger the profit? -
Quantifying the interaction between the pollinator *Manduca
sexta* and *Nicotiana*-flowers**

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III. List of Abbreviations

DCM	dichloromethane
GC-EAD	gas chromatography coupled with electroantennographic detection
GC-MS	gas chromatography coupled with mass spectrometry
MPI	Max Planck Institute
rpm	revolutions per minute
RT	retention time

1. Abstract

Considering the optimal foraging theory by that foraging strategies include decisions to maximize the energy intake while keeping down the expenditures cost-benefit analyses are extremely useful to estimate the degree of mutual dependence. The aim of this study was to give an energetic analysis of the pollinator-plant interaction between males of *Manduca sexta* and different *Nicotiana*-species with emphasis on the pollinator view. For this purpose the energy expenditure of the moth was measured by the use of respirometry for each plant species to have a look if there are differences and if these differences are correlated with morphological, such as the match of the moth's proboscis with the corolla tube, and as well with olfactory aspects, like the presence of specific volatiles in the floral headspace that can be detected by the moth's antenna. The results show that there is no significant difference for the costs when foraging on different species of *Nicotiana*. However, the costs seem to be influenced by the individual match of proboscis and the floral tube. The costs were balanced again the energy content of the nectar provided by the flowers and the energy net gain was calculated and compared among the different species. It was hypothesized that *N.alata* is the most profitable flower for *Manduca sexta*, because only flowers of this species show no significant difference between corolla and proboscis length. Surprisingly *Manduca* moths display the better energy balance with *N.pauciflora* that might be a potential hummingbird-pollinated flower. But not only the energy net gain has to be taken into account, also the probability of flower visitation by itself as well as the success rate play an important role for the estimation of the degree of mutualistic interaction of pollinator and plant. In summary, it can be stated that the costs for foraging correlates with the difference between the moth's proboscis and the corolla length. Based on electrophysiological measurements and the fact that the flowers of this species were visited by all responding moths it can be assumed that the 'good match' with *N.alata* might be signaled by a range of hawkmoth-attracting volatile compounds as linalool and geraniol. Also for the floral headspace of *N.bigelovii* two compounds (farnesol and linalool) that evoke antennal responses have been detected, which leads to the assumption that this flower species is also pollinated by hawkmoths. At the end it has to be said, that a better match seems to be correlated with lower energy expenditures, but in order to the small sample size further investigations were needed to confirm the results of this study.

2. Introduction

2.1 Interaction between pollinators and plants

2.1.1 Cross-fertilization – the benefit of the plant

For a long time researchers deal with the question how the interaction between pollinators and plants work. Contrary to popular belief at that time that seed-setting is a result of self-fertilization, C.K. Sprengel demonstrated in his book *Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen (The Secret of Nature revealed in the Structure and Fertilization in Flowers, 1793)* that the most angiosperm flowers usually require pollinating animals in order to set seed (Schoonhoven *et al.* 2005). More than 60 years later Charles Darwin revisited this topic and on the basis of Sprengels observations he brought up the question about the biological meaning of cross-fertilization and ascertained that this is critical for an increase in variation (Darwin 1859, Schoonhoven *et al.* 2005). Furthermore he underlined with additional experiments that pollinators are of crucial importance for plants.

From experiments which I have tried, I have found that the visits of bees, if not indispensable, are at least highly beneficial to the fertilisation of our clovers; but humble-bees alone visit the common red clover (*Trifolium pratense*), as other bees cannot reach the nectar. Hence I have very little doubt, that if the whole genus of humble-bees became extinct or very rare in England, the heartsease and red clover would become very rare, or wholly disappear. (Darwin, 1859)

2.1.2 Rewards for the pollinator

About two-third of all flowering plants, and consequently the majority of our food-sources are pollinated by insects (Schoonhoven *et al.* 2005, Abrol 2012). It cannot be supposed, that the transfer of pollen is given for free. So, the question arises what the plant offers in exchange for this service (Schoonhoven *et al.* 2005, Abrol 2012). As a quid pro quo the pollinator obtain profitable rewards from the flower.

The most common rewards are nutrients (Abrol 2012). Pollen by itself is a highly nutritious and well-balanced food source for some insects. It contains not only protein, but also a sizeable amount of other essential substances such as starch, sugars, fat or oil, minerals, antioxidants, vitamins and amino acids (Abrol 2012). Another common reward provided by the plant is nectar, which might be even more important than pollen (Barth 1985). Nectar

consists mainly of sugars, but also contains other essential elements such as proteins, organic acids, vitamins, enzymes and amino acids (Barth 1985, Schoonhoven *et al.* 2005). The sugar concentration can vary extremely among different species. In case of *Fritillaria imperialis* the nectar contains only 8% sugars while *Organium vulgare* shows sugar concentrations up to nearly 80% (Barth 1985). Other rewards can be resins and waxes produced by the plant, that can be used as nest-building materials (Abrol 2012). Also heat which is produced by flowers may act as rewards (Stanton & Galen 1989).

Since both parties benefit from this association and can barely or not survive without each other, this interaction is a classic example of mutualism (Schoonhoven *et al.* 2005). The degree of this interdependence can vary enormous between two extremes. The one extreme is an interaction of pollinators and plants were both were highly specialized and depend completely on each other. An example for this degree of interdependence is the relationship between figs and fig wasps, whereby each fig species has to be pollinated by its own species of wasps (Cook & Rasplus 2003, Schoonhoven *et al.* 2005). The other extreme is when only one of both partners benefits from this interaction, as is the case with flowers that cheat on the pollinator. For example some orchids allure pollinators by emitting odors that mimic the insect's female sex pheromone but offer no reward for flower visitation (Schoonhoven *et al.* 2005)

2.2 Requirements for making the interaction work

According to Schoonhoven *et al.* (2005) the interaction between flowers and insects is an example *par excellence* of mutualism because both partners benefit from this association. It has been intensively discussed which conditions were required to make pollinator-plant interaction work, so that it is profitable for both the pollinator and the plant.

Heinrich (1981) assumed three major requirements: advertisement, morphological fit and the movement of the pollinator between conspecific flowers.

2.2.1 Advertisement – visual and olfactory cues

At first an attraction is needed to ensure that the potential pollinator will visit this particular flower. Diverse signals can be used for this advertisement. Not only visual stimuli such as shape and color, but also an alluring odor bouquet can guide the pollinator to its mutualistic partner (Schoonhoven *et al.* 2005). It is suggested that a combination of both visual and olfactory cues govern the foraging behavior of the pollinating animals (Raguso & Willis 2002, 2005, Goyret *et al.* 2007, Balkenius *et al.* 2009, Balkenius & Dacke 2010). An example for this interplay of different sensual cues is given by Raguso & Willis (2002) who found out that floral scent attract the hawkmoth *Manduca sexta* at a long distance while visual cues stimulate to feed at a short range. Furthermore leaf volatiles seem to play a role in the attraction of pollinators because Kárpáti *et al.* (2013) assumed that a species-specific coaction of flower and leaf blends enables the moth to analyze the food site quality in a more detailed way.

Since visual stimuli are mostly ineffective to attract pollinators in the dark it is obvious that floral scents play a major role as signals (Knudsen & Tollsten 1993, Miyake *et al.* 1998, Jürgens *et al.* 2002, Raguso *et al.* 2003). This assumption can be made by the fact that the fragrance of plants pollinated by nocturnal animals contains specific odors during the night, whereas volatiles emitted at the daytime were less distinct (Raguso *et al.* 2003). A wide range of plant species flower only during one part of the day, thus they rely on pollinators which are active at this time. In dependence on the time at which they blossom the visitors of the flowers were either nocturnal or diurnal animals. Some plants however were visited by both (Miyake & Yahara 1998, Young 2002, Jürgens *et al.* 2003, Morinaga *et al.* 2008).

Raguso (2003) investigated the fragrances of nine different *Nicotiana*-species and ascertained that the nocturnal odor composition varied markedly between the flower species in contrast to the almost unnoticeable differences of the diurnal volatile bouquets, which leads to the assumption that the plant species were pollinated by different animals. Hawkmoth-pollinated flowers were not only characterized by a bright white visual display, long and narrow corolla tubes and abundant sucrose-rich nectar but also by a strong fragrance (Raguso *et al.* 2003, Abrol 2012). It is expected that there are specific volatiles that attract exclusively hawkmoths. Typical hawkmoth-attractants are benzenoid esters,

nitrogenous compounds and terpenoid alcohols (Knudsen & Tollsten 1993, Raguso *et al.* 2003, Andersson 2006).

Additionally to visual and olfactory advertisement there might be some more cues that play a role in the attraction of pollinators. Thom *et al.* (2004) assumed that the high level of CO₂ emitted by flowers of *Datura wrightii* could indicate food-source profitability.

2.2.2 Morphological fit

A second prerequisite which is needed for a successful pollinator-plant interaction is that the flower morphology must be appropriate for the pollinator (Heinrich 1981) not only to obtain the reward (success of the moth) but also to become dusted with pollen that potential can be transferred to a conspecific flower (success of the plant). So the match of proboscis and corolla tube especially plays an important role for pollination efficiency in the interaction of pollinators with those flowers that produce the nectar at the base of the tube (Alexandersson & Johnson 2002, Whittall & Hodges 2007, Boberg & Ågren 2009). In the case of competition for food resources long-tongued pollinators prefer flowers with deep corollas and short-tongued pollinator preferentially forage on shallow flowers (Rodríguez-Gironés & Santamaría 2007). Where it is the aim of the pollinator to reach the nectar, the plant aspires that the pollinator takes up pollen while feeding on the flower. Hence, the flower morphology effects the close-in behavior of the pollinator with a view to increase the percentage of cross-pollination events per given food reward provided (Macior 1974).

As yet, studies considering the effect of other morphological aspects on the pollination efficiency are rarely. Boberg & Ågren (2009) examine the influence of the perianth size on the pollination success of *Platanthera bifolia*, but not if the size of the perianth has an influence on the handling time.

2.2.3 Movement of the pollinator

Beside an effective advertisement and a morphological fit it is essential for the plants success that the pollinators move between different flowers of the same species so that the pollen can be transferred to the pistil of another flower or even better of another plant to

elevate the possibility of outcrossing (Heinrich & Raven 1972). As it was said above (2.1) the plant offers nutrients that were necessary for the pollinator. The amount of the nectar produced by the plant is a critical point in the interaction between plants and pollinators because if the flower offers too much nectar, there is no need for the pollinator to forage further and consequently there is no transfer of the pollen. On the contrary the reward has to be sufficient to attract the pollinator (Klinkhamer & Jong 1993), because there is a competition for pollinators between flowers of different plant species. So, the pollinators prefer those flowers that provide the most reward (Heinrich 1981, Schoonhoven *et al.* 2005). That means, not only the quality also the quantity of the reward determines the pollinator-plant interaction.

2.3 Flower constancy

A wide range of studies are dealing with questions concerning the energetic aspects of pollination from the perspective of the animal's (May 1988, Wolf *et al.* 1999, O'Brien 1999, Sprayberry & Daniel 2007, Ayala-Berdon *et al.* 2011, Moll *et al.* 2012, Abrol 2012) as well as from the plant's angle (Harder & Barrett 1992, Abrol 1992). The costs for the plant involve not only the production of the rewards such as pollen and nectar but also the sensory cues like scent or color of the petals (Leonhard *et al.* 2011). The reason why plants accept these expenditures is the consequent benefit of outcrossing (Heinrich 1981, Majetic *et al.* 2009, Leonhard *et al.* 2011). In order that the pollen get to the stamina of a conspecific flower with the result of fertilization, it is advantageous for the plant to be visited by 'plant-species-constant pollinators' (Schoonhoven *et al.* 2005). The tendency to visit only one or a few plant species while foraging is also referred as *flower constancy* (Schoonhoven *et al.* 2005). This phenomenon causes not only advantages to the plant. Even for the pollinator it can be profitable to forage only on a very few flower species because of its limited ability to learn and remember how to treat different blossom types (Waser 1986, Chittka *et al.* 1999). The conditioning to different floral features as a conspicuous coloration of the petals or a specific odorant seems to be the basis for flower constancy (Schoonhoven *et al.* 2005). Besides sensory cues there are different floral features that affect constancy such as nutrient rewards, the distance between individuals of the same

plant species and morphological aspects of the flower. Since the pollinators avoid other rewarding flowers in consequence of constancy, the benefit provided by the plant has to be so lucrative that it cannot be resisted. This profitability can be achieved by a less costly flower handling or a more profitable reward.

2.4 Energetics of foraging

According to the optimization theory both parties of the pollinator-plant interaction try to maximize their chance to survive as well as the reproductive success (Schoonhoven *et al.* 2005). That means that pollinators and plants have to balance their costs and benefits for each activity. Since this study focuses on the perspective of the pollinator *Manduca sexta* this chapter will address to the energetics of the moth during foraging.

2.4.1 Energetic expenditures

Since flying is one of the most demanding locomotion types (Reinhold 1999), foraging on flowers that offer none or only little rewards will lead to a negative energy balance of the pollinator. For that reason distant food sources were only visited if the reward is profitable enough (Schoonhoven *et al.* 2005). Larger insects often have to hover in front of the flower while feeding on it. The hawkmoth *Manduca sexta* for example has to feed on the wing due to the fact that it is too large to land on the flowers (Casey 1976). The costs for hovering are extremely as seen in the fact that the metabolic rates of hovering hawkmoths are up to 100 times higher than their standard metabolic rates (Casey 1976). Furthermore *Manduca* moths fly at high speed and cover long distances during their flower visits. The time needed to reach the nutrient reward is affected by the flower morphology where complex flowers requires more complicated handling methods from their pollinators as shallow, open-cup ones (Schoonhoven 2005). So, not only distance between the flowers but also morphological aspects have an influence on the energy balance. All this comes along with high energy expenditures that have to be compensated with relatively high energy intake. For the application of cost-benefit analyses it is necessary to consider also factors that have an influence on the metabolic rate such as temperature (Chown & Nicolson 2004, Heinrich 2004), water ability and size (Chown & Nicolson 2004).

To measure the energetic costs different methods can be used. Probably the most current method is respirometry which measures the rates of the respiratory gas exchange (Halsey 2011). Based on the rate of consumed O₂ or liberated CO₂ and the respiratory quotient (RQ) the energy expenditures can be calculated. Besides respirometry, further methods can be applied to estimate the costs of foraging, e.g. direct measurement of food consumption, use of isotope-labeled water and measurements of the body temperature (Abrol 2012).

2.4.2 Energy intake

To compensate the enormous expenditures the hawkmoth can either feed on only a few blooms that offer huge amounts of nectar, or it has to visit a plurality of flowers with less nectar during foraging. The energy intake of the moth is determined by the energy provided by the flower and as well from the opportunity to reach the offered reward. It is assumed that flowers that are difficult to track not only increase handling time but also lead to lower energy intake (Sprayberry & Daniel 2007). To make up a cost-benefit balance the energy intake has to be known. Nectar of different flowers does not only differ concerning sugar concentrations but also the ratio of the main sugars glucose, fructose and sucrose can vary (Abrol 2012). Pollinators with high requirements such as hawkmoths prefer sucrose rich flowers (Raguso *et al.* 2003, Abrol 2012). If the nectar composition is known the caloric content can be estimated, whereby it is assumed that 1 mg of sugar yield about 4.0 cal (Abrol 2012).

2.4.3 Net energy gain

After researching what the pollinator gives and what it gets during the interaction with the flower, the net energy gain can be estimated. It is suggested that nectarivorous animals visit especially flowers where the energy yield of the reward offered by the plant meets the energy expenditures of the pollinator (Ayala-Berdon *et al.* 2011). So due to the fact that the scent emitted by moth-pollinated flowers contains specific compounds it might be suggested that the pollinator *Manduca sexta* is capable to identify a priori these flowers where the energy yield exceeds the energy expenditures that must be raised while foraging on these. Or in other words, the moth smells where it gets the most for its effort.

2.5 Interaction of *Manduca sexta* and several *Nicotiana*-flowers – a win-win situation?

This study addresses the interaction of *Manduca sexta* and different *Nicotiana*-species whereby the pollinator's perspective will be focused. To investigate if the interaction with flowers of several *Nicotiana*-species is profitable for the moth, the energy costs of flower-handling of a single flower visit and the energy provided by this flower has to be taken into account. Based on both the costs and the profit the net energy gain can be computed. To estimate on which flowers *Manduca* feeds in the most profitable way the calculated net energy gain has to be compared among the different species.

Based on the observation of May (1988) that the energy contents of flowers were positively correlated with the length of the floral tube, it can be presumed that flowers of the five different *Nicotiana*-species will offer different amounts of nectar. Further it might be that longer corollas increase handling-time (May 1988). Due to differences of the morphological fit and the amount of nectar provided by the plant, it is assumed that some of the interactions are more profitable for the moth than others. It is suggested that the match of corolla length and proboscis length were correlated with the net energy gain. Since *N.alata* seems to be the best candidate for a perfect fit of proboscis and floral tube length, it is assumed that *Manduca* will show the best energy balance when foraging on flowers of *N.alata*.

As it is the aim of the study to investigate the strength of the mutualistic relationship, which is defined as an association from which both partners benefit (Schoonhoven *et al.* 2005), the success of the plant must also be considered. So one question is, if the moth's visit of flowers of different *Nicotiana*-species leads to a win-win situation. In this study the success of the plant will be measured by quantifying the amount of pollen that is taken up by the moth during probing and feeding on the flower.

Based on these considerations the following hypothesis will be examined:

The strongest mutualistic relationship of *Manduca sexta* is with those *Nicotiana*-flowers where the net energy gain is the highest.

- a) The energy balance of the pollinator *Manduca sexta* is the highest with those *Nicotiana*-flowers where the where the hawkmoth proboscis and the corolla tube length match.
- b) The strength of the interdependence between *Manduca sexta* and *Nicotiana*-flowers is associated with the presence of specific volatiles in the nectar.

To investigate the first part of the hypothesis the following steps will be realized:

1. Comparing the morphology of moth and flower (analysis of morphological aspects of the flower like shape, diameter and length of the flower tube; analysis of the proboscis length)
2. Measuring the energy effort while the moth is foraging on the different *Nicotiana*-flowers by the use of respirometry (energy expenditures)
3. Quantifying the energy providing by the plant by analyzing the nectar of the different flower-species (energy provided by the plant)
4. Balancing the energy expenditures against the energy yield to examine the success of the moth (net energy gain)
5. Measuring the pollen that was taken up by the moth during feeding (pollen load)

To analyze if the strength of the mutualistic interrelationship correlates with the presence of specific volatiles of the floral headspace the following experiments will be performed:

1. Sampling of the floral headspace of different *Nicotiana*-species (Flower headspace analysis)
2. Identification of volatile compounds in the floral headspace of the different species that evoke antennal responses (electroantennography)

3. Methods and Materials

3.1 Study animals and plants

3.1.1 Animal rearing

Moths were taken from a culture maintained at the Max Planck Institute (MPI) for Chemical Ecology and reared as described by Grosse-Wilde *et al.* (2011). Adult moths were put into a flight cage containing a *Nicotiana attenuata* plant for oviposition as well as artificial flowers providing a sugar solution from which moths could feed. Eggs were collected and transferred in small boxes to a climate-controlled chamber with a temperature of 27°C and humidity of 70%. After hatching the larvae were housed in new boxes with diet lying on metal lattices until the fifth instar level. For pupation the caterpillars were placed into wood blocks until one week before eclosure. Female and male pupae were sorted by sex and placed in climate chambers under a 16h light:8h dark photoperiod at 25°C and 60% relative humidity during day period and at 23°C and 70% relative humidity during night period. All experiments were carried out with starved naïve three days old, males during the second activity peak of the moth.

3.1.2 Plants

For my study plants from five different *Nicotiana*-species (*N. alata*, *N. pauciflora*, *N. bigelovii*, *N. repanda* and *N. obtusifolia*) were grown in the glasshouse of the MPI. After elongation plants were transferred to a climate-controlled chamber which was synchronize with respect to its photoperiod to the chamber where the moths were kept (60% relative humidity at day and 80% at night, temperature 25°C at day and 23°C at night, 16h light : 8h dark photoperiod). For habituation plants were kept in this chamber for at least one week before being used in the experiments. Plants were watered with 200ml of nutrient solution. All experiments were performed with newly opened flowers.

3.2 Energetic measurements

3.2.1 Respirometry

To measure the metabolic rate of *Manduca sexta* a flow-through respirometry system (Fig.1) was used. This method allows estimating the energy expenditure of an animal by measuring rates of respiratory gas exchange (Halsey 2011). In this study rates of the CO₂ were used to estimate the energy expenditures of the moth while foraging on flowers of different *Nicotiana*-species. The measurements were performed with a push-pull system (Fig.1). Purified compressed air was pumped through a closed glass cylinder (49 x 34cm) at a constant rate of 2 l min⁻¹ regulated by a flow meter. Subsequently, the air was sucked out and CO₂ concentration was measured using a CO₂-analyzer (Li-820, Licor GmbH, Bad Homburg, Germany). In addition, temperature and humidity were analyzed (T&F Sensor Sensorion SHT75). Changes of CO₂ levels, temperature and humidity were recorded at 5s intervals. The measurement was operated via LabVIEW (National Instruments).

Given that temperature and humidity influence the metabolic rate of *Manduca sexta*, the measurements were performed in a climate chamber under constant temperature and humidity level. Moths and plants were brought to the experimental chamber for acclimation at least one hour before measurements. The moths were kept separated in small cylindrical cages (15 x 14.5cm) and brought to a sealed chamber (66 x 81 x 61cm) to ensure they would not perceive floral odors before the experiments. To avoid contamination only one *Nicotiana*-species was tested per day.

For the measurements, resting moths were placed into a glass chamber, in which animals could move freely. The chamber was illuminated with 20 % infrared light and 8% white light. The behavior of the moth was monitored by two video cameras (Logitech C615). One of the cameras was placed above the experimental chamber, which allowed analyzing the over all behavior of the moth within the chamber, while the second camera was placed on the side, which allowed analyzing the handling of the blossom by the moths. Moths were allowed to fly for 4 more minutes within the chamber. Animals that did not started to flight within 5 min after being placed in the chamber were discarded from the experiment. If the moth was feeding on the flower the experiment was stopped immediately after the proboscis left the floral tube. After the recordings, moths were weighted and the length of the proboscis was measured.

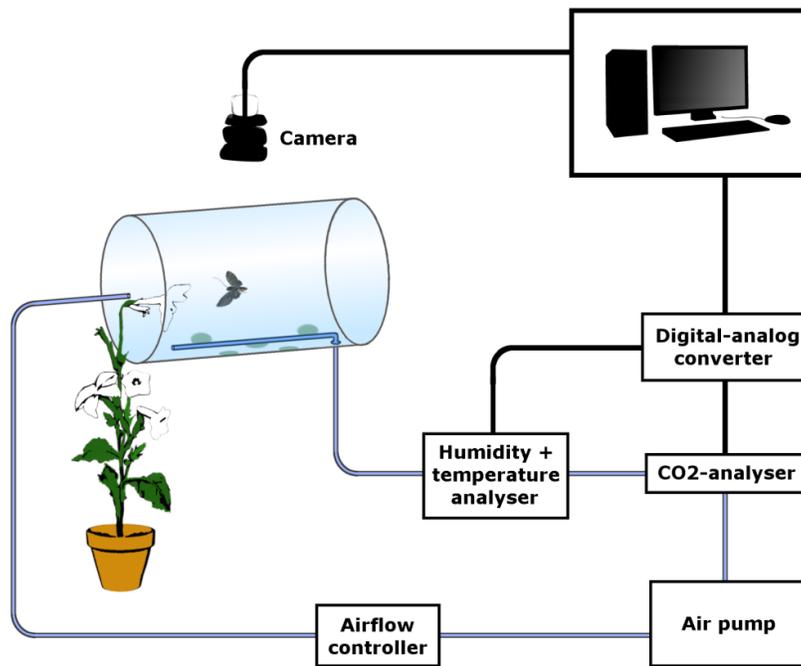


Fig. 1 Experimental setup used for measuring the metabolic rate of the moth (flow through respirometry system).

Purified, compressed air (blue) was pushed into a closed glass cylinder at a constant rate of 2 l min^{-1} regulated by a flow meter. The concentration of CO_2 produced by the moth (CO_2 -Sensor LI 820) as well as humidity and temperature inside the respirometry chamber measured (via T&F Sensor Sensorion SHT75). The behavior of the moth was observed from outside the climate chamber via two video cameras (Logitech C615) one located above (overview) and a second one at one side of the glass cylinder (detailed view on the flower).

3.2.2 Energy expenditures

The costs of probing and feeding from flowers were calculated and compared among the five different *Nicotiana*-species. The total of the expenditures for probing and feeding were summarized as costs of flower-handling in total that are needed for the cost-benefit analyses for one particular flower visit. The data from the CO_2 -measurements were classified into the different activities of the moth during the experiments (fan, fly, flower contact, feeding) and the energy expenses for the different activities of an individual moth were calculated. The energy consumption during an activity was computed from the increase of the CO_2 during this activity. The CO_2 -level value at the beginning of a given activity (e.g. probing the flower) was subtracted from to the CO_2 -level at the end of this activity to obtain the CO_2 production rate for each activity. The result was converted from

ppm to ml using a standardization curve previously generated by injecting defined amounts of CO₂ into the respirometric chamber. To calculate the costs of the moth while probing and feeding on flowers CO₂ production rate was converted into energy units. Hence, assuming 6.606cal ml⁻¹ CO₂ and 4.187 J cal⁻¹ (Moll *et al.* 2011), as well as a respiratory quotient (RQ) of 0.71 (Lighton *et al.* 1987, Moll *et al.* 2011) 1 ml CO₂ corresponds to 27.659 J (Moll *et al.* 2011). The used respiratory quotient (RQ) of 0.71 was assumed for leave-cutting ants (Lighton *et al.* 1987, Moll *et al.* 2011) but several studies show that the respiratory quotient of Lepidoterans seems to be also around 0.7 (Zebe 1954, O'Brien 1999).

Because the body mass differ among the individual moths the energetic costs were divided by the weight. For estimation of the expenditures independent of the time which was spent on flower-handling the costs were also divided by the time needed for a specific foraging activity e.g. feeding.

3.2.3 Calculation of the energy provided by the flower

To estimate the energy content of different *Nicotiana*-species their nectar was analyzed with respect to their sugar composition. For nectar collection flowers were removed from the plants. The top part of the corolla was cut off and the flowers were put into pipette tips upside down. These pipette tips were put into 50ml plastic falcons and centrifuged for 2 min at 1000 rpm so that the nectar could run out of the flowers through the pipette tips and concentrated on the bottom of the falcons. The nectar was pipetted into 4ml glass vials, weighted (fresh weight) and freeze dried at -80°C and 0.014 mbar. The dried nectar was weighted (dry weight) and solved 1mg: 1ml in pyridine. For the derivatization aliquots of 40µl were pipetted into 1.5ml glass vials containing 50µl BSTFA (N, O-bis(trimethylsilyl)-trifluoroacetamide) supplemented with 1% TMCS (trimethylchlorosilane). 10µl of internal standard was added and the sample was then shaken for 90 min at 37°C and 225 rpm. Afterwards the sample was diluted with 900µl pyridine. Phenyl-β-D-glucoside (Phenyl-β-D-glucopyraniside) solved in 1mg : 1ml pyridine was used as internal standard.

To estimate the amounts of glucose, fructose and sucrose in the nectar samples standards of these sugars were analyzed. Therefor 1mg of standard was solved in 1ml pyridine and different concentrations (1µl, 2.5µl, 5µl, 10µl, 15µl and 20µl) were prepared in the same way as the nectar samples. Three replicates were prepared for the nectar samples as well as for the different standard concentrations.

Samples of the different *Nicotiana*-species and sugar standards were analyzed by GC-MS (Agilent Technologies 7890 A) with helium as carrier gas (flow rate: 1.1ml min⁻¹). The GC was equipped with a non-polar HP5 column. Aliquots were injected splitless (injection port at 240°C) and the program started at 60°C and held for 3min. Temperature increased by 4°C per minute to 300°C. Identification of the main nectar compounds (glucose, fructose and sucrose) was performed by analyzing the retention times and comparing them with the retention times of the standards sugars. The peak areas of total ion chromatograms (TIC) were integrated with Chemstation software. To estimate the amounts and ratio of the sugars the peak areas of the samples were quantified by comparing them with standards at different concentrations.

Since nectar is basically sugar water (Barth 1985), the amount of sugar that it contains can be estimated after freeze-drying. To calculate the energy provided by the different flower species the amounts of the different main sugars were multiplied with the energy content that are provided by these compounds. The energy content for these compounds was assumed as follows: 15.56 kJ/g for glucose, 15.61 kJ/g for fructose and 16.48 kJ/g for sucrose (Livesey & Elia 1988). This value was multiplied with the ratio of fresh weight : dry weight of the nectar and the average amount of the nectar (fresh). So the energy content of the nectar could be calculated for each flower species.

$$energy\ intake = \frac{amount\ sucrose * 16.48kJ + amount\ fructose * 15.61kJ + amount\ glucose * 15.56kJ}{\left(\frac{dry}{fresh}\right) weight\ (mg) * fresh\ weight\ (mg)}$$

3.2.4 Estimation of the net energy gain

To estimate the net energy gain of the moth the calculated energy expenditures (costs of flower-handling in total per g moth) were deducted from the assumed average energy intake (total energy content of the nectar). After the tested moths fed on several *Nicotiana*-species, it was analyzed by removing the receptacle if the flower still contains nectar. In all cases there was no nectar left. That's why, the energy content for the average nectar amount can be considered as the potential energy intake of the moth when feeding on these flowers.

3.3 Behavioral analysis

For the analysis of the moth's behavior the videos from the respirometric measurements were used. To analyze the attractiveness of the flower for the moth the tested animals were scaled into the categories *response* (at least wing flapping) and *no response*. Additionally the activities of the responding moths were allocated to five different categories 1. *fan* (warming up), 2. *flying*, 3. *proboscis extension*, 4. *probing* and 5. *feeding*. To estimate the attractiveness of the flower more precisely the duration from the start of the flight till the first contact of the proboscis (*probing*) with the flower was measured. The number of contacts and the time spent by the moths on the flowers was quantified. In case the moth was successful on feeding on the flower (*feeding*) the duration of this period was also measured. For the evaluation of the flower-handling time the time spent by the moth probing the flower with the proboscis and the feeding time per se were summed (flower-handling in total) and compared intra- and interspecifically.

3.4 Morphological analyses

To estimate the accessibility of the nectar as well as the possibility to take up pollen while sucking the flower morphology was considered. Flowers that elicited a response by the moth were examined with respect to weight and corolla length. In order to get an idea of the position of the reproductive organs (stamina, pistil) and to estimate the accessibility of the nectar, cross sections of the different *Nicotiana*-flowers were made. Additionally, the diameter of the flower tubes was measured. To estimate the morphological fit of *Manduca*

and flowers of different *Nicotiana*-species the proboscis length was compared with the floral tube length.

3.5 Identification of active compounds of the floral headspace

3.5.1 Collection of the floral headspace

For absorbing the volatiles of the floral headspace SuperQ-filters were used. To avoid contamination of the samples with volatiles from the surrounding or other plant species SuperQ-filters and the equipment was cleaned before each sampling. The flower boxes were inserted overnight in Labosol, washed with ethanol and distillate water and dried for at least 2 hours at 55°C. Depending on the material adapters and other parts of the set up were either washed with DCM, ethanol and distillate water and dried at 200°C or only with ethanol and distillate water and heated together with the flower boxes at 55°C. The SuperQ-filters were washed with methanol, chloroform, acetone, dichloromethane and hexane before they were used.

For collecting the floral headspace from the different *Nicotiana*-flowers a push-pull headspace collection system was used. This kind of system allows the sampling of volatiles of flowers from intact growing plants (Tholl & Röse 2006). One flower of an intact growing plant was fixed in a small box. Purified compressed air was pushed through into this box at a controlled flow rate of 0.5 l min⁻¹ regulated by a flow meter. Before entering the box the inflowing air was cleaned by a charcoal filter. A vacuum pump was used to pull the air in the box through a volatile adsorbent SuperQ-filter at a rate of 0.4 l min⁻¹. The volatiles were eluted by washing the SuperQ-filters three times with 100µl of DCM. The samples were stored at -20°C till they were analyzed by GC.

The floral headspace collection was performed from 2.30 to 4.30 pm which corresponds to the time the experiments were performed. Similar conditions were used to make sure that the collected volatiles were similar to the ones emitted by the plants while conducting the experiments. Since the samples of the 2-hour sampling were not concentrated enough to use them for the EAD, the odor was collected for a second time. The sampling was performed as it was described above with the exception that the floral headspace was collected over 8 hours. Floral headspace samples were analyzed by gas chromatograph

coupled mass spectrometer (Agilent 6890N Network GC-System). The GC was equipped with a non-polar HP5 column. Helium was used as carrier gas with a flow rate of 1.1 ml min⁻¹. The oven program began with splitless manual injection (1µl) at 50°C and constant temperature for 2 min. Within 15 min the temperature increased to 250°C and finally was held for 5 min.

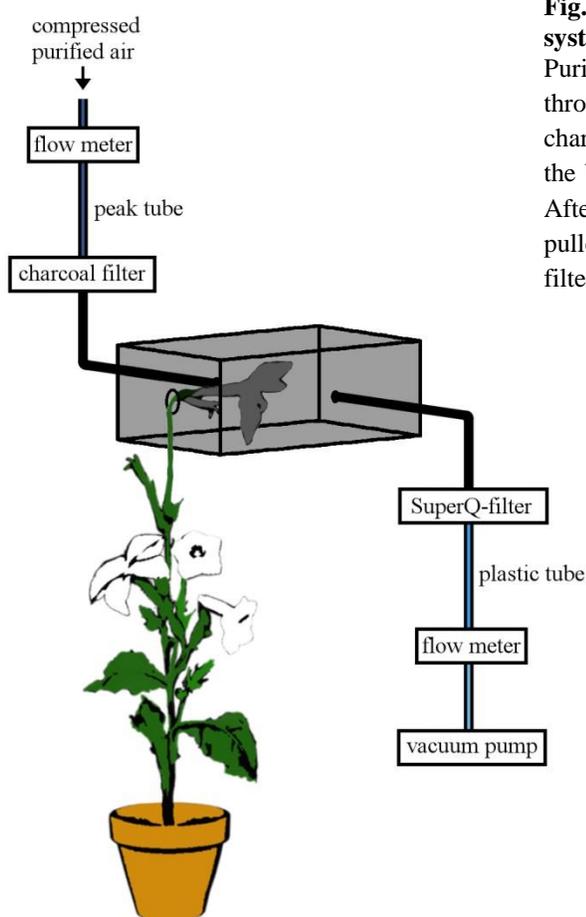


Fig. 2 Push-pull headspace collection system.

Purified, compressed air was pushed through a peak tube and passed a charcoal filter which was connected to the box containing the *Nicotiana*-flower. After passing the flower the air was pulled through an absorbent SuperQ-filter also directly related to the box.

3.5.2 Electroantennography (EAD)

GC-EAD (gas chromatography coupled with electroantennographic detection) was used to identify the compounds in the floral headspace of the different *Nicotiana*-species eliciting responses of the antenna of *M. sexta*. The antenna was excised as near as possible to the base, and from the thinner end the first three segments were cut off. Both ends were stick into glass capillary electrodes with hemolymph ringer (for composition see appendix) and silver wires which provided electrical contact via a Syntech 10x universal probe pre-amplifier (EAG-probe, Syntech, www.syntech.nl) to a Syntech IDAC-4 USB-converter. In Fig. 3 the setup is shown that was used for the electrophysiological measurements.

The odor samples collected from five *Nicotiana*-species were tested on three different antennae. For the stimulation 1 μ l of floral headspace samples was injected into an Agilent 6890 GC equipped with a non-polar HP5 column. The program started at 50°C after manual injection. The temperature was held for two minutes and increased within 15 minutes to the final temperature of 250°C and was held for 5 minutes. The GC was equipped with a 1:1 effluent splitter which enable the concurrent flame ionization detection (FID) and EAD of the separated compounds. The compounds that were separated through GC were introduced into a charcoal-filtered, humidified air flow at a constant rate of 1 l min^{-1} which ended about 1 cm of the antenna. The antenna was orientated into the same direction and with the thinner end oriented proximally to the air flow. The signal was visualized and recorded by using a suitable software program (GC-EAD 32 4.6 Syntech software). Both, the signal from the antenna and the FID were recorded simultaneously. Before and after each injection the responsiveness of the antenna was tested by puffing known odor attractants (benzyl alcohol, linalool and geraniol at concentrations of 10^{-3}). The responses of the moth's antenna to the floral headspace were filter at 5Hz and the recorded data was exported to MATLAB and analyzed manually.

Since the odor of the floral headspace was collected for only two hours to get data comparable to those from the other experiments the concentration of the volatiles in the samples was not high enough to use them for the electroantennography. For that reason a second, 8 hour collection was conducted to get more concentrated samples. Additionally the samples were evaporated to 20 μ l from which 1 μ l was used for manual injection. Finally

the volatile compounds were identified which were present during the two hours where the experiments were performed by comparing the analyzed data of both collections.

To identify the compounds that evoke a response of the *Manduca* antenna mass spectra and Kovats retention time indices were compared to authentic reference compounds to those published by the National Institute of Standards and Technologies.

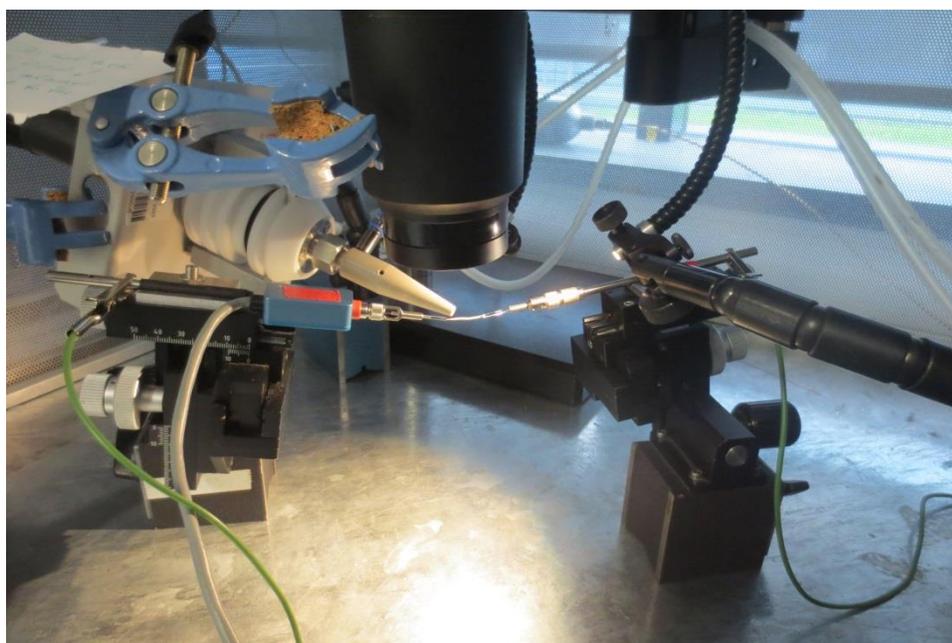


Fig. 3 Electrophysiology setup.

The setup for electrophysiological measurements based on deliberations of Olsson & Hansson (2013). The *Manduca* antenna was excised at the base, the first three segments of the thinner end were cut off and both ends were inserted in glass capillary electrodes containing hemolymph ringer and silver wire. A constant flow of humidified and filtered air was located ca. 1cm away from the thinner end of the antenna and orientated into the same direction.

3.6 Pollen load

To quantify the success of the interaction for the plant the amount of pollen that stuck on the proboscis after feeding on the flower was estimated. Therefore the proboscis of the moth was washed immediately after feeding. For the washing 10 μ l Tween 20 was added to 990 μ l distillate water. This milliliter was used to wash the proboscis of the moth three times. 10 μ l Safranin were added to the sample to color the pollen and afterwards the solution was centrifuged at 11000 rpm for 2 minutes so that the pollen could sink to the ground of the

reaction tube. After the supernatant was removed an amount of 100µl distilled water was added. 10µl of the solution were pipetted into each side of a Neubauer-chamber and the pollen in the four arrays on both sides was counted through by a light microscope. The result was multiplied by 10000 to recalculate the amount of 1ml and divided by the number of the arrays to get the pollen number for the whole sample.

3.7 Statistics

All statistical analyses were performed in MATLAB. Although the majority of the data was non-parametric they were analyzed by an ANOVA because in some cases the sample size was insufficient for analyses by Kruskal-Wallis-test. The ANOVA has been found to be more robust to small sizes and to a certain extent also to non-parametric distribution (Matlab-Statistics), even though the results have to be treated with caution. Analyses with an ANOVA were followed by a *post hoc* Tukey-Kramer HSD test. Only the differences between the floral tube length of several *Nicotiana*-species and the moth's proboscis were analyzed by using the Kruskal-Wallis test, followed by a Wilcoxon rank sum test corrected for multiple comparisons, because here the sample size was appropriate. Sample correlation coefficients were computed by performing a Spearman correlation.

4. Results

4.1 Morphological analyses

The flowers of the five tested *Nicotiana*-species vary in length of the corolla (Fig.4) whereat *N.obtusifolia* has by far the shortest flower tube ($2.00 \pm 0.131\text{cm}$). In comparison *N.alata* has the longest corolla ($7.714 \pm 0.573\text{cm}$). The flowers of the other three *Nicotiana*-species differ not so heavily (*N. pauciflora* $6.031 \pm 0.322\text{cm}$, *N. repanda* $5.342 \pm 0.728\text{cm}$, *N. bigelovii* $4.973 \pm 0.744\text{cm}$). All regarded flowers show significant differences ($P < 0.001$) to the length of the moth's proboscis (7.47 ± 0.574) excepted *N.alata* (Fig.4). Flowers of the regarded *Nicotiana*-species vary not only in length and shape of the corolla but also the diameter of the floral tubes (Fig.5) differ among the plant species. In the course of the morphological analyses it was noticeable that *N.pauciflora* and also *N.obtusifolia* to a lesser extent stand out from the flowers of the other species concerning the shape of the corolla, which is broader than the narrow flower tubes of the other three species, whereas *N.repanda* is the narrowest of all ($0.263 \pm 0.041\text{cm}$). Furthermore the pistil and as well the stamens of *N.pauciflora* stick out and do not line up with the floral tube as in the other flower species.

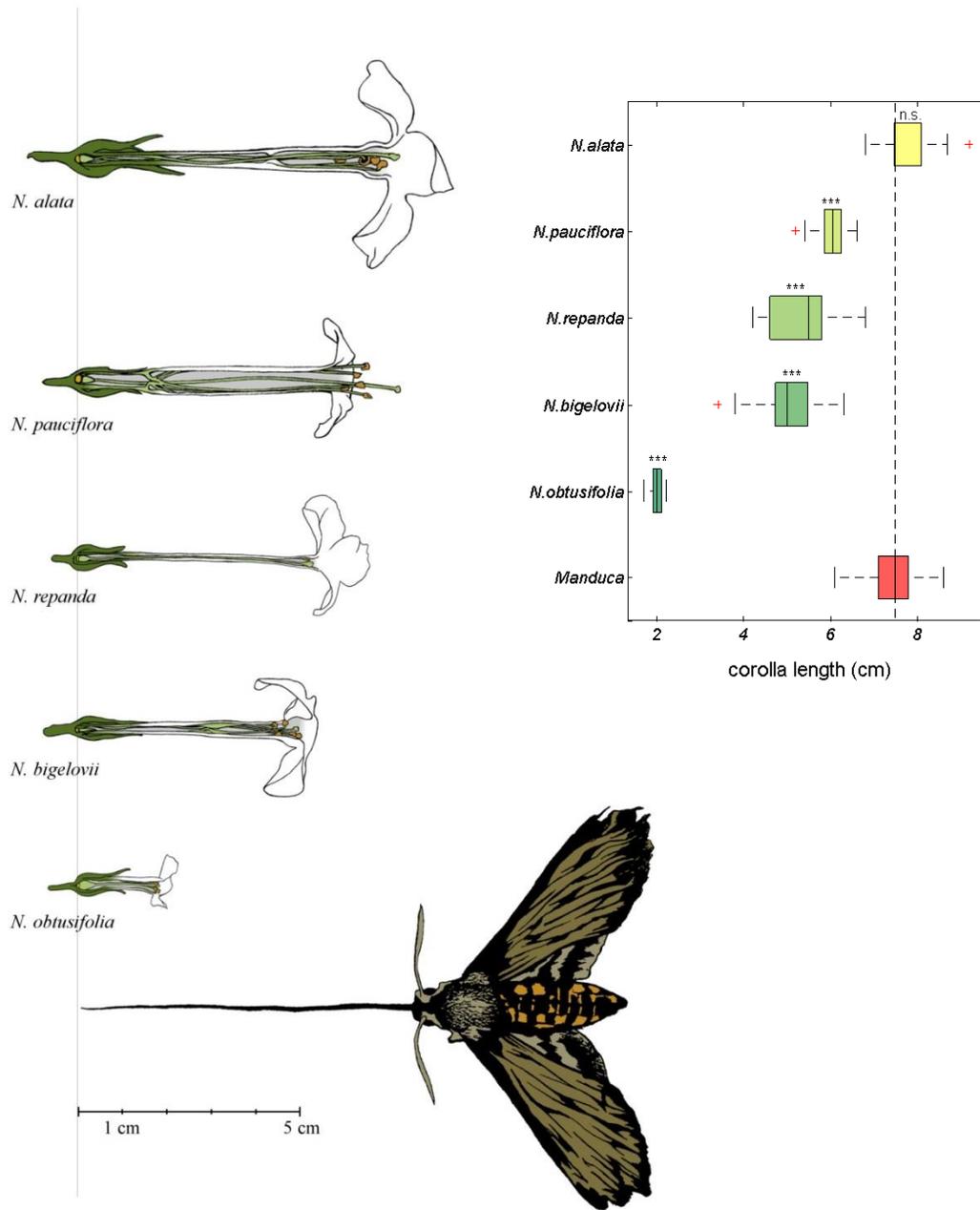


Fig. 4 Morphology of the flowers.

Flowers of the *Nicotiana*-species differ in corolla length (*N. alata* $7.714 \pm 0.573\text{cm}$ (mean \pm SD), *N. pauciflora* $6.031 \pm 0.322\text{cm}$, *N. repanda* $5.342 \pm 0.728\text{cm}$, *N. bigelovii* $4.973 \pm 0.744\text{cm}$, *N. obtusifolia* $2.00 \pm 0.131\text{cm}$), position of the reproductive organs (pistil, stamen) and diameter of the floral tube. a The difference between the floral tube length of different *Nicotiana*-species and the length of the moth's proboscis was strongly significant (Astrixes indicate significance levels according to a Kruskal-Wallis test ($P=5.43536e-28$), followed by a Wilcoxon rank sum test corrected for multiple comparisons) for all flower species except for *N.alata*. Stars represent significant differences (* $P<0.05$, ** $P<0.01$, *** $P<0.001$)

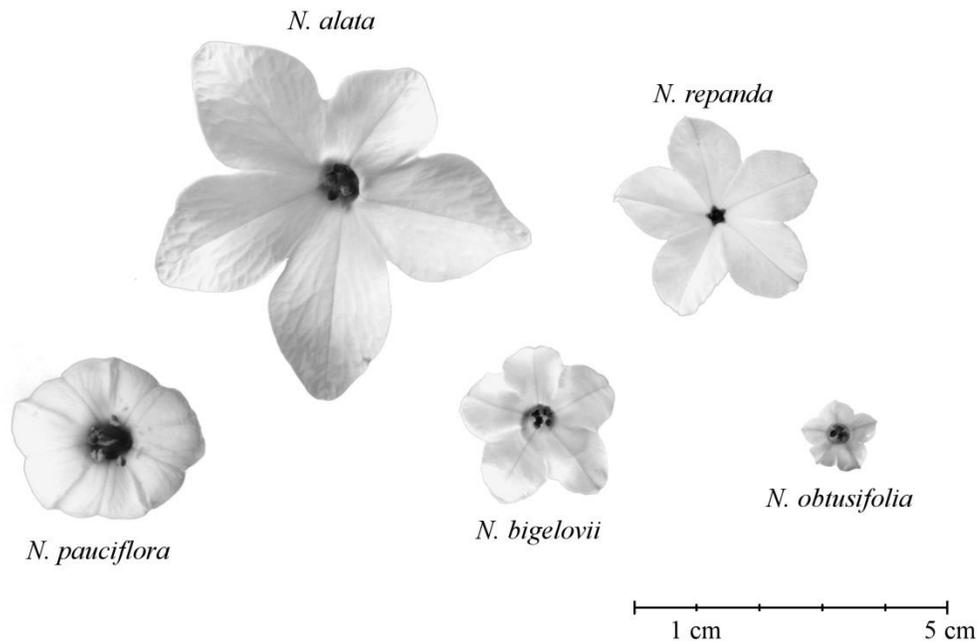


Fig. 5 Top view to flowers of different *Nicotiana*-species.

The flowers differ in shape of the corolla and diameter of the floral tube (*N. alata* $0.725 \pm 0.078\text{cm}$ (mean \pm SD), *N. pauciflora* $0.695 \pm 0.058\text{cm}$, *N. repanda* $0.263 \pm 0.041\text{cm}$, *N. bigelovii* $0.383 \pm 0.029\text{cm}$, *N. obtusifolia* $0.342 \pm 0.045\text{cm}$)

4.2 Behavioral analyses

The percentage of the moth that shows a response was analyzed among the different species to find out, if there are flowers that attract the moth more than others. For all flower species approximately 50% respond by at least flapping its wings, so that there was no difference. However, the percentage of the moths that contact the flower with the proboscis (Fig.6) seems to be more decisive for answering the question, how attractive the various flowers appear to the moth. For *N.alata* the percentage of the responding moths that probe the flower was 100%, whereas for *N.obtusifolia* it was only 36.36%. The probing rate of the other three flower species was similar (75%). From the perspective of energetic the success rate for the moth while foraging on the different species is of particular importance. The probability to acquire the reward was the highest when the moth visited flowers of *N.repanda* (83.33%), followed by a

success rate of 66.67% for *N.pauciflora*, 60% for *N.alata* and 50% for *N.bigelovii*. Only 25% of the moths that probe flowers of *N.obtusifolia* were successful.

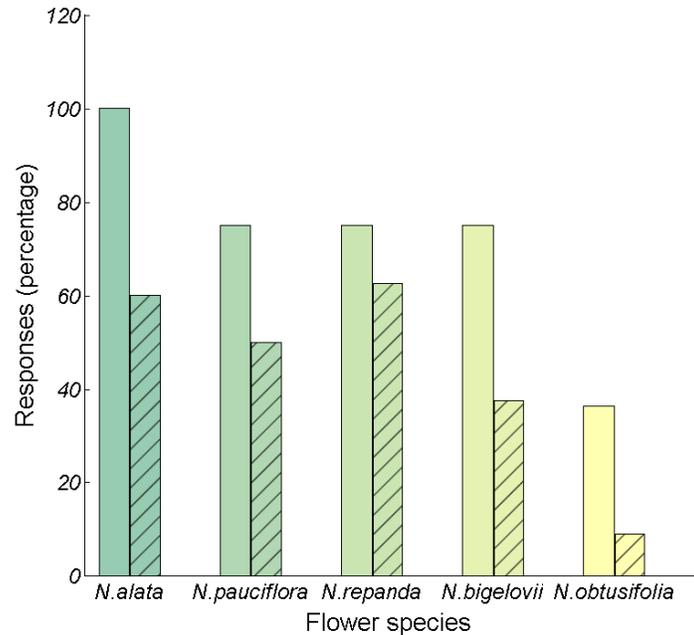


Fig. 6 Percentage of probing and feeding on different *Nicotiana*-species in relation to all responding moths.

For each flower species the first bar displays the percentage of the moths that probe the flower with its proboscis. The striped bar shows the percentage of the responding moths that feed for the different *Nicotiana*-species.

Furthermore the time that the moth spent on flower-handling on various species was compared for all moths (Tab.1) and also for the successful ones that finally feed on the flowers (Tab.2). For both considered groups the time expended for probing the flower did not differ significantly, whereas the differences in feeding time among the five *Nicotiana*-species are significant ($P=0.0027$).

Tab. 1 Flower-handling time (s) on five different *Nicotiana*-species (all moths).
Comparison of expended times for probing, feeding and total flower-handling (mean \pm SD).

Flower species	probing (s)	feeding (s)	total flower-handling (s)
<i>N. alata</i>	17.641 \pm 13.723	6.322 \pm 2.421	23.69 \pm 14.92
<i>N. pauciflora</i>	17.936 \pm 16.404	15.544 \pm 2.340	27.26 \pm 12.76
<i>N. repanda</i>	20.945 \pm 17.127	4.210 \pm 1.679	21.27 \pm 17.18
<i>N. bigelovii</i>	4.919 \pm 3.977	7.603 \pm 1.079	14.42 \pm 6.92
<i>N. obtusifolia</i>	18.180 \pm 6.725	10.173	20.723 \pm 9.158

Statistics

p-value (ANOVA)	0.5539	0.0027	0.7644
Significance	n.s.	significant (**)	n.s.

Tab. 2 Flower-handling time (s) on five different *Nicotiana*-species (successful moths).
Comparison of expended times for probing, feeding and total flower-handling (mean \pm SD)

Flower species	probing (s)	feeding (s)	total flower-handling (s)
<i>N. alata</i>	12.703 \pm 5.204	6.322 \pm 2.421	19.147 \pm 4.758
<i>N. pauciflora</i>	10.678 \pm 11.375	15.544 \pm 2.340	26.222 \pm 9.016
<i>N. repanda</i>	19.678 \pm 18.938	4.210 \pm 1.679	23.887 \pm 18.29234
<i>N. bigelovii</i>	2.957 \pm 2.385	7.603 \pm 1.079	10.560 \pm 2.056
<i>N. obtusifolia</i>	21.962	10.173	32.135

Statistics

p-value (ANOVA)	0.5123	0.0027	0.5198
Significance	n.s.	significant (**)	n.s.

4.3 Energetics of Foraging

4.3.1 Net energy gain

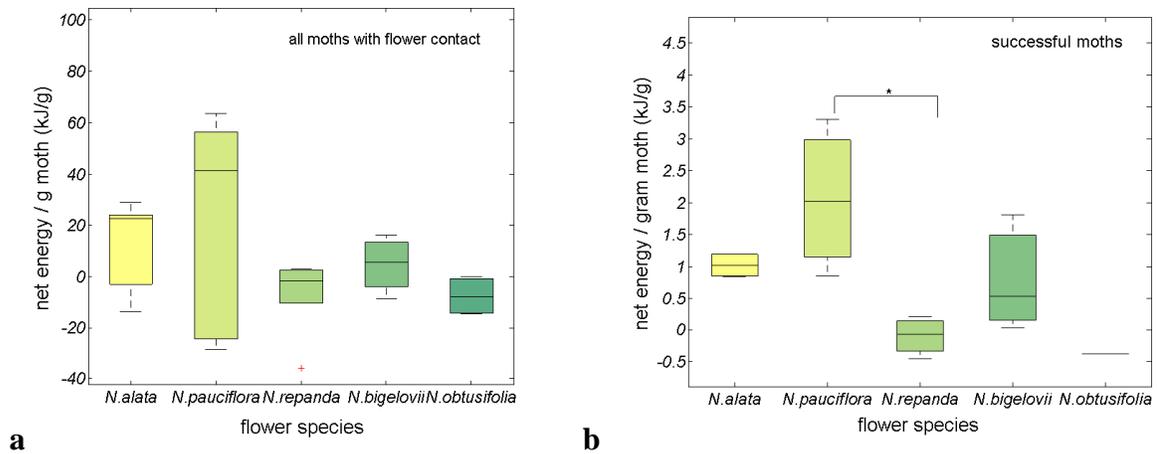


Fig. 7 Net energy gain of the moth.

(a) The net energy gain/body mass of all moths with flower contact show no significance ($P=0.2482$) (b). If only successful moths were taken into account the net energy gain differed significantly among *N. pauciflora* and *N. repanda* (Astrixes indicate significance levels according to an ANOVA ($P=0.0215$), followed by a Tukey-Kramer HSD test.)

The net energy gain of all moths that contact the flower with the proboscis was calculated by subtracting the energy costs from the energy provided by the plants. The costs of flower handling were computed by summarizing the costs of probing and feeding. For the moths that were not successful the feeding costs as well as the energy intake was zero. These moths had only costs for probing but also obtained no profit, so that at the end the energy the balance of the pollinator for the regarded flower was negative. The net gain of this moths show no significance (Fig. 7a). If only successful moths which were rewarded for its effort independent of how much energy they spent on flower-handling were taken into account, the net energy gain differed significantly among *N. pauciflora* and *N. repanda* ($P=0.0215$) according to an ANOVA, followed by a *post hoc* Tukey-Kramer HSD test (Fig.7b).

4.3.2 Energy expenditures

The energy expenditures (Fig.8 a-c) that the tested moths spent on the flower-handling on different *Nicotiana*-species were compared among each. Flower-handling costs were defined as the total of costs of probing the flower and the costs for feeding on it. Since the data for successful moths were less variable in comparison to all moths only the successful ones were considered for the analyses of the energetics of flower-handling. Neither the expenses of probing ($P=0.6336$) nor the costs of feeding ($P=0.0746$) on different flower-species showed significant differences. Also the total costs of flower-handling did not differ significantly among the regarded *Nicotiana*-species ($P=0.8384$). Because in the beginning it was hypothesized that a better match of proboscis and flower lead to a favorable energy balance, the relation of the difference proboscis – corolla length to costs of probing, feeding and total flower-handling per g moth and unite time (s) was examined via SPEARMAN correlation coefficient (Fig.8 d-f). The analyses showed a correlation for costs of probing ($r=0.7972$, $P=0.0032$), but not for expenses of feeding ($r=0.2520$, $P=0.3649$). However, flower-handling strongly correlates with the difference of length of proboscis and corolla ($r=0.7417$, $P=0.0015$).

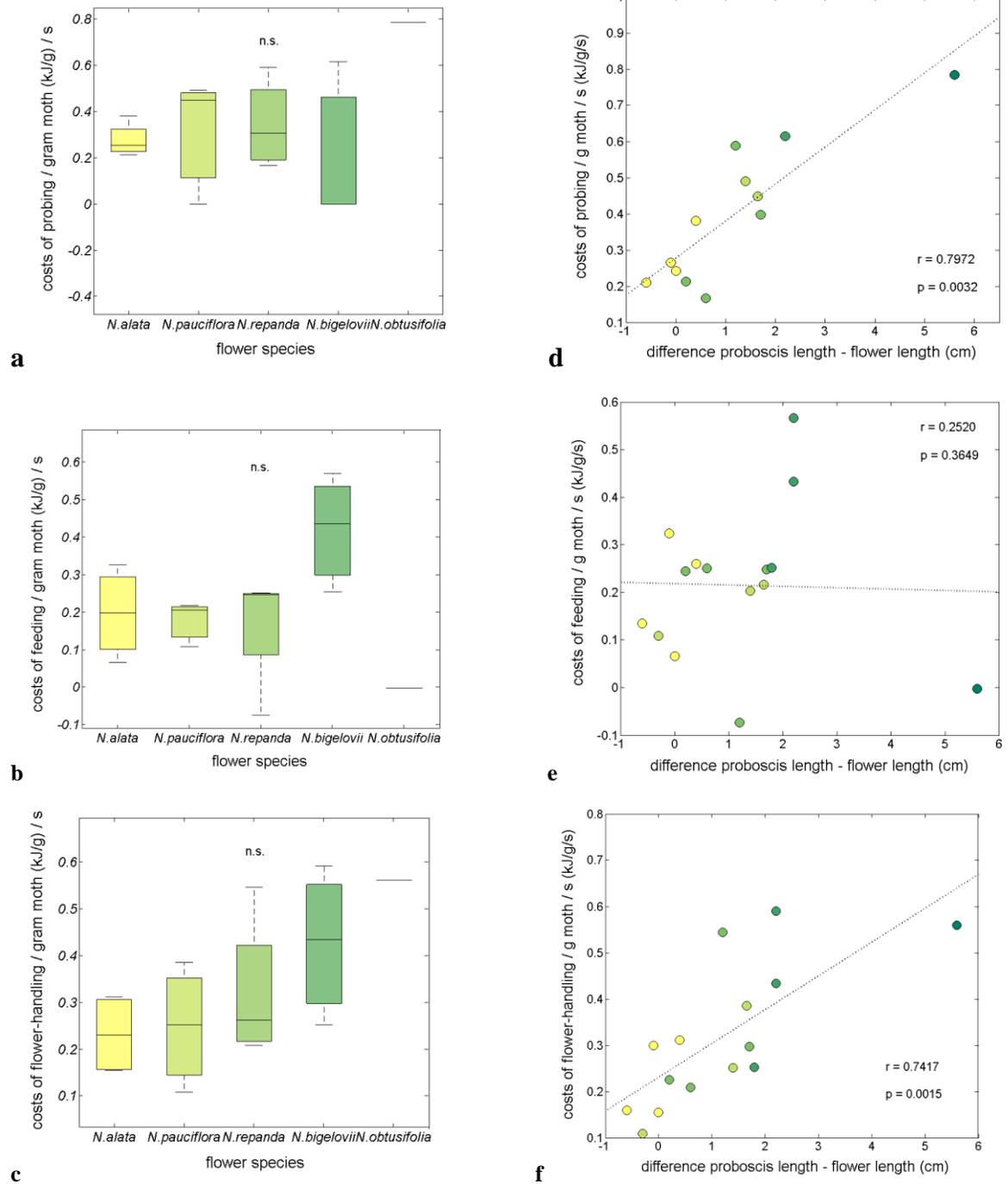


Fig. 8 Energetics of probing, feeding and flower-handling of successful moths.

The boxplots (a–c) display the costs of probing, feeding and total flower-handling per gram moth per time (kJ/g/s) of all successful moths on five different *Nicotiana*-flowers. The costs of successful moths were tested with an ANOVA and were not significant for probing ($P=0.6336$), feeding ($P=0.0746$) and total flower-handling ($P=0.8384$). (d–f) The costs of probing ($r=0.7972$, $P=0.0032$) and flower-handling ($r=0.7417$, $P=0.0015$) correlate with the difference between proboscis and flower length, whereas costs of feeding show no correlation with the proboscis – flower length difference ($r=0.2520$, $P=0.3649$).

4.3.3 Total energy provided by the flower

The energy provided by the flowers differs significantly among the regarded *Nicotiana*-species (Fig. 9a). ANOVA analyses show that the energy content of flowers of *N.pauciflora* have significantly more nectar than *N.alata* ($P<0.01$), *N.repanda* ($P<0.001$), *N.bigelovii* ($P<0.001$) and *N.obtusifolia* ($P<0.001$). Between *N.alata* and *N.bigelovii* there was no significance shown, but the energy provided by flowers of *N.alata* was significantly higher than by flowers of *N.obtusifolia* and *N.repanda* ($P<0.01$). The flowers with less nectar (*N.repanda*, *N.bigelovii* and *N.obtusifolia*) did not differ significantly from each other concerning the total energy content of the nectar. Additionally, the relationship between the potential energy intake for the moth and the costs for acquiring this reward was examined. There was no correlation of the total energy provided by the flower and the costs while feeding on the flowers.

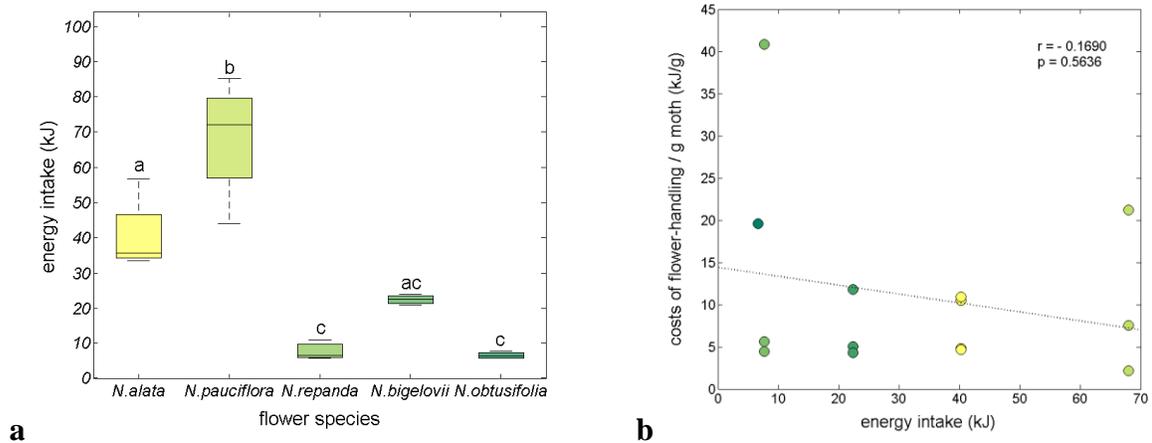
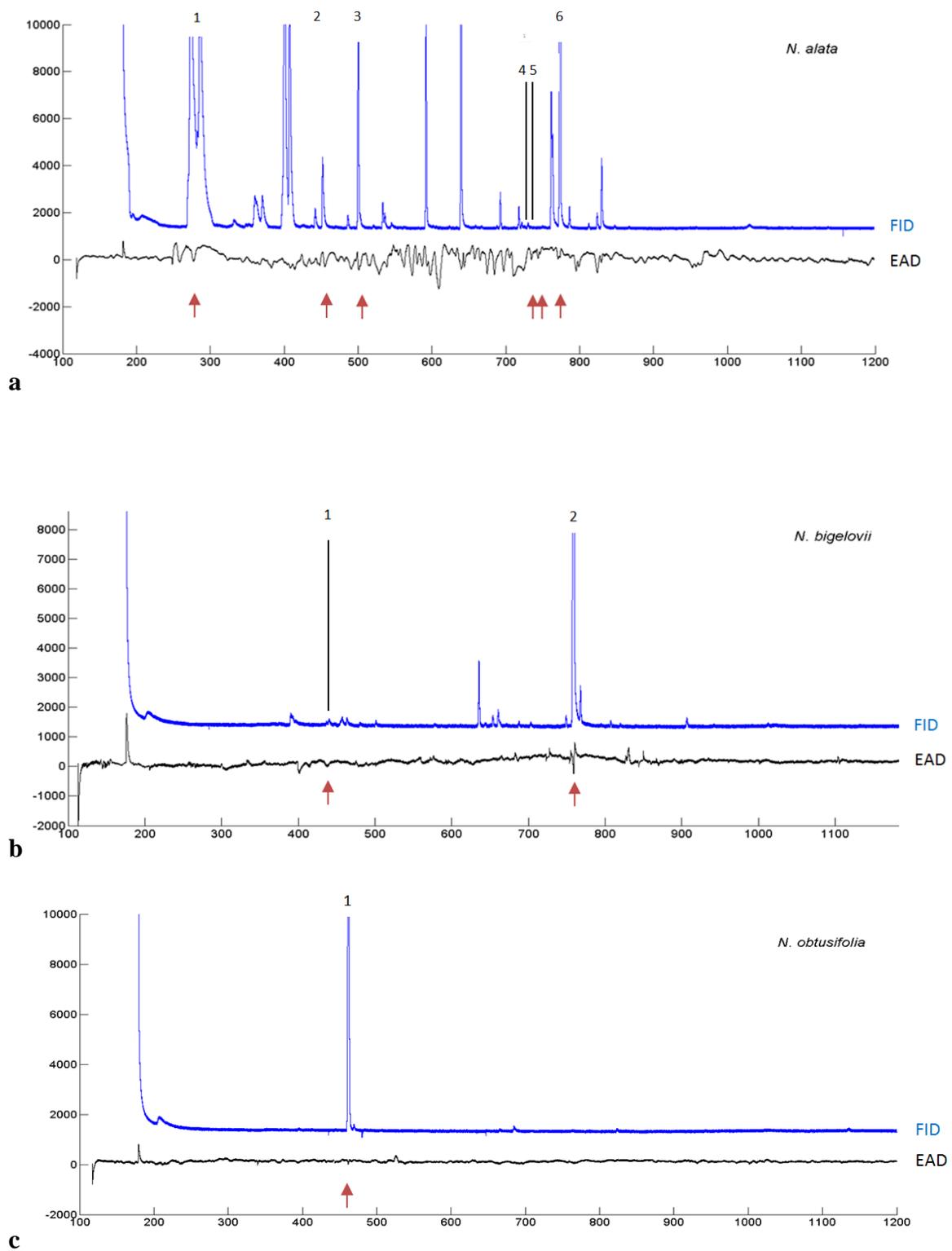


Fig. 9 Total energy provided by the flower.

(a) The figure shows the total energy provided by the flowers of five different *Nicotiana*-plants that differ significantly among the species ($P=9.2046e^{-8}$, letters indicate which groups differ significantly (at least $**P<0.01$)). (b) The energy intake displays no correlation ($r = -0.1690$, $P= 0.5636$) with the costs for flower-handling on these flower-species.

4.4 Antennal responses to the floral headspace of different *Nicotiana*-species



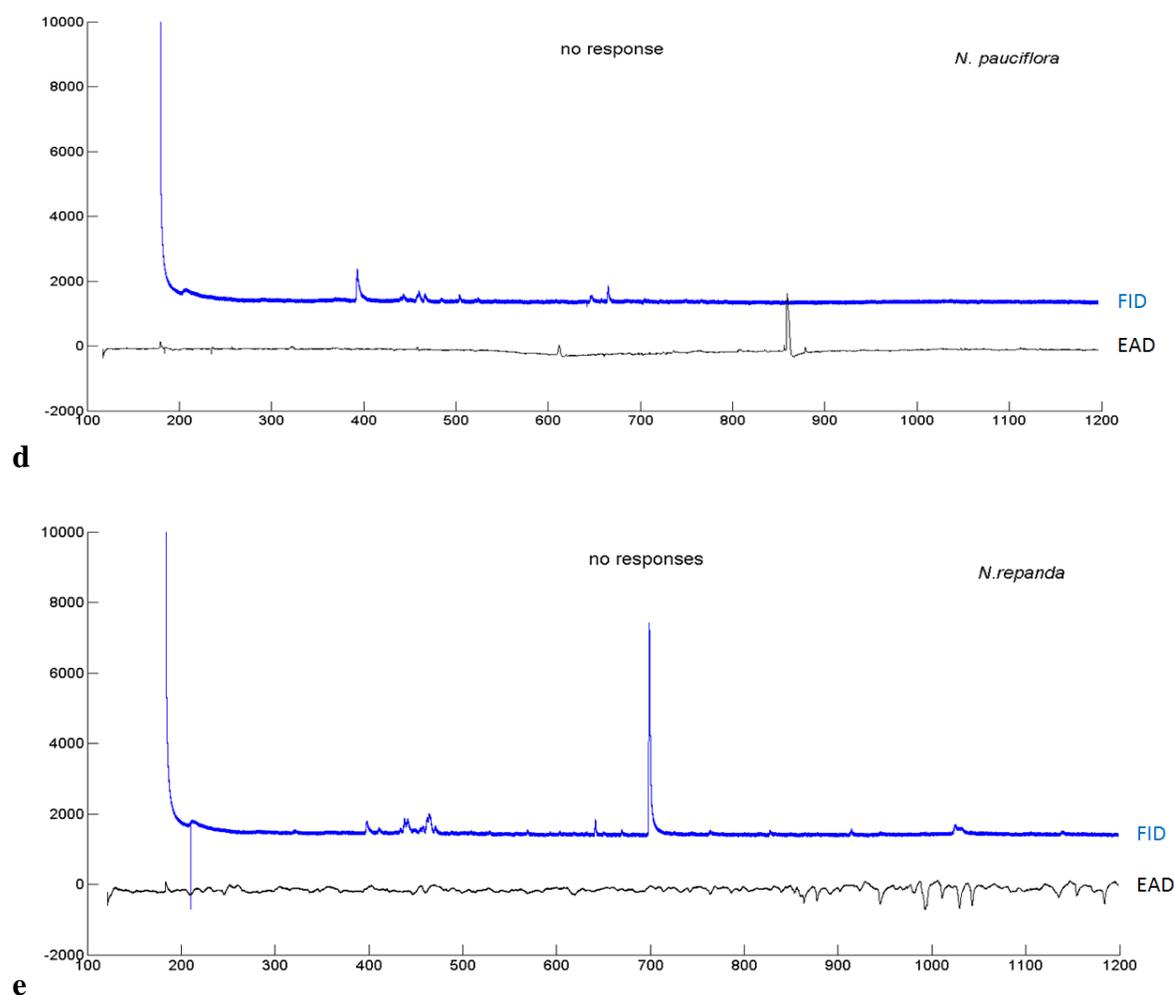


Fig. 10 FID and EAD signal for the floral headspace (8h) of five different *Nicotiana*-species. (a) The antennae of *Manduca* showed responses for six compounds of the 8-hour headspace sample of *N. alata*. Moreover two responses to floral headspace of *N. bigelovii* (b) were measured, whereas the flowers of *N. obtusifolia* caused only one response was noted (c). For the floral headspace samples of *N. pauciflora* (d) and *N. repanda* (e) no active compounds were found. All active compounds of *N. alata*, *N. bigelovii* and *N. obtusifolia* were listed in Tab.3.

GC-EAD was used to identify these compounds that cause measurable responses by the antenna of *Manduca*. The antenna is much more sensitive than the EAD, so there might be some other compounds that can be detected by the *Manduca* antenna but not by EAD due to limits of the method. For three of the five tested *Nicotiana*-species antennal responses were detected (Fig.6). In the floral headspace of *N. alata* six active compounds were identified. Furthermore the moth's antenna responded to two volatile compounds of *N. bigelovii*. The floral headspace of *N. obtusifolia* contained one volatile compound that

evoked antennal responses. However, this volatile (RT = 8.089) could not be identified. Not all of the detected compounds were present in the 2h-collection which was performed at the same time as the other experiments (Tab.3). Nearly half of the volatiles that evoke antennal responses were synthetically confirmed.

Tab. 3 Active compounds in floral headspace of *Nicotiana*-species.

The table listed the active compounds and relevant retention time and match. Also it is displayed which volatiles were confirmed with synthetic odors and if the active compounds were present in the 2h-odor collection.

Flower species	Volatile	Match	RT	Confirmed	2h-sampling
<i>N.alata</i>	1 Butyl aldoxime, 3-methyl-, syn-	831	4.783	*	+
	2 Linalool	906	7.716	*	+
	3 Phenylethyl alcohol	947	7.909		-
	4 alpha-Terpineol	941	8.749		-
	5 cis-Geraniol	877		*	-
	6 trans, trans-Farnesal	921	13.488		+
<i>N.bigelovii</i>	1 Linalool	906	7.716	*	-
	2 trans, trans-Farnesol	921	13.311		-
<i>N.obtusifolia</i>	unknown		8.089		+

4.5 Pollen load

To estimate the success for the plant the pollen that was taken by the moth's proboscis during feeding was estimated and compared between the different *Nicotiana*-species. The pollen load differed significantly but according to the Tukey-Kramer HSD test there was no difference between the groups. The relation of pollen load and the difference between length of proboscis and corolla was examined, but no correlation was given.

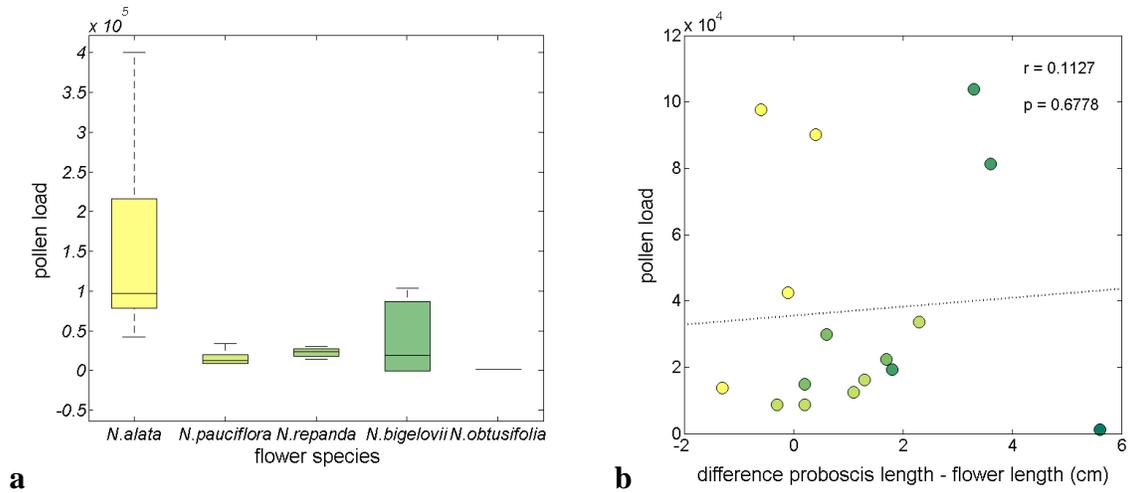


Fig. 11 Pollen load.

(a) The amounts of pollen that were washed from the moth's proboscis after feeding differ significantly between the five flower-species ($P=0.04$, there was no difference between groups according to the Tukey-Kramer HSD test). (b) Pollen load did not correlate with the difference between the length of the flower and the proboscis length.

As it was hypothesized in the beginning that in a strong mutualistic relationship the success of both the plant and the moth has to correlate, the relation between pollen load and net energy gain of the moth (kJ/g/s) was examined. As shown in Fig. 10 there was no correlation for the success of the moth and the success of the plant.

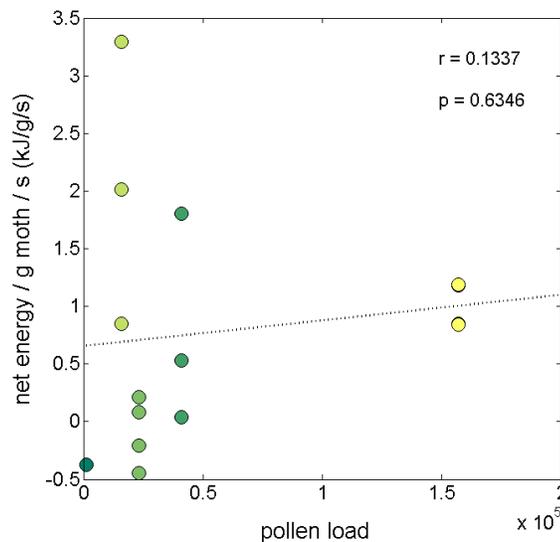


Fig. 12 Relationship between the success of the moth and the plant.

The measured net energy of the moth displays no correlation with the amount of pollen that was taken up during feeding.

5. Discussion

5.1 Influencing factors of energetic measurements

Measuring the energy expenditures of animals is a challenging task because the metabolic rate of an animal is affected by a huge range of internal e.g. activity level, and external factors such as temperature and gas-concentration (Lighton & Halsey 2010). To estimate the energetic costs for a single visit of various flower species the CO₂ consumption of the moth during several activities was measured using a respirometry system, which is a popular current method to calculate energy expenditures (Lighton & Halsey 2010). In this study a flow-through system was used. Such a system has to contain a chamber in which the experimental animal can move freely. Finding the right size is really difficult because on the one hand a chamber of small size might not ensure natural behavior of the moth. On the other hand it becomes more difficult to avoid leaks if the chamber size increases (Lighton & Halsey 2010). For the experiments, the chamber of the respirometric system might be too small to enable naturally foraging behavior of *Manduca* but the size was chosen to minimize potential measuring errors.

Changes of CO₂ levels, temperature and humidity were recorded at 5s intervals. In case the moth probe the flower only for a little while, e.g. for 2s, it was difficult to calculate the CO₂ consumption for this activity, because it has to be decided if the interval counts or not. The missing possibility to work with more precise times for an activity might lead to inaccuracy of the calculated rates of CO₂.

Since external factors such as humidity and temperature have an effect on the foraging behavior of the moth (Heinrich 2004, Schoonhoven *et al.* 2005, Contreras *et al.* 2013) in this study the conditions were held at a constant level. Humidity does not only influence the foraging behavior (Contreras *et al.* 2013) but also can affect the measuring of the CO₂, because the CO₂ sensor measures the light absorption of the air in the test tube. Because CO₂ and H₂O absorb light at a similar wave length there might be an error, the humidity was tried to keep constant so that the error afterwards could be subtract out. Also the temperature was hold at a constant level to avoid variations of the conditions among the different trails.

5.2 The individual match makes the difference

In the beginning it was hypothesized that the moth will show the most profitable energy balance with those *Nicotiana*-flowers where the hawkmoth proboscis matches the floral tube length the best. To analyze the best morphological match the length of proboscis and flowers of different *Nicotiana*-species were compared. The length of all flowers of the regarded tobacco plants differs from the length of the moth's proboscis with the exception of *N.alata*. This led to the assumption, that *Manduca* moths show the best energy balance with flowers of *N.alata*. Against this expectation the estimated net energy gains of successful moths show significant differences between *N.repanda* and *N.pauciflora*, but not for *N.alata*. The net energy gain was calculated from the energy expenditures of the moth and the energy provided by the flower for visitation. So at first the energy that the moth raises to feed on the flower has to be taken into account. Surprisingly the costs did not differ significantly among the species either for probing or for feeding, but the costs of probing and flower-handling in total correlate with the difference between the length of proboscis and corolla. Since the floral tube length varies strongly within the *Nicotiana*-species as well as the length of the moth's proboscis, the individual match might be more determining for the energy expenditures than differences between the flower species in general. So if the proboscis shows a good match with the floral tube, regardless of flower species, the costs of flower-handling were lower in relation to moth-flower pairs that show a morphological mismatch. A possible explanation for this correlation is that the moth can hang on the flower if the proboscis and the flower tubes match. This behavior was observed for all *Nicotiana*-species excluding *N.obtusifolia*. Even if the proboscis and floral tube varies strongly, the both *M.sexta* and *N.obtusifolia* cannot match in a perfect way. For the other species it is possible that, if the proboscis and flower tube is of the same length, the moth can hang on the flower and reduce the wing beat frequency or in some cases even stop to flap its wings (Fig.13). This might lead to a difference concerning the costs of flower-handling, because the moths did not have to hover in front of the flower the whole time. Hovering is one of the most costly locomotion activities of animals (Casey 1976) and metabolic rates of sphinx moths during hovering flight are up to 100 times higher than the standard metabolic rate (Zebe 1954, Heinrich 1971, Casey 1976). Since the costs of hovering are enormous it can be accepted that the moth can save energy when hanging on the flower. The reduced costs for the moth when foraging on flowers that matches the length of

the proboscis might be an explanation for the correlation of energy expenditures and corolla-proboscis match.



Fig. 13 Screenshots of the close-in behavior of moth and flower during the experiments.

(a) The moth's proboscis did not match the flower length of *N.obtusifolia* and so the moth has to hover the whole time while feeding. (b-c) In the case of a good match the moth could hang on the flower and reduce its wing beat frequency whereby the costs of flower-handling might decrease.

The match between proboscis and floral tube might also play a role for the plant's success. Even if the results show no correlation between pollen load and the difference of proboscis and corolla, it is more likely that the moth take up huger amounts of pollen if the lengths match. Maybe not the whole amount of pollen that was taken up while feeding on the various *Nicotiana*-species could be measured by washing the proboscis. The flower might use different parts of the moth's head to transport pollen (Heinrich 1981), especially if the moth had to put the head into the floral tube to reach the nectar. In this case, the pollen amount estimated by the washed pollen is not convincing.

5.3 Further aspects that might impede flower-handling

Another potential influencing factor could be the diameter of the floral tube. As it was seen in the morphological analyses the corolla tube of *N.repanda* was the most filiform of the tested species and the diameter was only around 0.26 cm. Although in this study there was no correlation found between the diameter of the floral tube and the probing costs, it might be an interesting question for further investigations if flowers with such filiform floral tubes are less attractive to the moth than flowers with wider corolla tubes.

Fulton & Hodges (1999) found that changing the orientation of flowers from upright to pendant reduce visitation of hawkmoths by an order of magnitude. In this study the flowers used for respirometric measurements also varied in orientation of the corolla tube (Fig.14).

Perhaps upright- or pendant-orientated flowers impose different requirements on the moth when probing or feeding on the flowers which might lead to differences of flower-handling costs.

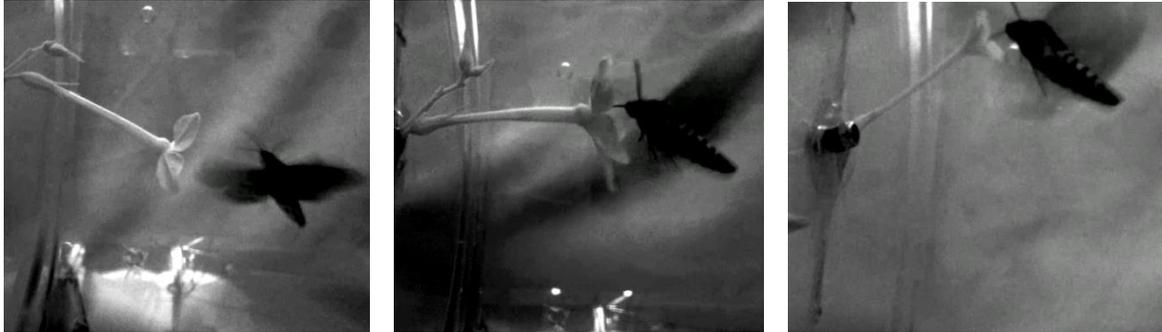


Fig. 14 Orientation of the flower.

The screenshots show different orientations (pendant, horizontal and upright) of the flowers during respirometric measurements. This orientation might influence the attractiveness of the flower to *Manduca* as well as the costs of probing and feeding because of more challenging flower-handling.

5.4 The bigger the reward, the better the profit

Kaczorowski *et al.* (2005) found out that in general, the nectar volume was positively correlated with floral tube length within the *Nicotiana* section *Alatae* (Solanaceae). In this study *N.alata* has the longest flower tubes but only offer the second largest nectar amount (11.5mg). The flower-species with the most nectar (19.57mg) was *N.pauciflora* which has a little shorter and broader floral tube. This various amounts of nectar offered by the regarded *Nicotiana*-species and the fact that there was no significantly difference between the costs that were spent on flower-handling bring about a slightly better energy balance for the interaction of *Manduca* and the flowers of *N.pauciflora* which offers the biggest reward. So the moth has a significantly better net energy gain when foraging on *N.pauciflora* than on *N.repanda*, but for the other species no significance was displayed. This is surprisingly because not only *N.alata* is not the most profitable flower as it was assumed, also *N.obtusifolia* was not the least profitable food source. Flowers of *N.repanda* and *N.obtusifolia* offer only little amounts of nectar so that it is more likely that the expenses of the moth exceed the energy intake. Since the costs for more and less rewarding flowers were nearly the same the amount of energy provided by the flower was determining for the net energy gain.

5.5 Anything is better than nothing

The results of the study show that the expenditures and the provided energy by the flower effects the net energy gain to a different degree because the amount of nectar seems to have a greater effect in this study. Because there were no significant differences concerning the costs of flower-handling among the tested species, the size of the reward seemed to decide on the level of profitability. However, it has to be asked for reasons why the energy costs differ not significantly among the species. Does it really make no difference for the expenses on which flower *Manduca* forage? Or does the missing lack arise from the laboratory conditions? With respect to the model of optimal foraging a moth should be more selective in its choice of suitable flowers when food is common, but feed most efficiently if it forages on all existing flowers when food is scarce (Emlen 1966, Krebs 1977). That means that when food supply is low, it is better for the moth to accept less rewarding flowers. In this study the moths had been starved since eclosion, so it might be assumed that *Manduca* accepted all presented food source, no matter how rewarding they are and how much energy the moth has to spend on flower-handling. This might be an explanation for the fact that the moth has comparable handling costs for flowers of all *Nicotiana*-species.

5.6 The role of learning behavior for foraging efficiency

An alternative explanation for the lack of differences concerning the amount of time and energy spent on flower-handling when foraging on flowers of different *Nicotiana*-species is that naïve moths were tested. Since insects often forage more profitably when they restrict their visits to only a few plant species (*flower constancy*) and that this strategy is assumed to increase foraging efficiency (Schoonhoven *et al.* 2005), the question arises to which degree foraging efficiency is determined by innate and by learning behavior. The fact, that there were no significant differences for the times and costs of flower-handling when *Manduca* was foraging on flowers of different *Nicotiana*-species, might lead to the assumption that these differences were missing because the moth has not learned till now how to reach the nectar. A range of studies addressed the role of innate and learning behavior for foraging efficiency (Heinrich 1979, Leonard & Papaj 2011, Brandenburg *et al.* 2012). Since, a bumblebees need up to 100 flower encounters before they had fully

developed the skills that were needed to extract the nectar from flowers of *Impatiens biflora* (Heinrich 1979) it can be assumed that experience plays an important role for foraging efficiency. This might indicate that, even if the moth could smell where it gets the most for its effort, the moth not know how to exploit the flower in an efficiently way till it gets more experience.

5.7 Potential cues that indicate profitability

In this study it was assumed that the presence of specific odorant attractants signalize the moth which flower is the most profitable. So, one question of this study was if there were flower species that seem more attractive to the moth. A result of the behavioral analyses was that there were no differences for the percentage of moths that show a response. But in reference to the percentage of the responding moths that finally fly towards the flower and probe it with the proboscis differences between the tested species were observed. In case of *N.alata* all responding *Manduca* moths fly towards the flower in marked contrast to *N.obtusifolia* that was visited only by 36.36%, whereas the visitation rate of the other species was similar (75%). This leads to the expectation that flowers of *N.alata* are highly attractive to the hawkmoth. If so it has to be asked why flowers of this species were more attractive than others. Because it was presumed that specific volatile compounds might act as cues for profitability, the responsiveness of the *Manduca* antenna towards the floral headspace of various *Nicotiana*-species was measured.

For the floral headspace of *N.alata* six volatile compounds were detected that elicit responses of the *Manduca* antenna, whereas for the other species less or none response could be found. Among these six responses some were evoked by typical hawkmoth-attracting volatiles such as linalool and farnesal (Raguso *et al.* 2003). *N.bigelovii* also emits putative hawkmoth-attractants in this case farnesol and linalool, hence it can be assumed that both are hawkmoth-pollinated species. Since farnesol was not found in the 2-hour collection of both species, it might be that this volatile compound is irrelevant for attracting *Manduca*. Both *N.bigelovii* and *N.alata* not only display similarities concerning the odor bouquet but also boast resemblances in morphology such as shape of the corolla and the relation of tube length and tube diameter. The distinctions in the temporally emission of the two similar volatile compounds of *N.bigelovii* and *N.alata* that evoke responses of the

Manduca antenna might indicate differences concerning the pollinator-plant interaction. Various assumptions can be made concerning the question why these volatile compounds were not found in the shorter odor sampling. First, it can indicate that the flowers of *N.bigelovii* attract hawkmoths that are active during another time of day as *Manduca*. Also the compounds might be emitted during the other activity peak of the moth to minimize interspecific competition with other flower species (Heinrich 1981). Another explanation for the lack of the volatiles might be that they were emitted in a lower rate and the SuperQ-filter was not sensitive enough to absorb them.

For the other flower species there were no responses detected, except for *N.obtusifolia*. One volatile compound in the floral headspace of *N.obtusifolia* was detected that evoke antennal responses, but it could not be identified. It would be interesting if the compound in question is a putative hawkmoth attractant or not. If *N.obtusifolia* in general is hawkmoth-pollinated its flowers might be more attractive to hawkmoths that are smaller and have shorter proboscises.

The absence of typical hawkmoth attractants in the floral headspace of *N.pauciflora* as well as the observed ability of self-pollination indicates that this flower may not rely on hawkmoth-pollination. Since hummingbirds in general ignore floral scent during foraging (van Riper, 1960) and the fact that flowers relying on hummingbird-pollination rarely emit large amounts of fragrance (Knudsen 1993, Levin *et al.* 2001), it can be assumed that *N.pauciflora* might be pollinated by hummingbirds.

It was conspicuous that *N.alata*, the only flower species that show no self-pollination, emits the most hawkmoth-attracting volatile compounds that evoke antennal responses. This corresponds to the observation, that all *Manduca* moths that show a response during the respirometric measurements also probe the flower. This leads to the assumption that under natural conditions *N.alata* seems to be the most attracting of the tested flower species, if only considering attraction by floral scent. There might be some other factors that attract *Manduca* or other hawkmoths such as floral CO₂ (Thom *et al.* 2004, Goyret *et al.* 2008) or visual cues. Since *N.alata* show no self-pollination it can be presumed that it depends to a higher degree on *Manduca* than the other species. For that reason *N.alata* probably invest more energy in attracting *Manduca* or other hawkmoths.

5.8 One good turn deserves another?

It is surprisingly that *Manduca* displayed a better energy balance with *N.pauciflora*, a potential hummingbird-pollinated flower, than with *N.alata* even if the difference between both was not significant. But not only the net energy gain has to be taken into account when estimating the degree of mutualistic interaction of pollinator and plant, also the probability of flower visitation by itself as well as the success rate play an important role (Schoonhoven *et al.* 2005). It makes no sense if a flower is incredibly profitable for the pollinator if it cannot be perceived under natural conditions, e.g. because of missing olfactory cues. For *N.pauciflora* there was no active volatile compound detected. So it is not sure, whether the moth will forage on flowers of this species under natural conditions.

In this study there was no correlation found for the success of the moth (net energy gain) and the success of the plant (pollen load) but this missing correlation could also be a consequence of the fact that only the pollen load of the proboscis was examined. In the case that the proboscis and the flower length were similar the moth has to put the head into the flower tube to reach the nectar and the pollen might be taken up by other parts of the moth's head. Another possible explanation for the missing correlation is that this study focus only one flower visitation. In addition to the energetics of this one specific foraging event, the probability of following visitations of a conspecific flower has to be taken into account, because it doesn't matter, whether the flower is hugely profitable but the moth is not attracted by specific sensory cues. From the flower's perspective not every insect that feed on it is an effective pollinator (Fenster *et al.* 2004). If pollinating animals feed on a wide range of flower species, the chance that the pollen taken up by the moth is transferred to a conspecific species is very low. In summary, it can be said that there was no correlation found between pollen load and the profit of the moth, but a single flower visit is influenced by many factors and does not always reflect the degree of mutualistic interaction.

6. Outlook

This study focused the energetics not for pollination in general but for a single flower visit. Besides analyzing the influence of the morphological match on the costs of a single flower visit, this study aimed to examine if *Manduca* can predict on which flowers it gets the most for its effort with the aid of sensory cues. Since the odor plays an important role in locating suitable food sources it was examined that a potentially good match and energy balance is signaled by the presence of specific volatile compounds in the floral headspace. The results show that the individual match of proboscis and floral tube actually has an effect on the flower-handling costs. Nevertheless, in this study no significant difference of the energy expenditures among various *Nicotiana*-species was shown. However, it has to be considered that the sample size in most cases was very little, so that the results have to be treated with caution. So with view to further investigations a confirmation of the results of this study with a considerable sample size has top priority. Afterwards it might be interesting if there are significant differences when considering *Nicotiana*-species with flowers that are still more different from each other concerning the corolla tube length, to see if predictions can be made with which flowers the moth shows the best energy balance. A potential candidate for this examination is *N.longiflora*, which has flowers with about 10 cm long floral tubes. Hence, it can be assumed that this flower is probably too long for the *Manduca* proboscis and that's why lead to a negative energy balance. Another flower species that might shed more light on the effect of the morphological match for the decisions of the pollinator is *N.attenuata* because the flower tube length of this species can be located in between the flower length of *N.obtusifolia* and *N.bigelovii*.

Even if *Manduca* shows not the best energy balance with *N.alata* in this study, it does not mean that there is no strong mutualistic relationship between both. To make assumptions about the strength of pollinator-plant interaction not only energetic aspects has to be taken into account. In addition to it, the rate of success when foraging on several flowers as well as the probability that the moth can recognize the flower as a suitable food source in general has to be included into considerations about mutualistic interactions. In this study the moth had no choice between more and less rewarding flowers and seemed to be less selective because of low food availability, so that only in combination with the results of electroantennography assumptions can be made which flowers might be potential

interaction partners of the moth beyond the artificial conditions in the respirometry chamber. On this account, it seems worthwhile to observe the visitation rate of several *Nicotiana*-flowers under more natural conditions, so that morphological features, energetic aspects, and perceptibility can be brought together with observations of natural behavior to get a more detailed insight into the nature of mutualistic relationships. Investigations of specific aspects of this interrelationship as the energetic aspects of flower visitation are of prime importance and might lead to new insights concerning the accomplishment of mutualistic pollinator-plant interactions, but it has to be in mind that those relationships are influenced by a huge range of factors and that all has to be brought together after analyzing it separately.

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9. Declaration of Original Authorship

Ich versichere,

dass ich die vorliegende Arbeit selbstständig verfasst und nur unter Verwendung der gegebenen Literatur und Hilfsmittel verfasst habe.

Sämtliche Stellen, die anderen Werken entnommen sind, wurden unter Angabe der Quellen als Entlehnung kenntlich gemacht.

Jena, den 29.08.2014

10. Appendix

Artificial diet

Ingredients: 46g of agar, 144g of wheat germ, 140g of corn meal, 76g of soy flour, 75 g of casein, 24 g of salt, 36 g of sugar, 5 g of cholesterol, 12 g of ascorbic acid, 6 g of sorbic acid, 3 g of methylparaben, 9 ml of linseed oil, 60 ml of 37% formalin, 30 mg of nicotinic acid, 15 mg of riboflavin, 7 mg of thiamine, 7 mg of pyridoxine, 7 mg of folic acid, and 0.6 mg of biotin per 1.8 ml of water (Grosse-Wilde 2011)

Hemolymph ringer

NaCl	150mM	8,77g/l
CaCl ²	3mM	0,33g/l
KCL	3mM	0,224g/l
TES	10mM	2,29g/l
Sucrose	25mM	8,56g/l

pH-value 6,9 (1M NaOH)