THE EFFECT OF NECTAR REDUCTION IN *PETUNIA AXILLARIS* ON FORAGING BEHAVIOR OF NOCTURNAL HAWKMOTHS, OBSERVED IN LABORATORY AND FIELD BEHAVIORAL ASSAYS

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Anna Brandenburg

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Prof. Redouan Bshary, directeur de thèse
Prof. Cris Kuhlemeier, directeur de thèse
Prof. Giorgina Bernasconi, rapporteur
Prof. Lars Chittka, rapporteur

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by Anna Brandenburg

Dipl. biologist, Free University of Berlin, Germany

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Jury:

Prof. Redouan Bshary, thesis advisor
Prof. Cris Kuhlemeier, thesis advisor
Prof. Giorgina Bernasconi
Prof. Lars Chittka

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FACULTE DES SCIENCES
Secrétariat-Décanat de la faculté
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IMPRIMATUR POUR LA THESE

The effect of nectar reduction in *Petunia Axillaris* on foraging and pollination behavior of nocturnal hawkmoths, observed in laboratory and field behavioral assays

Anneken BRANDENBURG

UNIVERSITE DE NEUCHATEL

FACULTE DES SCIENCES

La Faculté des sciences de l'Université de Neuchâtel,
sur le rapport des membres du jury

Mme G. Bernasconi Fusi, MM. R. Bshary (directeur de thèse),
L. Chittka (Londres, UK) et C. Kuhlemeier (Berne)

autorise l'impression de la présente thèse.

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Le doyen :
F. Kessler

UNIVERSITE DE NEUCHATEL
FACULTE DES SCIENCES
Secrétariat - décanat de la faculté
Rue Emile-Argand 11 - CP 158
CH-2009 Neuchâtel

Felix Kessler

■ Téléphone : +41 32 718 21 00 ■ E-mail : secretariat.sciences@unine.ch ■ www.unine.ch/sciences

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Summary

A key component shaping plant-pollinator interactions is nectar. Its volume can regulate the length and frequency of pollination events. Nectar provisioning can be costly for the plant. Once secreted by the nectaries, the sugar-rich solution is usually consumed by a floral visitor and lost for "recycling" within the plant. Nectar reduction should thus be advantageous for the plant: non-secreted carbohydrates can be reallocated within the plant to other structures promoting growth, reproduction or attraction. However, most angiosperms provide nectar. It is assumed that certain pollinator behaviors, so called partner control mechanisms, favor nectariferous over deceptive plants and ultimately prevent the spread of "cheaters". The partner control mechanisms identified in the context of plant-pollinator mutualisms are **avoidance** of nectarless species, **reduction** of **drinking time** and **number of flowers** visited on nectarless plants. Not all behaviors are performed simultaneously, and external conditions such as plant density as well as intrinsic factors of the foraging insect can determine to which extent certain behaviors are exerted.

In the present study, we analyze foraging behavior of nocturnal hawkmoths on cheating *Petunia axillaris axillaris* plants under several conditions. The aim of this thesis was to assess which partner control mechanisms are executed by pollinators facing nectarless/low nectar plants. We observed hawkmoth behavior in two field sites and conducted experiments with naïve and experienced hawkmoths *Manduca sexta* under controlled conditions. We investigated which of the foraging decision rules might potentially reduce the fitness of cheaters and thus limit their spread in a population.

In field assays, we observed that the density of naturally occurring *P. axillaris* plants and the presence of alternative food sources can influence hawkmoth behavior on nectarless Petunias: only when food plants were abundant and dense, pollinators would reduce the number of flowers on nectarless Petunias, whereas in the lower density there seemed to be no selection against cheaters.

In learning experiments under controlled conditions, we observed that none of the tested behaviors (reduction of drinking duration on nectarless plants, avoidance of nectarless plants, reduction of number of flowers visited on nectarless plants) were improved over the course of the experiment. However, in all learning trials there was a significant reduction of drinking duration on nectarless plants, indicating that this control mechanism of hawkmoths is always exerted innately. Learning might therefore not be of major importance in discrimination against cheaters in our system.

We constructed a plant with extremely high phenotypic similarity to *P. axillaris* yet only a third of the regular nectar volume (F25). Genotyping of F25 revealed a high genotypic similarity to its parental plant but failed to answer questions about the genetic background of low nectar volume. The low nectar line was used in behavioral experiments with *Manduca sexta.* A major goal was to find out how pollination behavior affects female reproductive success of F25. Analogous to previous experiments, we found that the drinking duration was significantly reduced on cheaters. In hand pollination assays, F25 produced significantly more seeds than *P. axillaris*, however this effect was neutralized when pollinated by *Manduca sexta*. The benefits of nectar reduction are thus counterbalanced by a change in pollinator foraging behavior. In the

future, we would like to assess which other fitness parameters are concerned when a plant ceases its nectar production.

Altogether, we were able to show which foraging rules are exerted by hawkmoths on cheating *P. axillaris* and how one partner control mechanism, namely drinking duration, affects seed set of a plant with reduced nectar offerings. We hope that this work has contributed to answering questions about the costs and benefits of cheating.

Keywords: mutualism, nectar, pollination, *Petunia axillaris*, hawkmoth, cheating

Mots clés: mutualisme, nectar, pollinisation, *Petunia axillaris*, sphinx, tricher

General Introduction

General introduction

The history and significance of pollination science

Angiosperm flowers are characterized by an amazing diversity. This diversity in architecture, colour, scent and other traits reflects the wide range of mechanisms that flowers have adapted to attract certain pollinators. It is assumed that animal-mediated pollination is the major driving force of diversification and evolution in angiosperms. A great part of the plants, fruits and plant-related products that we consume daily are angiosperms.

One of the oldest agricultural methods to obtain fruits is the manual pollination of plants: The first record of artificial plant fertilization to induce fruit set is shown on an Assyrian bas relief, dating to 1500 B.C. (Real 1983). It would however be too time consuming and expensive to fertilize each plant in a plantation by hand. Farmers exploit the beneficial behavior of insects and other pollinators that feed on nectar and pollen of a large number of flowering plants. Thereby, they carry parts of their load to adjacent flowers where they drop pollen grains on the stigma and pollinate the plant, the prerequisite for a plant to produce fruits and seeds. The advantage of employing these “professional” pollen vectors is their directionality and their fast, reliable, long-distance service. It has been estimated, that 35 % of the global crop supply depends on animal-mediated pollination (Klein et al. 2007). Pollinators are thus crucial in the maintenance of the world’s vegetable and fruit supply. The biology underlying the interaction between pollinators and plants has received great attention from a large number of scientists, including ecologists, ethologists, geneticists and many more.

The pioneering work in this field mainly followed a descriptive approach, including detailed observation of floral mechanisms and visitor diversity. One of the first naturalists to show that pollinators are rewarded with nectar (and therefore implying mutualism) was Sprengel (in Real 1983). Darwin focused on the evolutionary processes influencing pollination. He described that natural selection is the driving force of the evolution of floral traits (reviewed in Real 1983). Recently, scientists began to reveal general principles of pollination and plant reproduction in an ecological and evolutionary context (Baker and Baker 1983). Today, the following themes dominate the field of pollination biology: functional ecology of floral traits (pollination syndromes), dynamics of pollen transport, competition for pollinator services, niche relationships, community ecology of pollination and the persistence of mutualisms (Mitchell et al. 2009).

The persistence of mutualisms

Mutualisms are interactions between unrelated individuals that both derive a net benefit (Bronstein 1994, 2001). In the pollination mutualism, pollinators transport male gametes and fertilize plants, which in turn provide a reward to the pollen vector. Typically, mutualisms involve investment costs that either one or both partners have to pay (Bronstein 2001). The production of floral nectar is a costly expenditure into plant-pollinator mutualisms (Southwick 1984). To save costs, a nectar reduction would be beneficial for the plant, if the pollination was still reassured. Energy that is not spent on the production of nectar can be reallocated, e.g. to mature seeds (Pyke 1991). Some flowering plants, namely the deceptive orchids, are examples of animal-pollinated plant species that do not provide any reward to pollinators. The fertilization of deceptive

orchids is accomplished by pollinators lacking foraging experience, which are lured to the rewardless flower by sensory exploitation of innate pollinator preferences (Dafni 1984, Schiestl 2005).

In spite of the example of the deceptive orchids, surprisingly few angiosperms (ca. 4%) actually do not provide any reward to their pollinators (Renner 2006). The paucity of rewardlessness is assumed to be due to specific mechanisms employed by pollinators that prevent the spread of rewardless species, termed partner control mechanisms. The major questions that we need to understand are the conditions under which partner control mechanisms select for nectar production and the conditions under which plants may circumvent control mechanisms and evolve into cheaters.

Nectar is indisputably a key trait in plant-pollinator interactions, however little research has been conducted concerning its role in the stabilization of mutualisms. In this thesis, I studied pollinator responses (potential partner control mechanisms) on plants with reduced or no nectar offerings. In order to understand which conditions favor and which select against rewardlessness, I have conducted several behavioral experiments: in the laboratory with naïve pollinators and with pollinators that were exposed to nectarless flowers in several successive trials, and in the field with native pollinators. I will briefly present these experiments in the order of their appearance in this thesis. Altogether, I hope to contribute to understanding which pollinator behaviors might promote nectar production of the plant and under which conditions rewardless plant species can spread.

Nectar production and composition

In order to understand evolution and stability of mutualisms, one has to understand the production of nectar first. Nectar is produced and released from nectaries into the floral tube. These specialized glands occur in or around vegetative or reproductive tissues; in eudicots they are usually associated with reproductive organs (Nepi 2007). Nectaries vary widely in morphology and cellular structure, and are a term that describes function rather than the origin (Pacini et al. 2003). They must fulfill three functions: (1) import primary reduced carbohydrates from source tissues, (2) carry out metabolic reactions to allow for local storage, diversification and concentration of end products, and (3) secrete nectar into the extracellular environment through modified stomata, epidermal secreting cells or secreting trichomes.

Nectar is a complex mixture of substances belonging to diverse biochemical classes and its chemical composition is highly variable (Brandenburg et al. 2009). The main constituents are three sugars: the hexoses glucose and fructose and the dissacharide sucrose. In the past, the particular composition of these three sugars was believed to determine the pollinating guild (Baker and Baker 1983). However, due to recent advances in nectar research, this paradigm has been revised. Chapter 1 (The sweetest thing) will review all topics, problems and new methodologies that have become relevant in the science of nectar in the last two years.

The cost of nectar

Nectar production and secretion require energy; starch which is broken down to sugars is lost once consumed by a pollinator. Little is known about the true cost of nectar. The cost

of sugar may be low for a photosynthesizing plant, in contrast to nitrogen containing compounds such as amino acids. It remains unclear how amino acid and protein content of nectar contributes to the total costs of nectar production.

According to Southwick (1984), 4 to 37 % of the photosynthetic products are secreted in nectar as sugars in *Asclepias*, secretion costs not included. In other studies, removal of nectar resulted in replenishment and reduced seed production, indicating that a substantial amount of the plant's resources are used for nectar production (Pyke 1991, Ordano and Ornelas 2005).

Unused nectar can be reabsorbed by the nectaries and energy that is saved can be reallocated to growing ovules or new flowers (Nepi and Stpiczynska 2008). The fact that nectar is worth saving suggests that it is expensive. However, more studies under controlled conditions in multiple model organisms are needed to support such generalized statements.

Nectarless flowers

It is generally assumed that in the long term, flowers providing no reward to their visitors will suffer severe fitness losses and eventually go extinct. However, deceptive orchids provide an example how cheating can be an evolutionarily stable strategy (Dafni 1984). One third of all orchids lure visitors to their empty flowers by mimicking either the floral display of a common co-flowering species, the perfume of a female mating partner, or without mimicry (generalized food deception). Plants that do not produce any nectar are thought to gain a fitness advantage by reallocating resources to other floral organs that promote growth, reproduction or attraction (Nepi and Stpiczynska 2008). However, a

literature survey revealed, that nectarless orchids generally have a lower reproductive success than rewarding ones (Neiland and Wilcock 1998). It is assumed that certain pollinator behaviors might reduce the reproductive success of nectarless flowers (Smithson and Gigord 2003).

Conventional approaches to study plant-pollinator mutualisms rely on nectar removal and addition to observe pollinator response and effects on plant fitness. Nectar supplementation studies have shown that rewarding plants can have greater pollen removal and generally higher reproductive success (Jersakova et al. 2008), but can lead to an increase in self-pollination (Jersakova and Johnson 2006). A major drawback of nectar supplementation studies is that they neglect the costs that might be involved in nectar production and secretion, and do not allow any conclusions about net fitness consequences. Reduced nectar production may incur costs due to a changed pollinator response but also benefits due to the reallocation of resources. Therefore we pursued a novel approach in which we can measure the trade off between costs and benefits in one plant-pollinator system. We have bred a *Petunia* line with naturally reduced nectar volumes and observed pollinator behavior on rewarding vs. no or less rewarding plants. One parameter of pollinator behavior, namely drinking duration was always significantly reduced in low nectar plants. We analyzed the impact of reduced drinking duration on single flower seed production of both cheaters and mutualists in hand- and insect-pollinated flowers. The results are presented in chapter 2.

Low nectar introgression lines

The prerequisite for conducting a study that comprises costs and benefits of nectar reduction was the establishment of a line with low nectar volume. In the present study, I introgressed the low nectar phenotype of *P. integrifolia* into the *P.axillaris* genetic background. In a backcross (BC) breeding design using *P. axillaris* as recurrent parent, I have obtained one line (F25) in the third BC generation with 30% of the nectar volume of *P. axillaris* yet a high phenotypic similarity. Genotyping with 66 co-dominant PCR-based markers of the low nectar line revealed, that surprisingly, none of the *P. integrifolia* alleles were retained. This is in sharp contrast to the 12.5% heterozygous markers expected in the low nectar line F25 to *P. integrifolia*. This deviation from the theory might be due to the breeding and selection process which was very much biased towards *P. axillaris*. Further genotypic analysis, including multilocus markers like amplified fragment length polymorphic (AFLP) markers will hopefully help us to find the *P. integrifolia* introgression responsible for the reduced nectar production in the low nectar line. The "cheater" line was used for behavioral assays with pollinators *M. sexta*. Increasing our knowledge about the genetic background of nectar production will be helpful to make more specific manipulations in future experiments. The phenotypic selection process and the genotyping of the low nectar line are described in chapter 3.

Density dependance and nectar distribution

An important advantage of using plant-pollinator systems to study the effects of cheating on the maintenance of the mutualism is that interactions of pollinators and flowers can be observed easily in the field. However, experiments conducted in the field can be

challenging, as not only the plant-pollinator interaction has to be taken into account but also the ecological settings both pollinators and study plants are embedded in. It is well known, that plant density influences pollinator foraging behavior (Bosch and Waser 2001). The situation becomes more complex, if there are cheaters intermingled with rewarding plants (Internicola et al. 2006). Do pollinators discriminate between cheaters and rewarding species? How does plant density affect the foraging behavior of pollinators? Currently there are two opposing hypothesis in the literature about plant density and cheating: The “magnet species” theory (Thomson 1978) describes the effect of increased reproductive success of non-rewarding species growing in the vicinity of a large bout of rewarding plants (the “magnet”). A local increase of pollinator abundance leads to enhanced pollination success of mistakenly pollinated deceptive flowers. This “facilitation” has been demonstrated in *Anacamptis mori*, which significantly increase their pollen removal and deposition when transplanted in habitats with rewarding species (Johnson et al 2003). Opponents of this theory propose that a high density would lead to a severe competition between plants (Callaway 1995), assuming that reproduction in flowering plants is pollinator-limited. Deceptive flowers should thus benefit from a sparse rewarding plant community and increase their pollination success in a remote habitat (Lammi and Kuitunen 1995). In line with this argumentation, there was a significant decrease in fruit set of *Dactylorhiza sambucina* in high density communities independent of whether the surrounding plants were rewarding or deceptive (Internicola et al 2006).

We conducted experiments in two Uruguayan *Petunia axillaris* populations differing in plant density and community composition. One population was dense and intermingled

with co-flowering food plants, while the other population was less dense and featured only *P. axillaris* as suitable foraging plant. Here, I used plants with manually extracted nectar. These experiments are described in Chapter 4.

Pollinator Learning

All insects tend to have an innate preference for a certain flower type (Lunau and Maier 1995), however with proceeding foraging experience, pollinators start to discriminate between rewarding and cheating flowers (Giurfa et al 1995). Visiting empty flowers is a waste of energy for foragers; therefore pollinators avoid species if the food gain obtained is below the amount needed to maintain their metabolisms (Heinrich and Raven 1972), even after a single flower visit (Dukas and Real 1993). Avoidance learning has been demonstrated mainly in hymenopterans (Internicola et al. 2007). Phenotypic similarity between rewarding and empty flowers has been shown to slow down the learning process (Internicola et al. 2009, Dyer and Chittka 2004), leading to an increased error rate of foragers (Gigord et al. 2002, Johnson et al. 2003, Internicola et al. 2007). Therefore, avoidance learning might not be a pollinator foraging decision rule if rewarding and nectarless species are phenotypically not discriminable.

Other foraging decision rules like the number of flowers visited per inflorescence or drinking time reduction on nectarless plants have been far less investigated. However, these two decision rules are extremely important as they might limit the spread of cheating individuals in a plant population. Therefore, studying the learning of pollinator decision rules is essential if we want to understand how plant pollinator mutualisms are maintained stable.

Other learning mechanisms that pollinators use to avoid nectarless species, is spatial learning (Burns and Thomson 2006). Floral visitors associate landmarks or celestial cues with rich food sources (Menzel et al. 1996). However, spatial learning might be constrained by the unpredictable distribution of nectar rewards in space and time. Nectar volumes can be (temporarily) decreased by recent depletion, evaporation or decreased water supply; to name a few. This variation might lower foraging efficiency but is impossible to learn.

In Chapter 5, I investigate the role of learning for partner control mechanisms in *Manduca sexta*. The experiments with manually depleted versus wildtype *P. axillaris* were conducted in a greenhouse-based flight arena.

The study sytem *Petunia*

The genus Petunia has been studied widely (Gerats and Strommer 2009). It is endemic to South America, and today, populations of Petunia are found throughout Uruguay, Brazil, Paraguay, Bolivia and Argentina (Stehmann et al. 2009). The genus comprises at least 14 species. These can be classified in three groups pollinated by distinct pollinator guilds: the purple colored *Petunia* complex, comprising the majority of *Petunia* species, e.g. *P. integrifolia*, *P. mantiqueirensis* and *P. scheideana* displays many characteristics that are involved in hymenopteran pollination: purple corolla, short tube with wide opening and low nectar volumes. The second complex consists of at least three subspecies: *P. axillaris axillaris*, *P. axillaris parodii* and *P. axillaris subandina*. These three subspecies are classical hawkmoth pollinated plants: white corolla, long narrow floral tube and large amounts of nectar. The last group is constituted of a sole species: *P. exserta*, a putative

hummingbird pollinated species displaying a red corolla with flexed petals, a long floral tube and fairly large amounts of nectar (Figure 1).

Figure 1: Different *Petunia* species feature highly diverse floral traits that are involved in pollinator attraction (images from Stehmann et al. 2009)

Left: *Petunia axillaris parodii*, a hawkmoth-pollinated plant
Middle: *P. exserta*, a putatively hummingbird-pollinated plant
Right: *P. integrifolia*, a hymenopteran-pollinated plant

Hybridization in natural habitats occurs between *P. exserta* and *P. axillaris*, but has not yet been observed in *P.integrifolia* and *P.axillaris*. It is assumed that a divergent pollinator preference causes the reproductive isolation between these species (Galliot et al. 2006, Hoballah et al. 2007). However, they can be manually crossed and yield fertile offspring. Floral traits are extremely diverse in these two species, e.g. there is a difference in nectar volume of ca. 30 µl between *P. integrifolia* and *P. axillaris*. This offers the possibility to cross traits of interest such as low nectar volume of *P. integrifolia* into the background of *P. axillaris.*

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Chapter I

The sweetest thing- recent advances in nectar research

Anna Brandenburg, Alexandre Dell'Olivo, Redouan Bshary and Cris Kuhlemeier

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The sweetest thing

Recent advances in nectar research

Anna Brandenburg[1,2], Redouan Bshary[2], Alexandre Dell'Olivo[1] and Cris Kuhlemeier[1*]

[1]Institute of Plant Science, University of Bern, Altenbergrain 21, CH-3013 Bern,

[2]Institute of Biology, University of Neuchâtel, Emile Argand 13, CH-2009 Neuchâtel

[*]corresponding author: cris.kuhlemeier@ips.unibe.ch

Summary

We all appreciate the beauty of flowers, but we seldom consider their function in the life cycle of the plant. The function of flowers is to advertise the presence of nectar. Floral nectar is the key component in the mutualism between flowering plants and their pollinators. Plants offer nectar as a reward for the transport of pollen by animal vectors. Studying nectar is challenging because of its complex physiology, complex polygenetic structure, and strong environmental variability. Recent advances set the stage for exciting future research that combines genetics and physiology to study ecological and evolutionary questions.

Introduction

Floral nectar is a key innovation of angiosperms that evolved as a reward to visitors that transport pollen in return. It is a sugar-rich fluid dominated by the hexoses glucose and fructose and the dissacharide sucrose. Nectar allows flowers to "outsource" the pollination business to animal vectors, which assure a directional, accurate and efficient transfer of pollen compared to wind pollination. The establishment of animal-mediated pollination solves a problem but also creates new ones. First, nectar production is costly in terms of photoassimilate allocation (Southwick 1984, Pyke 1991). Second, the sugar solution does not only attract pollinators. Nectar robbers and microbes may consume the reward without transferring pollen. Third, pollen may be deposited at the wrong recipient, i. e. a different plant species. While this latter problem can be reduced with the evolution

of more exclusive relationships with few or even only one pollinator species, plants using this strategy limit their potential distribution to the distribution of their pollinators, which may increase extinction risk (figure 1).

Figure 1. Closely related species attract different pollinators.
Left, *Petunia exserta* with *Hylocharis chrysura*; right, P. *axillaris* ssp *axillaris* with hawkmoth *Manduca diffisa*. Nectar production is similar in the two species. Differences in color, fragrance and architecture of the flower determine the specificity of the interaction.

Most floral traits are likely to be genetically complex, and few of the genes involved have been isolated so far. The identification of such genes will allow for a genetic analysis of floral traits involved in plant pollinator interactions. Downregulation of relevant genes can give information about the effect of single gene mutations on pollinator behavior (Baker and Baker 1983, Liu et al. 2007, Irwin and Adler 2008, Kessler and Baldwin 2007). Marker-assisted breeding (near isogenic lines) and transgenic plants can provide useful material for field assays (Kessler et al. 2008, Hoballah et al. 2007)

We will briefly present the recent key advances in nectar research related to the following topics: 1) the physiology of nectar sugar production, 2) nectar composition, in particular the functions of primary and secondary compounds, and 3) the genetics of nectar

production. We will conclude with propositions for important future research questions on nectar.

The physiology of nectar sugar production

Figure 2. Floral reward and floral display. Longitudinal section through a flower of *Petunia axillaris*. The nectaries (arrows) are concealed at the base of the gynoecium, favoring access to specific hawkmoth pollinators and restricting access to unwanted visitors.

The site of nectar production, secretion and release are the nectaries (figure 2). These specialized organs occur in or around vegetative or reproductive organs (Wist and Davis 2006, 2008, Nepi and Stpiczynska 2008). In evolutionary terms, the variability in location reflects the broad diversity of pollinators and their foraging behavior. The specification of nectaries does not depend on the ABC genes that control the specification of all other floral organs. This lack of genetic constraints may explain the flexibility in position. (Baum et al. 2001).

Although nectaries may have active chloroplasts, carbohydrates for nectar production are mostly imported. Sucrose is transported from source tissues via the phloem and stored in the nectary parenchyma as starch (De la Barrera and Nobel 2004, Cawoy et al. 2008).

Ren et al. (Ren et al. 2007a) recently demonstrated in *Nicotiana* that starch-breakdown in nectary plastids does not only produce nectar sugars but in addition causes an influx of sucrose into the nectaries. The expression of genes involved in starch synthesis and breakdown are tightly linked to nectary developmental stages, where starch catabolism is correlated with nectar release prior to anthesis (Ren et al. 2007b).

It was originally assumed that the production of glucose and fructose resulted from the hydrolysis of sucrose (Lüttge 1961). However, the ratio may deviate significantly from the expected 1:1 in many species. This discrepancy between theory and data was recently resolved. (Wenzler et al. 2008): after the hydrolysis of sucrose, the hexoses are partially cycled through various biochemical pathways before being secreted into the lumen of the nectary. This more complex metabolism could explain a deviation from the 1:1 ratio. In addition, microbial degradation can alter nectar composition (Herrera et al. 2008). To counteract degradation and protect reproductive organs from microbial attack, some plants secrete antimicrobial hydrogen peroxide into the nectar (Carter and Thornburg 2004).

Functions of nectar

From the plant's perspective, in an ideal scenario, pollinators carry the maximum amount of pollen from one plant to the stigma of a conspecific while consuming minimal nectar. This entices pollinators to forage on a larger number of flowers and enhance pollen distribution. Plants make a preselection by luring certain pollinator guilds via advertising floral traits like scent (Raguso 2008), petal pigmentation (Tanaka et al. 2008) and other

floral structures (waxes, cell shape…). Recently, Goyret and Raguso (Goyret et al. 2008) demonstrated the importance of CO_2 emission as an attractant. *Datura wrightii* emits large amounts of CO_2 at anthesis when nectar volume is highest, provoking a strong attraction of the hawkmoth *Manduca sexta* towards the carbon dioxide source. Only insects with CO_2 sensing organs can receive this signal and choose the flowers with highest rewards. Species identity of the visitor as well as length and frequency of visits are thus crucial factors for plant reproductive success.

Both length and frequency of foraging bouts are regulated by the composition and concentration of primary and secondary metabolites in the nectar. The long-standing dogma that pollinator preference is the driving selective force for nectar sugar composition (Baker and Baker 1983) has been repeatedly supported (Chalcoff et al. 2008, Lotz and Schondube 2006, Kromer et al. 2008, Wolff 2006). Lotz and Schondube (2006) provide an extreme case for the importance of sugar composition by demonstrating that two passerine bird clades cannot digest sucrose. In parallel, however, several authors recently provided evidence for the importance of sugar concentrations and nectar volume for pollinator preferences: for example, several species of birds consistently switched from a hexose preference in diluted nectars to a sucrose preference in a concentrated diet (Johnson and Nicolson 2008, Fleming et al. 2004, 2008).

The primary function of secondary compounds in the nectar is to repel less specialized or even illegitimate visitors such as nectar robbers and pathogens. However, secondary compounds may also regulate the duration of pollinator visits and as a consequence the number of plants visited. Irwin & Adler (2008) demonstrated that the occurrence of the alkaloid gelsemine in nectar of *Gelsemium sempervirens* significantly decreased both

frequency and length of pollinator visitations but increased the number of flowers visited. A model demonstrates that under specific ecological conditions, plants can thus favorably influence pollen distribution patterns and promote outcrossing with alkaloids (Irwin and Adler 2008). Kessler and Baldwin (2007) found that nicotine in nectar repelled pollinators and decreased their visitation (drinking) times. In addition, they found that plants may counterbalance this effect with increasing amounts of the major volatile attractant, benzylacetone (BA). In subsequent field experiments, Kessler and colleagues (Kessler et al. 2008) utilized plants where nicotine synthesis was knocked down, which resulted in an increased visiting time on fewer flowers. In contrast to that, transgenic plants with reduced BA emission received shorter visits on more flowers. Plants emitting both attractant and repellent produced more seeds than any of the manipulated experimental groups (Kessler et al. 2008).

Some angiosperms, in particular orchid species, have evolved an alternative pollination strategy that involves no nectar production but still relies on pollinators. These species deceive their visitors by mimicking a mating partner or a rewarding species, often exaggerating attractiveness relative to models (for overviews see: (Schiestl 2005, Jersakova et al. 2006, Schluter and Schiestl 2008). Sexually deceptive orchids, like *Ophrys exaltata* fool their victims by producing female bee pheromones but actually in different relative proportions than found in bees. Apparently, the plant exploits a mating decision rule of male bees that makes them prefer novel pheromone combinations as an outbreeding strategy that promotes mating with immigrated females (Vereecken and Schiestl 2008). With respect to food deceptive species, Peter and Johnson (2008) demonstrated that the mimic *Eulophia zeyheriana* differs in only 0.03 units in bee colour

space from its model, which implies according to bee vision studies that model and mimic are indistinguishable to the pollinator. Pollinators alter their flower visitation patterns if they encounter empty flowers: they switch plants faster and move larger distances between consecutive visits (Jersakova et al. 2006, Jersakova 2008). These changes actually provide some benefits to the mimic in the form of enlarged pollen dispersal radius and prevention of inbreeding (Anderson and Johnson 2006, 2008). Nevertheless, recent experiments on the deceptive orchid *Dactylorhiza sambucina* demonstrate that plants supplemented with nectar receive more visits and pollen (Jersakova et al. 2008). The authors conclude that for the mimics the benefits of nectar production must be outweighed by the cost of nectar production in a deceptive species. The main concepts of the maintenance of plant-pollinator interactions mediated by nectar are summarized in figure 3.

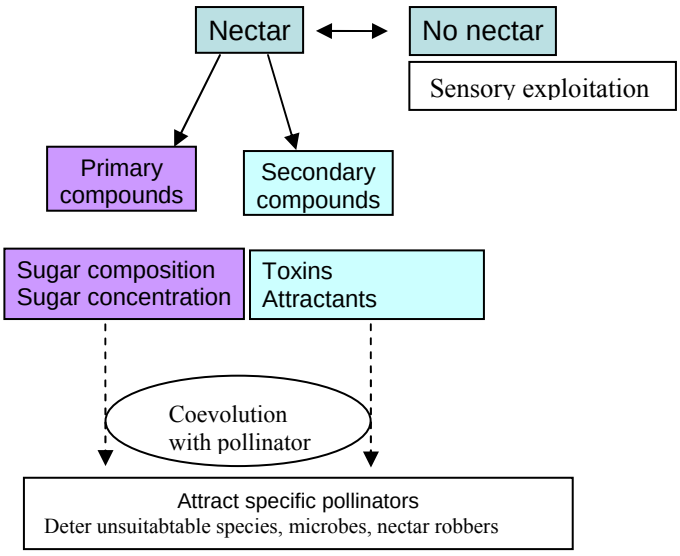

Figure 3: Functional relationship of nectar and floral visitors. Key strategic options how a plant may maximize its lifetime reproductive success by adjusting nectar quantity and composition. The first decision is whether to reward pollinators or to cheat through sensory exploitation of the pollinator's nervous system.

In the case of nectar production, coevolution with preferred pollinators should lead to specific compositions of primary and secondary compounds that optimize visitation by pollinators help to reduce the number of unwanted visitors. Physiological and molecular approaches will play a major role in testing this evolutionary scenario

Nectar genetics

Experimental manipulation of floral traits, such as supplementation/depletion of volatiles or sugars can give an indication of how these traits affect pollinator behavior and plant fitness. However, such experiments will rarely be conclusive. They do not account for the cost of production, and experiments are necessarily short-term. Nor do they give insight into the underlying molecular and genetic mechanisms. Designing plants with genetically modified nectars as seen in the studies discussed above offers obvious advantages (Kessler et al. 2008, Kessler and Baldwin 2007). The production of such genetic material is challenging, however. Characteristic for nectar is its substantial environmental variability in concentration, composition and volume between populations (Leiss et al. 2004), plants (Herrera et al. 2006, Goulson et al. 2007, Canto et al. 2007); also genders (Carlson 2008), and even inter- and intrafloral variability from day to day (Smith et al. 2008, Martins and Johnson 2007).

Floral traits that affect pollinator behavior have the potential to lead to reproductive isolation. One of the most exciting aspects of plant reproductive biology is the fact that in many cases, plants with major phenotypic differences may be isolated in the wild but remain sexually compatible. A good example is the genus *Petunia* with species such as *P. axillaris*, *P. integrifolia* and *P. exserta* that are partly or even complete reproductively isolated in their natural habitats, yet are routinely crossed in the laboratory. Controlled

interspecific crosses make it possible to elucidate the genetic modifications underlying their contrasting pollination syndromes. Under controlled laboratory conditions, bee-pollinated *P. integrifolia* produces an average of 1.2 µl nectar, whereas in the moth-pollinated species *P. axillaris* it is as high as 13-23 µl (Stuurman et al. 2004, Galliot et al. 2006). Such clear differences between sister species offer unique opportunities to study the genetic changes that have led to the evolution of new pollination syndromes and reproductive isolation. Four minor QTL (*VOL* 4-7) were identified in an interspecific cross between the two *Petunia* species. The additive effect of *VOL 4-7* accounted for 30% of the difference between the parental lines (Galliot et al. 2006). This suggests that nectar production is strongly polygenic. A different situation was found in *Mimulus*: Half the phenotypic variance between two closely related species with an 80-fold difference in nectar volume could be explained by one single major QTL (Bradshaw et al. 1995). These few studies give first hints into the genetics of nectar traits. They demonstrate that that, in addition to strong environmental variation, there is also abundant genetic variation and thus a substantial opportunity for a response to selection on these traits.

Conclusions and future directions

The field of nectar research has evolved in recent years. Advances in analytical methods have changed our views on the function of both the major and minor constituents. In particular, the unexpected chemical complexity of secondary metabolites in floral nectar translates into new insights into their ecological significance. An important field for future research concerns the role of individual traits that make up pollination syndromes.

Can we untangle the specific function of nectar composition from other floral traits? Most experiments are conducted by conventional approaches such as nectar supplementation or depletion. Genetic manipulations in model organisms such as *Mimulus*, *Petunia* and *Nicotiana* will be invaluable. What will be the effect of genetically reducing nectar content or composition? Will such cheating plants have reduced fitness because they are avoided by pollinators, or will fitness be increased due to enhanced outbreeding? We look forward to the answers to these and many other exciting questions.

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BA enhances pollinator visits, N reduces drinking time; both are needed to maximize seed set and reproductive success.

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Orchids fool male bees by emitting the female bees' sex pheromones. Both female bees and orchids produce the same compounds to attract mating partners, but in different relative proportions. Male bees significantly prefer orchids to females, probably due to their sensory preference of "novel" signals.

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Nectar supplementation had an overall positive effect for reproductive success of deceptive orchids, regardless of petal color. It is assumed that a mutation resulting in nectar production (as observed in other orchid species) would be of benefit for the plant, but might not have occurred because enhanced nectar volumes can cause inbreeding and involve costs.

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The genetic architecture of differences in floral size and nectar volume are studied in two closely related Petunia species. An AFLP-based QTL map was established to define the genomic regions explaining for phenotypical variation. QTLs with moderate and small effects underlying nectar and size suggest the polygenic nature of these floral traits.

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Chapter II

Costs and benefits of low nectar provisioning for female reproductive success in *Petunia axillaris* N

Anna Brandenburg, Cris Kuhlemeier and Redouan Bshary

Unpublished data

Consequences of reduced nectar production on female reproductive success in *Petunia axillaris*

Anna Brandenburg[1,2*], Cris Kuhlemeier[1] and Redouan Bshary[2]

[1]Institute of Plant Science, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

[2]Institute of Biology, University of Neuchâtel, Emile Argand 13, CH-2009 Neuchâtel, Switzerland

*Corresponding author: anna.brandenburg@unine.ch

Summary

Most angiosperms provide food in exchange for pollen transport. Manual nectar removal has demonstrated that cheating reduces seed production. This approach ignores potential benefits due to saved energy. We provide a new approach that allows measuring net effects using *Petunia axillaris* and hawkmoth *Manduca sexta*. In a crossing design of *P. axillaris* and *P. integrifolia*, we obtained an introgression line producing ⅓ of the nectar volume of P. *axillaris.* There was no discrimination of cheaters prior to probing. The number of flowers visited per plant was similar. However, drinking duration on cheaters was significantly reduced. Similar results were obtained with plants with manually removed nectar. We assessed how hawkmoth behaviour influenced female reproductive success of low nectar lines. Hand pollination resulted in higher seed sets of low nectar plants compared to the wildtype. This apparent benefit of nectar reduction was neutralized when pollination was effected by hawkmoths, indicating that shorter visits reduce the reproductive potential of cheaters. Overall, cheating through nectar reduction seems to be selectively neutral with respect to female reproduction in our study system. Fitness effects on male reproductive success remain to be studied in order to understand why a nectar reduction appears to be under negative selection.

Key words: mutualism, cheating, nectar, *Petunia*, hawkmoth, fitness

Introduction

Mutualisms are co-operative interactions between two or more individuals from different species gaining a net benefit from their associations (Boucher et al. 1982). They appear in a great number of biological systems and are believed to be critical in shaping nearly every existing ecosystem (Bronstein 1994, Herre et al. 1999). Most of these interactions involve investment (a reduction of the actor's immediate payoffs to the benefit of a recipient) by at least one partner (Bronstein 2001, Bergstrom and Lachmann 2003, Bshary and Bronstein 2004). The existence of investments raises the question which factors stabilise the mutualism, preventing cheaters that reduce their investment to spread in the population. Game theory provides a number of scenarios how individuals may potentially control their partner's behaviour, and thereby promote cooperation (Axelrod and Hamilton 1981). The problem with the game theoretic approach is that it is extremely difficult to quantify the payoff matrices for specific behavioural options in naturally occurring interactions. Therefore, the standard approach is to describe short term consequences from which inferences are made on lifetime fitness consequences. What is lacking, however, are studies that measure the fitness consequences of an individual with reduced investment relative to the wildtype.

Plant-pollinator interactions are a model system to study the stability of a mutualism. Pollination mutualisms are usually asymmetrical interactions in the sense that only the plant invests in the production of a costly reward while the pollinator ensures reproductive success of the plant as a by-product of self-serving foraging decisions.

Therefore, a key question is what typically prevents plants from reducing or even stopping the investment in nectar. Deceptive orchids demonstrate that cheating may be an evolutionary stable strategy at least under some conditions (for overviews see Schiestl 2005 and Jersakova et al 2006). Furthermore, several studies found that reduction of nectar volumes had positive effects on the plant: outbreeding and pollen export efficiency in the deceptive species *Anacamptis morio* were increased due to a change in pollinator foraging behaviour (Johnson et al. 2004). In addition, resources necessary for nectar production and secretion appear to be moderate to considerable (Southwick 1984, Pyke 1991, Ordano and Ornelas 2005, Nepi and Stpiczynska 2008 but see Harder and Barrett 1992).

Manipulation of nectar quantities has been instrumental in deducing pollinator decision rules that predict the fitness of plants with reduced investment in nectar. Such plant-pollinator interaction studies were conducted on artificial (Internicola et al. 2008) or manipulated flowers, either by extraction of nectar (Pleasants 1981, Mitchell and Waser 1992, Hodges 1995, Smithson and Macnair 1997), by cutting nectar spurs (Ackerman 1994) or by manual supplementation of the flowers with artificial nectar (Johnson and Nilsson 1999, Wolff et al. 2006, Jersakova and Johnson 2007, Jersakova et al 2008). Generally, deceptive orchid species produce less seeds than rewarding ones (Neiland and Wilcock 1998). However, the approaches do not allow a proper cost-benefit analysis. In order to measure the net outcome of cheating, we need to compare plants with genetic variation in the amount of nectar produced. This will make it possible to integrate measured costs (e.g. in terms of reduced seed production) with the benefits of additional resources available for reallocation.

Here, we provide a framework in which net fitness consequences for plants with reduced nectar investments as a function of pollinator behaviour may be studied for the first time. In a backcross breeding design, we introgressed a low nectar locus from *Petunia integrifolia* spp. *inflata* into *Petunia axillaris* ssp. *axillaris* N, a species characterized by high nectar volumes. To ascertain that the introgression line and the recurrent parent differ specifically in nectar production we assessed all floral traits known to affect pollinator behaviour. In a next step, we studied the behavioural responses of a natural pollinator of *P. axillaris*, the tobacco hornworm moth *Manduca sexta*, to both our introgression lines and wild type plants with manually depleted nectar. We paired such 'cheaters' with standard wild type plants in choice tasks. If our introgression lines were similar to the wild type with respect to essential features other than nectar volume, we predicted that the pollinators would treat both types of cheating plants in similar ways in the experiments. Concerning the pollinators' behaviour, we were particularly interested in behaviours that may affect the fitness of a plant, focussing on three aspects: 1) Are the moths able to discriminate between flowers with nectar and flowers without or with reduced nectar? If so, we predicted that they preferentially choose the rewarding plants. 2) Are the moths able to adjust the number of flowers visited on a plant to the nectar quantities they encounter per flower? Based on optimal foraging theory (Pyke 1984), we predicted that they would visit more flowers on plants with nectar. 3) Are the moths able to adjust probing drinking duration to nectar volumes? We predicted that they spend more time on flowers with nectar. Finally, we report on a first experiment designed to test how one aspect of pollinator behaviour, namely drinking duration, may affect a plant's female reproductive success, measured as seed set per visit per flower. We compared seed set in

wild type plants and low nectar lines both after hand pollination (as indicator of maximal seed set, e.g. Oz et al 2009) and after a visit by a hawkmoth. We focussed on probing duration as a potential partner control mechanism employed by hawkmoths because there is some evidence that shorter visits may lead to a reduced fertilization of the flower (Thomson and Plowright 1980, Warren and James 2008). Game theoretic models propose that power – the premature ending of an interaction in response to cheating by a partner – may indeed be a suitable partner control mechanism to diminish the payoffs for cheaters (Johnstone & Bshary 2002, Bowles & Hammerstein 2003), as long as cheaters cannot easily find new partners. This condition is fulfilled in our system under natural conditions as hawkmoth population densities are generally low; several studies found that hawkmoth-pollinated plants are actually pollinator-limited (Vesprini and Galetto 2000, Luyt and Johnson 2001, Wolff et al 2003). Therefore, we considered it reasonable to assume that one pollinator visit per flower reflects natural conditions in our study system. We predicted that if power plays a role in stabilising our study system by selecting for stable nectar production, we should find a reduced seed set in our low nectar lines.

Material and Methods

The study system: *Petunia axillaris axillaris* N

Petunia axillaris axillaris N (later referred to as *P.axillaris*) (Solanaceae), is a self-compatible inbred line (Botanical Garden of Rostock, Germany) derived from a wild accession of *P.axillaris axillaris*. It was maintained in the greenhouses of the Institute of Plant Science (University of Bern) by selfing. The flowers display the typical

characteristics of a hawkmoth pollination syndrome (Faegri and van der Pijl 1979): showy white corollas, long, narrow floral tubes, emission of strong fragrance at night and large amounts of dilute nectar. Hawkmoth pollination has been observed repeatedly (Galetto and Bernadello 1993; Ando 2001; Hoballah et al. 2007). *P.axillaris* originates in South America, and has been found in Uruguay, Paraguay, northern Argentina and southern Brazil (Ando et al. 1995; 2001). Natural habitats are mainly found in disturbed environments (roads, construction sites; Stehmann et al 2009). *Petunias* are hermaphrodites with both male and female sexual function; some populations of *P. axillaris* are self-compatible, others and all *P.integrifolia* accessions are self-incompatible (Kokubun et al 2006). Plants were grown in peat-based soil, in 15 cm diameter plastic pots and kept under greenhouse conditions (supplementary light in winter months, minimum 14h light).

The study was conducted at the Institute of Plant Sciences (University of Bern), from mid-October 2005 until the end of December 2005 and from April 2007 until December 2007. All experiments were conducted in a greenhouse featuring a flight arena and one pollinator species (*Manduca sexta*)

Pollinator species*: Manduca sexta*

Manduca sexta (L.) (*Sphingidae*), the tobacco hornworm moth, occurs throughout the American continent. Female tobacco hornworm moths oviposit two days after mating on solanaceous species (e.g. *Datura* and *Nicotiana*), where herbivorous larvae are known as pests. Pupal stages last 19-23 days. Adult moths are effective and specialized pollinators

of solanaceous plants like *Nicotiana*, *Petunia axillaris* (Ando 1995) and *Datura* sp. (Raguso and Willis 2005).

For behavioural experiments, female pupae of *Manduca sexta* were obtained as pupae from NCSU Insectary (Raleigh), USA. Animals had been reared under laboratory conditions, described in detail elsewhere (Bell and Joachim 1976). Pupae were kept in BugDorm-3® insect tents at 24°C, with 60% air humidity and a 16/8 day/night cycle and controlled daily for eclosion of adults. Adult moths emerged 1-5 days before the trials, and were used unmated for experiments.

Establishment of a low nectar line (F25)

Petunia integrifolia ssp. *inflata* S6 (later referred to as *P.integrifolia*) was used in the breeding design to establish a low nectar line of *P. axillaris*. Flowers of *P. integrifolia* are purple, emit very little fragrance at night, contain low nectar amounts (1.35 ± 0.47 µl) and are pollinated by hymenopteran species. Both species have been observed growing in sympatry in Uruguay (Hoballah et al. 2007), are cross-compatible and routinely crossed by hand. However, no hybrids have been found in their native habitats, probably due reproductive isolation based on pollinator preference (Hoballah et al. unpublished data, Galliot et al 2006). To establish a low nectar line, a single F_1 progeny of an initial cross between *P.axillaris* and *P. integrifolia* was backcrossed (BC) three times with *P.axillaris* as recurrent parent. Note that this scheme selects for introgression of dominant *P. integrifolia* low nectar loci. Both parents used in our breeding design were kindly provided by Dr. Ronald Koes, Department of Genetics, Vrije Universiteit Amsterdam (The Netherlands).

Selection process (PASI)

We established a "*Petunia axillaris* similarity index" (PASI), where all phenotypic parameters (see electronic supplementary material) of backcrossed individuals were compared to *Petunia axillaris.* In a first step, we selected plants with nectar volumes below 7 µl. We then calculated the relative proportion of numeric phenotypic traits such as tube length and corolla size of BC lines to *P. axillaris.* For the presence of floral scent and white colour we added 1 point, for the absence (pink corolla, no detectable scent) 0 points. Thus, the lines with the highest PASI index had the closest phenotypic similarity to *P.axillaris.* This method helped to select suitable *Petunia* lines that could be used for further backcrossing. From each BC generation, 23 plants were selected that showed the highest similarity to the recurrent parent but the lowest nectar volumes. These 23 lines were crossed to *P. axillaris* and of the obtained seed, 25 seedlings were grown. All plants were screened for the highest PASI. In the third backcross population we found one line (F25) with high similarity to *P. axillaris*, except for nectar volumes (table 1). We decided to use this line in behavioural assays. It was vegetatively propagated by cutting.
After backcrossing three times with *P. axillaris* as pollen donor (BC_3), the predicted proportion of *P.axillaris* genome in a BC_3 generation is 87.5%. A set of 66 genetic markers discriminating between *P.integrifolia* and *P.axillaris* alleles was tested in the low nectar line to identify genomic regions of *P.integrifolia* introgressed into *P.axillaris* background. None of the markers had retained *P.integrifolia* alleles in the low nectar line, suggesting that the selection for multiple *P. axillaris* characters resulted in a much smaller introgression than expected based on chance alone (chapter 3).

Phenotypic measurements

All phenotypic measurements were replicated ten times on different days over a period of four months for every backcross population (BC_{1-3}). Phenotypic measurements included tube length, corolla diameter, nectar volume, nectar concentration, UV reflectance and fragrance emission. For technical details on the measurements, see the ESM.

(f) Behavioural experiments with hawkmoth Manduca sexta

For the behavioural assays, we used manually depleted *P.axillaris* plants ("no nectar") and cuttings of the low nectar introgression line ("F25") and compared them to *P.axillaris* ("with nectar"). For manual depletion, floral tubes were pierced at the bottom (above the gynoecium) and exuding nectar was removed. Their corresponding control plants "with nectar" were also pierced in the floral tube but above the nectar level to avoid loss of nectar while controlling for potential wounding effects. In the experiments involving low nectar lines, plants were not pierced.

In hawkmoth behavioural assays, two plants were presented simultaneously, one plant with nectar (control) and one with either low (F25) or no nectar (hand manipulated). The average number of flowers varied naturally and due to different growth rates. It was not manipulated in the experiments to avoid potential confounding effects of damaging the plant. The number of flowers per plant ranged from 5-10 (average: 7) for manually depleted plants and from 1-18 flowers per plant (average 3) for low nectar lines. In every trial, both plants had the same number of flowers. The plants were used once per evening and after visitation all flowers were removed. In the first set of experiments (October 2005- December 2005), we tested manually depleted against "with nectar"plants. The

establishment of the low nectar introgression line was completed in April 2007, and subsequent behavioural experiments took place from April 2007 to July 2007. Experiments were conducted in a flight arena (144cm height, 248x368 cm surface area), in the middle of a greenhouse. As a consequence, the flight arena was saturated with scent. This is a technical limitation of the experimental design, and not a personal choice. A discrimination of two plants that differ in scent would not possible under these conditions. The flight arena had three different moth entrance sites, which were randomly chosen to release the hawkmoth to exclude potential side bias. Hawkmoths were kept isolated in a bug-dorm® in the greenhouse. One pollination flight was done per moth. Moths were removed after having visited both plants ("no nectar / F25" and control plant) or latest after 300s. First approach was noted as the plant that hawkmoths first fed on. Drinking duration was recorded from the moment the hawkmoth inserted the proboscis until its retraction. We only recorded the first drinking event per flower, as flowers were supposedly emptied during the first drinking event. For each plant, we noted the total number of flowers each hawkmoth drank from. Revisits of flowers were not considered. If none of the plants were visited, the trial was annotated as no choice and not used for statistical analysis. If only one of the two plants was visited, we included the data for "first choice" but excluded it for "number of visited flowers" and "drinking duration". First approach, number of flowers visited and feeding time per flower were recorded with a dictaphone and analyzed the following day. The drinking duration was measured with a chronometer. The experiments started at dusk (in summer 2100, in winter 1700). A 40 Watt incandescence lamp outside the arena was used to illuminate, however not strongly.

This had no marked effects on hawkmoth foraging, while permitting a precise determination of behaviour. Experiments terminated at 2300.

Seed set

To compare female fitness of F25 with *P.axillaris*, we measured seed set capacities of both plants. We used 15 vegetative cuttings of both F25 and *P.axillaris*. We compared seed set obtained by hawkmoth pollination and compared it to seed set obtained by hand pollination. For pollinator induced seed set, two plants with single flowers were placed in the flight arena. Moths had to visit first a control *P. axillaris* plant to collect pollen and then visit either another control plant or a F25 (pollen receiver). The pollen receiver was emasculated two days prior to testing. Pollen transfer therefore could only take place from *P.axillaris* to the pollen receiver plant. If a hawkmoth did not perform the required behaviours in the right order, the plants were excluded from the analysis. Each moth was used only once. For hand pollination, the pollen receiver was emasculated and pollinated by the experimenter with *P. axillaris* pollen. Stigmas were maximally loaded with pollen by smearing 2-3 anthers on the stigma. After pollination, flowers were bagged and labelled and kept until seed maturation. Plants were allowed to ripen one seed capsule at a time; other flowers of the plant were not used for seed set experiments simultaneously. After harvesting the seed capsule, plants were used again. To measure seed set, 20 seeds from each capsule were counted and weighed. The number was divided by 20 to obtain the weight of one seed. Finally the total weight was measured and divided by the weight of one seed to obtain the number of seeds per capsule. The experiments were conducted from August to December 2007.

Statistical analysis

Normality was tested using Shapiro-Wilk analysis. Values are given as median with interquartiles for data sets violating parametric assumptions (MB emission (table 1) and behavioural data (figure 2-3)); while average values and standard deviations are given for data sets with normal distribution (all other phenotypical traits (table 1) and seed set data (figure 4)). Due to small sample sizes, non-parametric statistical tests (Mann-Whitney-U tests for independent data sets) are used to compare phenotypical traits between F25, *P. axillaris* and P. *integrifolia*. Mean feeding time per flower per moth and visitation rate (number of flowers per moth) were analyzed with Wilcoxon signed rank test for paired data sets. First approach was analyzed with χ^2-test ("Goodness of Fit"; Vassar Stats). Seed set of the two lines *P.axillaris* and F25, and their pollination method (hand and moth-pollinated) was compared using a non-transformed analysis of variance (ANOVA) to account for interaction effects (line and pollination method and pollination method nested in line). Seed set was the fixed factor.

For all non parametrical statistical analysis, SPSS 15.0 for Windows (SPSS inc.) was used. For the ANOVA, the statistical package jmp 8 (JMP®) was used. We performed a post hoc t-test to assess difference in seed set for each of the four treatments.

Results

Phenotype

Figure 1: comparison of corolla colour, diameter, tube length between low nectar line and *P.axillaris*

	Tube length D1 (cm)	Tube length D2 (cm)	Corolla diameter (cm)	Corolla UV reflectance	MB Emission (pptv)	Nectar volume (µl)	Nectar sugar proportion (%)	Nectar sugar concentration (%w/v)	Sugar (g)/ flower
P.axillaris	2.1±0.1	1.2±0.1	5.4±0.3	No	20019 Q_1: 16506.3 Q_3: 31603.5	34.7±6.8	G: 21 F: 22 S: 57	16.53±1.5	5.62 (100%)
F25	2.1±0.1	1.3±0.1	5.6±0.2	No	11257.3 Q_1: 3516.6 Q_3: 26908	10.4±4.1	G: 26 F: 26 S: 48	30.75±4.3	3.17 (56%)
Comparison *P.axillaris* - F25 (Mann-Whitney U)	p=0.35	p=0.35	p=0.28		p=0.181	p<0.001		p=0.02	
P.integrifolia	0.2±0.02	1.6±0.2	3.1±0.18	Yes	413.7 Q1: 254.5 Q3: 440.4	1.35 ±0.47	G: 32 F: 33 S: 35	37.5	0.5 (9%)
Comparison *P.integrifolia*- F25 (M-W-U)	p<0.001	p<0.001	p<0.001		p<0.001	p<0.001		p=0.262	

Table 1: statistical analysis of average phenotypic parameters of *P.axillaris* , F25 and *P.integrifolia*
All values are given as averages with standard deviation except for MB emission (median).

F25 and control plants differed significantly in nectar volumes, with F25 containing on average only 30% of nectar volume compared to *P.axillaris* and no overlaps in volumes between treatment groups (table 1). The two lines also differed in nectar sugar concentration and methylbenzoate (MB) emission. In F25, concentration and MB emission were nearly doubled. Note that the difference in scent emission is not significant

(table 1). All other measured phenotypic traits, that were significantly different between *P.axillaris* and *P.integrifolia* were similar between *P.axillaris* and F25 (figure 1, table 1).

Feeding time per flower

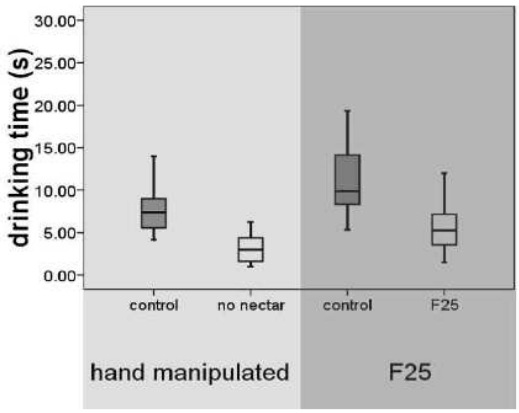

Figure 2: Box plots of feeding time per flower on hand manipulated no nectar plants/low nectar line F25 compared to control plants *P.axillaris*. The upper and lower limit of the box depict the 1st and 4th interquartile, the middle line is the median. Error bars depict the variance with maximal value on top and minimal value on the bottom.

On manually depleted *P.axillaris*, the feeding time per flower was significantly reduced compared to the control plants (Wilcoxon signed rank test, N=20, Z=-3.3, $p<0.001$) (figure 2). The median feeding time per flower on "no nectar" plants was 3 s as compared to 7.4 s in the control treatment. Feeding time per flower on F25 was significantly decreased compared to the control plant as well (Wilcoxon signed rank test, N=20, Z=-3.4, $p<0.001$). The median feeding time per flower on F25 was 5.25s compared to 9.85s in the control treatment (figure 2). This corresponds to a feeding time reduction of 60% in hand manipulated *P.axillaris* and 47% in F25 compared to *P.axillaris*.

Number of visited flowers

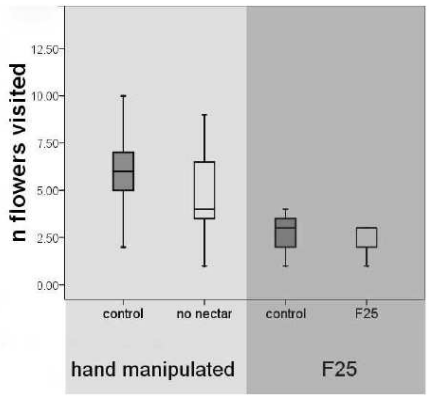

Figure 3: Box plots of visited flowers per plant and *M.sexta* flight in hand manipulated no nectar plants and F25 compared to control *P.axillaris* plants. The upper and lower limit of the box depict the 1st and 4th interquartile, the middle line is the median. Error bars depict the variance with maximal value on top and minimal value on the bottom.

The number of flowers visited was not significantly different between manually depleted flowers (median 4 flowers) and the control (median 6 flowers) (Wilcoxon Signed Ranks test; N=20, Z=-1.559, p=0.119). Also the number of flowers visited on F25 (3 flowers) and control plants (3 flowers) were not significantly different (Wilcoxon signed rank test; N=20, Z=-0.885, p=0.376) (figure 3). Note that differences in median between experiments are due to differences in the number of open flowers.

First approach

There was no significant difference in first approach between no nectar and control plants in the hand manipulated assay (9 vs. 11, χ^2 =0.06, df=1, p=0.8). Similarly, there was no significant difference between F25 (8 first approaches) and control plants (13 first approaches) (χ^2 =0.76, df=1, p=0.38).

Seed set per flower and single visit

	d.f.	ss	ms	F	*p*
line	1	13494	13494	0.485	0.488
pollination	1	11320	11320	0.407	0.526
line*pollination	1	138252	138252	4.967	**0.029**
error	78	2170976	27833		

d.f.= degrees of freedom
ss= sum of squares
ms= mean square
F= *F*-ratio
p= p-value

Table 2: ANOVA of seed set capacities including the effects of line (*P.axillaris*, F25), pollination method (hand-pollination, Manduca pollination) and pollination-line interactions.

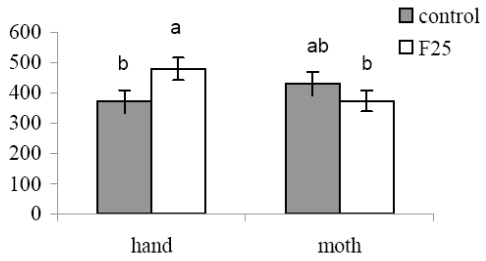

Figure 4: Mean seed set rate of low nectar line F25 and *P.axillaris* with standard error, both in hand- and *M.sexta* pollination treatments.

There was a significant interaction between line and pollination method (table 2): seed set was significantly higher (+29%) in F25 than in *P. axillaris* when hand-pollinated, but not when both lines were pollinated by moths. Additionally, hand-pollinated F25 had a significant increase in seed set compared to moth-pollinated F25 (+28%) (figure 4).

Discussion

A novel approach

Previous studies on nectar-pollinator interactions have been limited to nectar depletion or addition. While this approach is useful for testing changes in pollinator behaviour or either costs *or* benefits of a change, costs *and* benefits and hence fitness consequences

cannot be assessed. Our alternative approach may eventually allow us to measure fitness consequences of a *Petunia* introgression line with decreased nectar production.
The F25 introgression line was selected for low nectar but for similarity to the recurrent parent in all other measured phenotypic parameters. F25 was homozygous for *P. axillaris* at 70 randomly chosen marker loci, indicating that it retained less than the expected 12.5% of the *P. integrifolia* genome. The phenotype of the low nectar line flowers (F25) was similar to that of the *P. axillaris* parent in every other investigated aspect except scent emission. Most importantly, pollinators treated hand manipulated flowers and low nectar lines in similar ways in our experiments. Therefore, our introgression approach offers a valid method to investigate the net fitness consequences of reduced nectar volumes in an otherwise unaltered phenotypic background for the first time.

Potential benefits of reduced nectar production

Energy that is conserved due to nectar reduction can be reallocated within the plant (Southwick 1984). We found that two pollination-related traits in our low nectar line as candidates for signs of reallocated resources: the increased volatile production and the increased seed set. Low nectar lines emitted methylbenzoate in a 68 fold concentration compared to *P.integrifolia* and double the amount of *P.axillaris*. Seed set of *P. integrifolia* is approximately 20 % compared to our selection line, which makes it unlikely that the higher seed set in F25 is due to hitchhiking of *P.integrifolia* alleles linked to the nectar locus. Olfactory stimuli like methylbenzoate are known to play a role in hawkmoth orientation and elicit a feeding response (Hoballah et al. 2005, Raguso and Willis 2005). As our greenhouse was scent-saturated, future field tests are needed to

validate how the increased odour production in our low nectar line may affect pollinator behaviour.

Pollinator decisions as control mechanisms

In our experimental setup, we only found one behavioural adjustment of pollinators that may potentially act as a control mechanism against cheating plants: the probing duration of a flower was longer in interactions with rewarding plants. With respect to the two other non-significant variables we tested, we note that prior discrimination and avoidance of nectarless flowers by naïve insects seems to be generally rare (Thakar et al. 2003), with only a few partial exceptions (Goulson 1999). In contrast, the lack of difference in the number of flowers visited does not correspond to optimal foraging theory (Krebs 1977, Pyke 1984) and the results from other studies (e.g. Mitchell 1993, Hodges 1995), including results on *Petunia*-hawkmoth interactions (Brandenburg et al. unpublished). With respect to our search for pollinator behaviours that may select against reduced nectar production by plants, reducing the number of visited flowers on low nectar plants may have negative or positive effects on the plants' fitness. This is because the optimal number of flowers visited per pollinator for a plant depends on many variables like a plant's level of self-incompatibility (Levin et al. 2009), its level of sexual segregation (Harder et al. 2000), the population densities of both plant and pollinators (Ågren 1996, Barrett et al. 2004), the population densities of alternative plant host species (Raine et al. 2007), pollinator foraging strategies (Waddington 1983) and distance between patches (Cresswell 2000, Internicola et al. 2006). In the case of *Petunia*, pollinators visiting fewer flowers in response to low nectar should cause selection against reduced investments in

nectar as several studies found that hawkmoth-pollinated plants are actually pollinator-limited (Vesprini and Galetto 2000, Luyt and Johnson 2001, Wolff et al 2003). The occurrence of pollinators visiting fewer numbers of flowers on low-nectar plants may well depend on plant densities in our study system (Brandenburg et al. unpublished). Our experiment on seed set indicates that shorter visitations per flower reduce the female reproductive potential of the low nectar lines, suggesting that pollen deposition is a function of probing duration (Mitchell and Waser 1992). Previous studies have compared fitness of nectarless vs. nectariferous plants in different species and/or different habitats and hence could not look at costs *and* benefits (Montgomerie 1984, Harder 1986, Mitchell and Paton 1990, Mitchell and Waser 1992, Cresswell 1999, Collins 2008). Our cost-benefit analysis of the consequences of low nectar production on one fitness component (seed set/flower/visit), suggest selection neutrality. Therefore, low nectar plants must suffer a fitness reduction compared to average plants with respect to other fitness components so that cheating is under negative selection. Most importantly, the effects of the pollinators' behaviour on the male reproductive success of low nectar plants have to be incorporated. Links between low nectar production and pollen production as well as links between duration of visit and pollen uptake must be determined. Apart from a low uptake of pollen, pollinators may reduce a cheating plant's reproductive success by switching to a different plant species in response to low nectar quantities (reducing their flower constancy, Jacobi et al 2005). A final complication for a complete fitness analysis is that low nectar plants may also gain additional benefits that would not be detected in behavioural experiments, for example an edge in competitive abilities with neighbours

and/or the ability to produce a larger number of flowers per reproductive season/during their lifespan.

Conclusions

Understanding the causes for stable nectar production in most flowering plant species has been a major challenge. The new approach described here using low nectar introgression lines allows for the first time proper cost-benefit analyses within a species for each of the fitness-relevant components affected by nectar production and the interaction with pollinators. This approach will thus hopefully provide more comprehensive answers regarding the evolution and stability of plant-pollinator mutualisms as well as identification of the ecological conditions under which the mutualism breaks down, as has happened repeatedly and most famously in orchids (Jersakova et al. 2006).

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Chapter III

Genetic and phenotypic characterization of an introgression line derived from *Petunia axillaris* with reduced nectar volume

Anna Brandenburg and Cris Kuhlemeier

Unpublished data

Genetic and phenotypic characterization of an introgression line derived from *Petunia axillaris* with reduced nectar volume

Anna Brandenburg[1,2] and Cris Kuhlemeier[1]

[1]Institute of Plant Science, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

[2]Institute of Biology, University of Neuchâtel, Emile Argand 13, CH-2009 Neuchâtel, Switzerland

Introduction

The co-evolution between flowering plants and pollinators is considered to be one important aspect contributing to the wide diversity of angiosperms (Grant 1994, Galen 1996). Floral traits are adapted to certain guilds of pollinators that impose distinct selection pressures on traits like scent, color, shape and nectar. Specific combinations of these floral characteristics are known as pollination syndromes (Fægri and van der Pijl 1979). Nectar is one of the most important traits for the maintenance of the plant-pollinator mutualism. In evolutionary ecology, a major question is what forces the plant to produce costly nectar and under which conditions nectar reduction can be an evolutionarily stable strategy for the plant.

Volume, composition and secondary compounds of nectar define and regulate the length of the foraging bout and species identity of pollinators (Brandenburg et al. 2009). Plants that offer high nectar volumes are for example visited by pollinators with high metabolic rates like hummingbirds or hawkmoths (Heinrich and Raven 1972). The differences in nectar volume can be easily measured with a pipette. However, tremendous environmental variation constrains unraveling the genetic basis of nectar traits (Mitchell 2004).

The flower organ producing nectar is the nectary. These can not only be situated in various positions in angiosperms (Nicolson 2007), but also differ considerably in cytological and ecological aspects. Nectary is a term with ecological significance, describing the location where sugar fluids involved in interaction with floral visitors are produced and secreted (Pacini et al. 2003). Several transcription factors involved in

nectary development have been identified (Bowman and Smyth 1999, Ge et al 2000, 2001, Lee et al. 2005a, b) and a recent nectary transcriptome study identified 270 genes that were expressed in nectaries of *Arabidopsis* (Kram et al. 2009). In contrast to that, the genetic basis of floral nectar production remains relatively unclear.

The genus *Petunia* integrates the advantages of having been intensely studied for many years, being genetically accessible and being a decent system for behavioral studies. The presence of a number of traits determining divergent pollination syndromes within cross-compatible species makes *Petunia* an ideal model system to study the genetics of plant-pollinator coevolution.

The genus *Petunia*

The genus Petunia comprises at least 14 species (Stehmann et al. 2009) and is native to South America. Most of these species can be classified in two major groups based on floral traits that are important for pollinator attraction. *P.axillaris* subsp. *axillaris, parodii* and *subandina* have a large white corolla, a long floral tube, emit sweet fragrance at dusk and produce large volumes of nectar. These species are pollinated by nocturnal hawkmoths. Most *Petunia* species show a flower morphology similar to *P. integrifolia,* such as *P. mantiqueirensis, P. scheideana, P. saxicola* and *P. reitzii*. These species display purple colored limbs with a shorter tube and produce very little nectar. Main pollinators of *P. integrifolia* belong to the order of hymenopterans. Hybridization of *P. axillaris* and *P. integrifolia* has not been reported, presumably caused by reproductive isolation due to pollinator preference. Additionally, a species endemic to a small region

of Southern Brazil, *P. exserta,* displays all floral traits that infer ornitophily. However, hummingbird pollination has not yet been observed in native habitats of *P. exserta*.

Nectary development

One major regulator of nectary development in core eudicots is *CRABS CLAW* (*CRC*), (Lee et al. 2005 a,b). *CRC* is not expressed in nectaries of one basal eudicot species (*Aquilegia*), suggesting that it is not required for nectary development in this species. Phylogenetic analysis indicates that *CRC* (a putative transcription factor of the YABBY family) might have been recruited as a regulator of carpel development in the last common ancestor of angiosperms (Lee et al. 2005 a). Putative orthologues of *CRC* are expressed in gynoecium tissue of *Amborella trichopoda*, a flowering plant that represents one of the earliest diverging groups of angiosperms. Specialized nectaries are absent in these species, however, it is noteworthy that stigmas are highly secretory (Fourquin et al. 2005). In *Petunia hybrida*, *CRC* expression was found not only in nectaries but as well in developing carpels and stamens (Lee et al. 2005b). *crc* mutants of *Arabidopsis* lack nectaries (Bowman and Smyth 1999, Lee et al. 2005a) and thus fail to produce nectar. They furthermore exhibit strong anomalies in carpel development. *CRC* is probably a master regulator necessary in the initial steps of the carpel formation. It functions independently of the ABC(DE) genes that determine floral organ identity. Therefore nectaries can develop in the absence of the activity of ABC homeotic genes. However, they do influence nectary development as ectopic expression of *CRC* in conjunction with genes such as *UFO* results in development of ectopic nectaries at the basis of flower

pedicels (Baum et al. 2001). So far, *CRC* function in nectary development of *Petunia* has not been described and is currently being investigated.

Nectar production and nectar composition

Previous work has highlighted in *Echium* and *Nicotiana*, that nectar production is a quantitative trait characterized by relatively low heritability (Leiss et al. 2004, Kaczorowski et al. 2008). Only few genes involved in nectar production and composition have been identified. By differential display RT-PCR, a nectary-specific cDNA clone was obtained in *Petunia hybrida* and the deduced amino acid sequence revealed *NEC1*. It is assumed to play a role in nectar secretion and nectary development (Ge et al. 2000), due to its high expression in nectary tissue. However, partial downregulation of this gene resulted in a normal nectary phenotype with defects in anther dehiscence (Ge et al. 2001). NEC1 and two homologues termed NEC2 and NEC3 have been mapped in a *P.axillaris axillaris* N x *P.integrifolia inflata* S6 backcross 1 (BC_1) population. NEC1 was located on Chromosome VII, NEC2 on Chromosome IV and NEC3 on Chromosome VI (Gübitz et al. 2009).

The major constituents of floral nectar are mono- and disaccharides, mainly glucose, fructose and sucrose, derived mostly from the degradation of starch that accumulated in the nectaries during the early stages of carpel development and in part directly from the phloem (Ren et al. 2007). Recently, it was discovered that genes involved in starch metabolism in floral nectaries are tightly correlated with nectary development: starch anabolic genes (e.g. sucrose and starch synthases) are expressed early in developing nectaries, breakdown genes (e.g. starch debranching enzymes) are upregulated prior to

anthesis and nectar production (Ren et al. 2007 a, b). It was also demonstrated that the breakdown of starch resulted in an increased influx of sucrose into the nectaries.

Genetic control of nectar volume in Petunia

Nectar volume is regulated by multiple genes and is a genetically complex trait (Galliot et al. 2006). Genes that contribute to complex traits (also known as quantitative trait loci, or QTL) pose special challenges that make gene discovery difficult.

QTL analysis aims at identifying statistical associations between molecular marker genotypes and phenotypic traits in segregating progeny. This can be used to detect and map loci contributing to the expression of quantitative traits (Doerge 2002). A QTL is a region of the genome that is responsible for variation in the quantitative trait of interest, in this case nectar volume.

Previously, two studies on the genetic control of nectar production were conducted in *Petunia* (Stuurman et al. 2004, Galliot et al. 2006). In the study of Stuurman et al. (2004), backcross recombinant inbred lines (BILs) were generated using as donor parents *P. integrifolia inflata* S6 and *P. axillaris parodii* S7 and as recurrent parent *P. hybrida* W138 (Stuurman et al. 2004) because the two donor lines could not be directly crossed due to cross incompatibility. The aim was to introgress *P. integrifolia inflata* S6 loci (WI-BIL) respectively *P. axillaris parodii* S7 loci (WP-BIL) and to fix them by further selfing, to compare the phenotypic effects of species-specific genome introgressions in the common genetic background of *P. hybrida* W138. This line contains a highly active DNA transposon called d*Tph*1 which was often used to isolate genes in *Petunia* through transposon tagging (Gerats et al. 1990).

QTL mapping was done in WI-BILs and WP-BILS and two nectar volume QTL termed *VOL1* and *VOL2* on Chromosomes II and VI were identified in the WI-BILs. The summed additive effects of *VOL1* and *VOL2* (89% of 6 µl parental difference) are unexpectedly large considering the environmental variation in this trait. Small population sizes are one possible explanation for overestimating these QTL effects (Beavis 1996).

Galliot et al. (2006) found 4 minor QTL linked to nectar volume in an interspecific backcross (BC) population between *P. integrifolia inflata* S6 and *P. axillaris axillaris* N, using *P. integrifolia inflata* S6 as recurrent parent. These QTL mapped to chromosomes III, IV, V, and VI, and additively explained 70% of the difference in nectar volume. Interestingly, as previously described for *VOL1* (Stuurman et al. 2004), these nectar QTL were also closely linked to morphology QTL. Both studies mainly used amplified fragment length polymorphisms as molecular markers for QTL mapping. These two studies share only one QTL for nectar volume on chromosome VI.

Altogether, these studies demonstrate that nectar volume is a quantitative trait with complex genetic control. The high environmental influence on nectar volume poses an additional challenge to the phenotypic characterization and a precise estimation of the genetic effects (see also table 1). A major precondition to unravel the genetics underlying nectar volume is to conduct all nectar volume measurements under controlled conditions.

P. integrifolia inflata S6	P. *axillaris axillaris* N	*P. axillaris parodii* S7	N flowers per plants measured	Study
1.21+/-0.92	-	12.8 +/- 2.57	10-40/unknown	Stuurman et al. 2004
2.2 +/-1.5	16.8+/ -5.9	-	10/5	Galliot et al. 2006
1.35 +/-0.47	34.7+/ -6.8	-	10/2	Brandenburg et al. unpublished

Table 1: Nectar volume differs between Petunia species *P. integrifolia inflata* S6, *P. axillaris axillaris* N and *P. axillaris parodii* S7 but also within the species. The phenotypic measurements were conducted in a similar way. However, the age of flowers in the study of Galliot was variable. The variation in mean and standard variation underlines the high environmental variation in this trait.

Aim of this study

One aim of this study was to breed a low nectar line that displays all other floral characteristics similar to *P. axillaris axillaris* N. The genotyping of such lines is going to shed light into the genetic architecture of nectar production with the ultimate aim of identifying the genes underlying nectar volume QTL. An important question will be to assess whether the identified genes are so called speciation genes. Speciation genes code for isolating mechanisms that prevent gene flow between organisms and finally lead to reproductive isolation. If reproductive isolation is complete, two organism groups can be named species ("biological species concept", for an overview see Coyne and Orr 1998). If the reduction of nectar volume in a *P. axillaris* line can be an isolating mechanism is unclear. In a laboratory set up, we have detected that nocturnal pollinators do not discriminate against low nectar plants but reduce their visitation time and the number of flowers visited (chapter 5). Further studies are needed to evaluate whether nectar traits are an isolating mechanism.

The low nectar line was obtained by introgressing the low nectar volume phenotype from *P. integrifolia inflata* S6 into the *P. axillaris axillaris* N genetic background. This low

nectar line was used in behavioral assays with *Manduca sexta*. The major advantage of using lines with reduced volumes in contrast to previous approaches, where nectar was manually removed, is that this line enables us to do a proper cost-benefit analysis. We can assess the costs that are involved in nectar production to the benefits of nectar reduction e.g. in terms of female fitness, measured as seed set in hand-pollinated plants. We can compare these results to female fitness of low nectar lines that are pollinated by a hawkmoth. For a detailed description of the behavioral experiments and results, see chapters 2, 4 and 5.

Material and methods

Plant material

P.axillaris axillaris N and *P.integrifolia inflata* S6 were kindly provided by Ronald Koes, Department of Genetics at the Vrije Universiteit Amsterdam (The Netherlands). They have been maintained in the laboratory for many generations by inbreeding and sib mating respectively (Galliot et al. 2006). Both plants originate in South America and display distinct pollination syndromes (sensu Faegri and van der Pijl 1979): *P.axillaris axillaris* belongs to the group of hawkmoth-pollinated species, with a large white corolla, a long and narrow floral tube, UV absorbing petals and large nectar volumes whereas *P. integrifolia* displays all characteristics involved in bee attraction: the petals are smaller and purple-colored, the floral tube is short and its opening wide. Furthermore, these plants produce fairly low amounts of nectar (table 1, appendix). Despite overlapping geographical distributions of both species in some areas in Uruguay, both species are

reproductively isolated (Hoballah et al. 2007). A hybrid barrier is more likely explained by prezygotic barriers due to divergent pollinator preferences rather than a postzygotic hindrance. *P. axillaris axillaris* and *P. integrifolia* can be cross-fertilized in the lab and produce fertile offspring.

Growth conditions

Plants were grown in plastic pots in soil and kept in greenhouses under long day conditions (16 hours light). The backcross 1 (BC_1) population was grown in a different greenhouse than BC_2 and BC_2F_1/BC_3 populations. The temperatures in both greenhouses ranged from 15 to 37°C in summer. They were fertilized twice a week and watered daily, twice on very hot days (10 am and/or 4 pm).

Phenotyping of floral traits

Tube length and corolla diameter were exactly measured as described in Galliot et al. 2006. In contrast to Galliot, where nectar volumes were measured of open flowers with variable ages ("second last flower at anthesis"), flowers used in this study were marked at the day of opening (1^{st} day of anthesis) and measured the following day. Therefore, the floral tube was cut at two places: the point where the anthers detach from the tube and on the bottom below the gynoecium. The floral tube was transferred to a centrifuge tube (0.5 ml) with three holes pierced in the bottom. This tube was placed in a regular (1.5 ml) Eppendorf tube and centrifuged at 7000 rpm for 10 seconds. The nectar collected in the regular Eppendorf tube was measured with a 10 µl Eppendorf pipette and a calibrated tip. Nectar volume measurements for selected lines were repeated 10 times. Measurements

were distributed over 4 months and always done one day after anthesis. The nectar measurements were conducted at 6 pm or later, as initiation of nectar secretion coincides with scent production at dusk in *Saponaria officinalis*, a hawkmoth-pollinated species (Wolff et al. 2006).

Genotyping of the low nectar line

The molecular markers that were used for genotyping the low nectar line F25 were polymerase chain reaction (PCR)-based co-dominant markers. We used single sequence repeat (SSR) markers, derived from the EST library of the National Center for Biotechnology Information (NCBI), cleaved amplified polymorphic sequence (CAPS) markers, and cosmid (COS) markers derived from tomato orthologous sequences (Bossolini et al. submitted). We used 55 SSR, 10 CAPS and one COS marker to search for *P. integrifolia* introgressions in the low nectar line, thus in total 66 markers.

For detailed description of the primers used for amplification, the PCR settings and pictures of the gels see the appendix.

Results

Breeding design

An interspecific cross between *P. axillaris axillaris* N and *P. integrifolia inflata* S6 with *P. integrifolia inflata* S6 as pollen donor produced an F_1 from which a single plant served as seed parent for a backcross with *P. axillaris axillaris* N. Progeny plants were selected according to low nectar volumes and subsequently backcrossed to *P.axillaris axillaris* N.

This is in contrast to the study of Galliot (et al. 2006), where *P. integrifolia inflata* S6 was chosen as recurrent parent for backcrossing. Backcrossing with *P. axillaris axillaris* N as recurrent parent was necessary to establish low nectar lines that display all other floral traits of *Petunia axillaris axillaris* N that are known to be involved in hawkmoth attraction. A backcrossing design similar to that of Galliot would not have been suitable for this purpose. *P. integrifolia inflata* S6 has a mean of 1.35 µl and *P. axillaris axillaris* N 34.7 µl (appendix). The nectar volume of the F_1 was not measured.

Selection of low nectar lines

In the BC_1 population, 130 seedlings were grown and phenotyped. 24 plants with the highest similarity to *P.axillaris axillaris N* and nectar volumes below 7 µl were selected. These 24 plants were backcrossed to *P. axillaris axillaris* N, resulting in 24 BC_2 lines. 25 seedlings were grown for each of the 24 BC_2 lines (in total: 600 plants). These 600 plants were screened for nectar volumes lower than 11 µl. Three flowers were measured per plant. The 43 plants that had nectar volumes in all three measurements consistently under 11 µl were phenotyped ten times for all floral traits under investigation and are listed below. Out of these 43 BC_2 plants, the 5 with the highest similarity to *P. axillaris axillaris* N were selected. These were again backcrossed to *P.axillaris axillaris* N and also selfed, resulting in 10 BC_3 and BC_2F_1 lines from each of which 28 seedlings were grown. These 280 BC_3 and BC_2F_1 plants were again screened three times for nectar volume below 11 µl. 20 plants remained and were phenotyped 10 times. During the selection process with the "*Petunia axillaris* similarity index" (PASI; for detailed description see Chapter 2) we found one BC_3 line ("F25") with almost identical

phenotype to *P .axillaris axillaris* N and clearly reduced nectar volume. A detailed breeding scheme is displayed in figure 1.

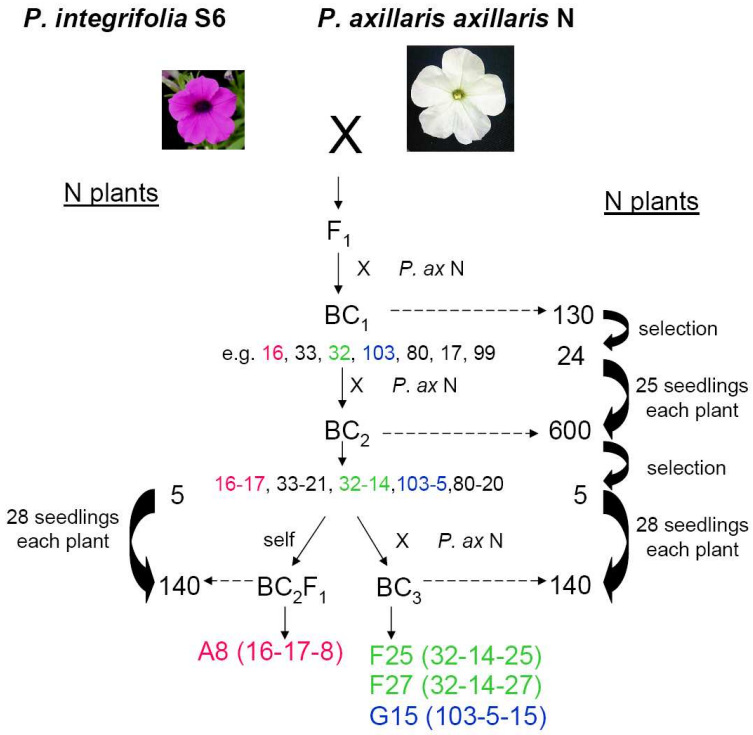

Figure 1: breeding scheme used to establish low nectar line F25. From a single F1 plant backcrossed to *P. axillaris axillaris* N (*P. ax* N), 130 BC_1 plants were obtained. From these, 24 were selected (7 shown in scheme) for backcrossing to P. ax N. 600 plants were obtained in the BC_2. From these, 5 plants (all shown in scheme) were selected for backcrossing to P. ax N and the same 5 for selfing. 2*140 (BC_2F_1/BC_3) plants were obtained. Out of the 280 plants, the low nectar line, a BC_3 (F25), was selected.

Distribution of phenotypes in the BC_1 population

During the phenotyping and selection process, the segregation of all measured phenotypes in BC_1 population and the segregation of nectar volumes in all other screened populations (BC_2, BC_3, BC_2F_1) were examined (figure 2 and 3). A visual display of segregation can give an idea about the inheritance of the trait in question.

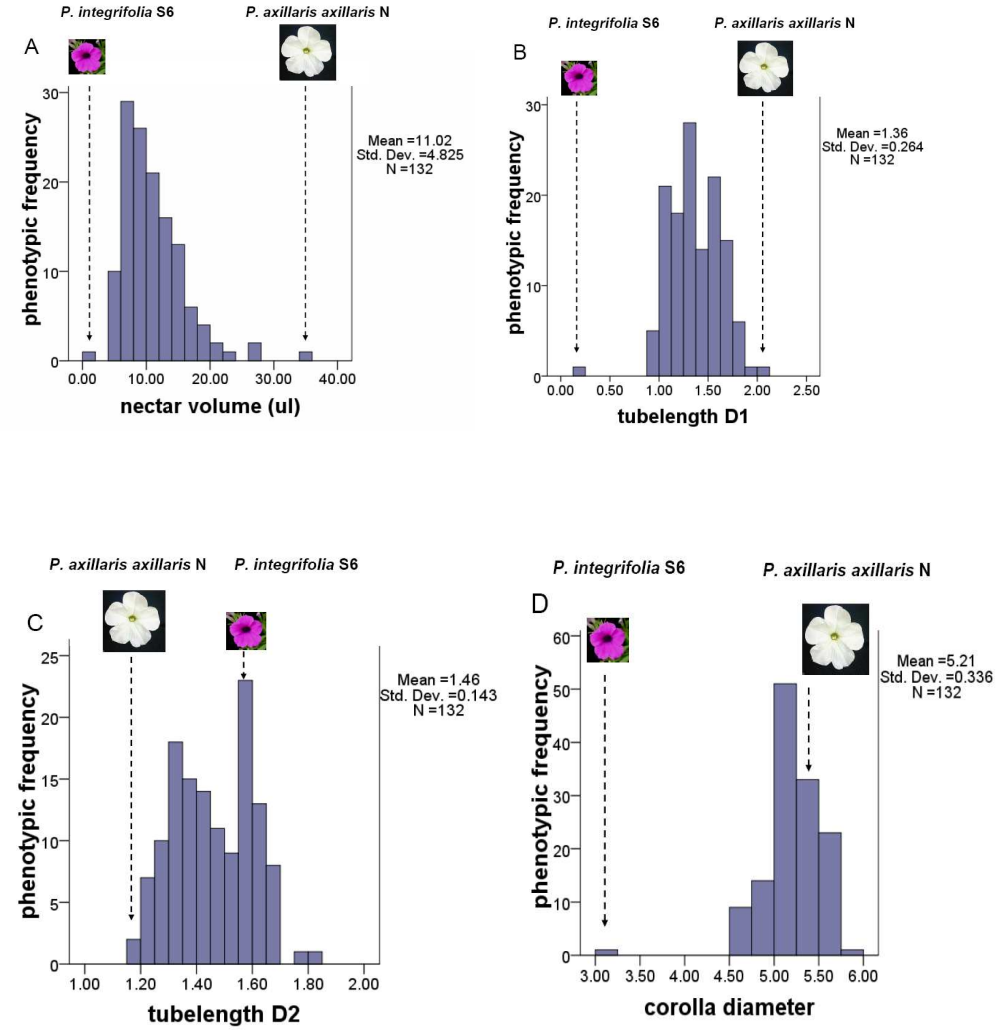

Figure 2: Segregation of phenotypic traits in the BC_1

A. Segregation of nectar volume. *P. axillaris axillaris* N and P. *integrifolia inflata* S6 had the largest and lowest nectar volumes respectively. The 24 plants that were used for the backcrossing had <7 µl
B. Segregation of tubelength D1. *P. axillaris axillaris* N and P. *integrifolia inflata* S6 had the longest and shortest floral tubes respectively. The 24 plants that were used for backcrossing had tubelengths D1 ranging from 0.92-1.67 cm
C. Segregation of tubelength D2. *P. axillaris axillaris* N had the shortest tubelength D2 (1.2 cm), and *P. integrifolia inflata* S6 had an intermediate tubelength (1.6 cm). Note that there are many transgressions in this category. The 24 plants that were used for backcrossing had tubelengths D1 ranging from 1.21-1.68 cm.
D. Segregation of corolla diameter. P. *integrifolia inflata* S6 had the smallest corolla and *P. axillaris axillaris* N an intermediate-large corolla (5.4 cm). Note that there are transgressions in this category.

Distribution of nectar volume in the BC_2, BC_2F_1 and BC_3

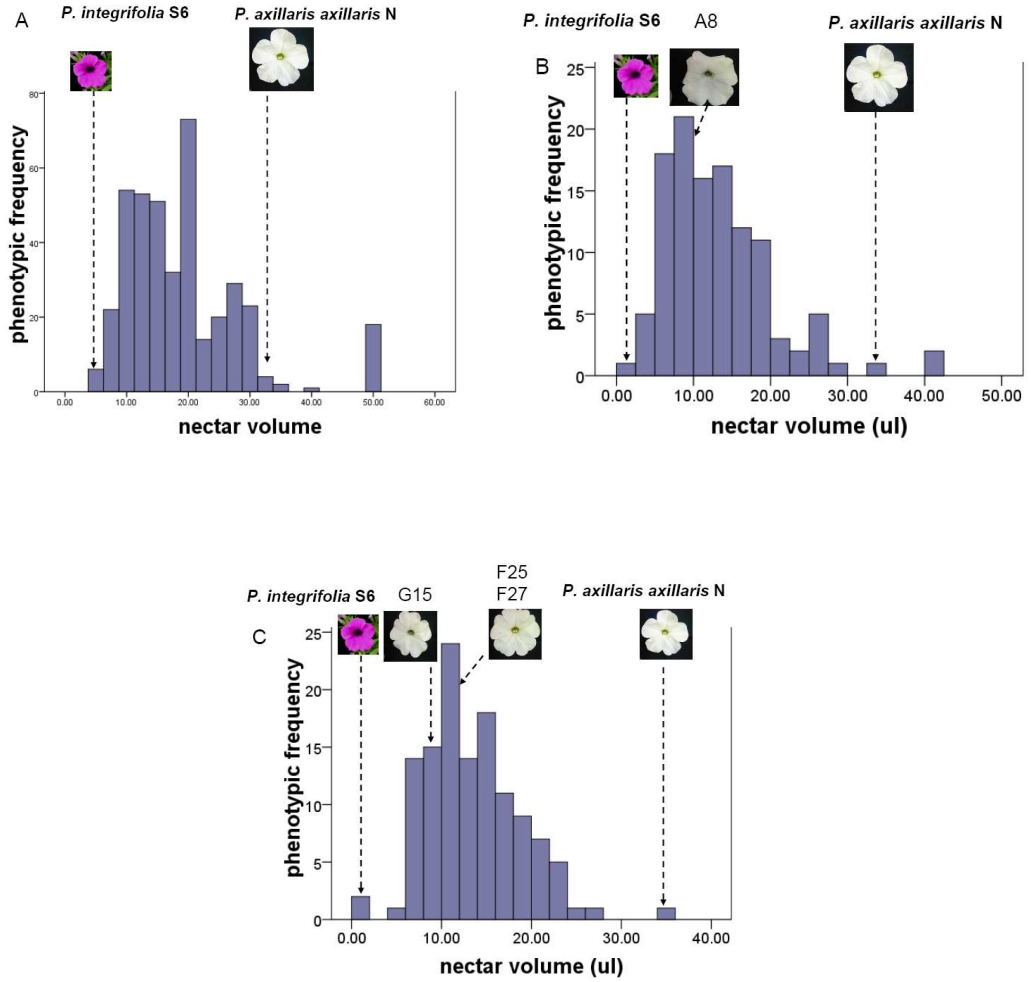

Figure 3: segregation of nectar volumes in backcross populations that were derived from low nectar plants

A. segregation of nectar volume in the BC_2 population
B. Segregation of nectar volume in the BC_2F_1 population, including one plant used in the genotyping (A8)
C. Segregation of nectar volume in the BC_3 population, including plants used in the genotyping (A27, G15) and the low nectar line F25

The low nectar line F25

Phenotypically, F25 resembles *P.axillaris axillaris* N except for nectar volume, as well as concentration and scent emission (table 2).

Plant	**nectar volume (µl)**	**tubelength D1(cm)**	**D2 (cm)**	**limb diameter (cm)**	**MB emission (pptv)**	**corolla color**	**Pollen color**
G15 BC_3	8.25+/-2.3	1.3+/-0.06	1.4+/-0.1	4.8+/-0.3	25723+/-4384	white	green
A8 BC_2F_1	8.4+/-4.7	1.4+/-0.07	1.3+/-0.05	5.1+/-0.3	36136.2+/-59791.04	white	green
F27 BC_3	8.85 +/-4	1.9+/-0.08	1.3+/-0.1	5.6+/-0.3	23982+/-6211	white	yellow
F25 BC_3	10.35 +/-4	2.1+/-0.05	1.3+/-0.1	5.6+/-0.2	29912+/-15345	white	yellow
***P. axillaris axillaris* N**	34.65 +/- 6.8	2.1+/-0.1	1.2+/-0.1	5.4+/-0.3	158075+/-479	white	yellow
***P. integrifolia inflata* S6**	1.35+/- 0.5	0.15+/-0.01	1.6+/-0.1	3.1+/-0.2	436+/-57	purple	blue

Table 2: mean phenotypic values of low nectar plants including low nectar line F25, as well as *P.axillaris* (*P.ax*) and *P. integrifolia* S6 (*P.int*)

The genotyping revealed no *P. integrifolia inflata* S6 allele in none of the 66 molecular markers tested in the low nectar line F25 (table 3). For the original gel images, see appendix.

Marker	PM7	PM8	PM10	PM15	PM17	PM19	PM32	PM37	PM40	PM42
P.axillaris	A	a	a	a	a	a	a	a	a	a
P.integrifolia	b	b	b	b	b	b	b	b	b	b
F25	a	a	a	a	a	a	a	a	a	a

	PM44	PM77	PM81	PM94	PM101	PM103	PM106	PM107	PM109	PM110
P.axillaris	a	a	a	a	a	a	a bad quality	a	a	a
P.integrifolia	b	b	b	b	b	b	b	b	b	b
F25	a	a	a	a	a	a	a	a	a	a

	PM111	PM114	PM119	PM120	PM121	PM128	PM130	PM132	PM134	PM137
P.axillaris	a	a	a	a	a	a	a	a	a	a
P.integrifolia	b	b	b	b	b	b	b	b	b	b
F25	a	a	a	a	a	a	a	a	a	a

	PM141	PM144	PM149	PM150	PM157	PM163	PM164	PM166	PM168	PM171
P.axillaris	a	a	a	a	a	a	a	a	a	a
P.integrifolia	b weak	b	b	b	b	b	b	b	b	b
F25	b	a	a	a	a	a	a	a	a	a

	PM183	PM186	PM188	PM189	PM190	PM191	PM192	PM193	PM195	PM197
P.axillaris	a	a	a	a	a	a	a	a	a	a
P.integrifolia	b	b	b	b	b	b	b	b	b	b
F25	a	a	a	a	a	a	a extra bands	a	a	a

	PM198	PM200	PM202	PM219	AN2	ADH1	ADH2	CHI A	BSMT 1	C4H 1
P.axillaris	a	a	a	a	a	a	a	a	a	a
P.integrifolia	b	b	b	b	b	b	b	b	b	b
F25	a	a	a	a extra bands	a	a	a	a	a	a

	F3H	SAMS 1	AN11	HF1	COS 13
P.axillaris	a	a	a	a	a
P.integrifolia	b	b	b	b	b
F25	a	a	a	a	a

Table 3: genotyping for 55 SSR markers (PM 7-219), 10 CAPS markers (AN2 – HF1) and one COS-marker (COS 13, from tomato) fail= failed PCR), extra bands (see discussion)
a stands for *P. axillaris axillaris* N and
b for *P. integrifolia inflata* S6.

Discussion

A low nectar line with high similarity to *P. axillaris axillaris* N was derived by recurrent backcrossing and selection of plants derived from an initial cross between *P. axillaris axillaris* N and *P. integrifolia inflata* S6. All other characteristics known to be involved in hawkmoth attraction show a high similarity to *P. axillaris axillaris* N (chapter 2).

The low nectar line F25 produces only a third of the nectar volume of *P. axillaris axillaris* N. The genetic introgression from *P. integrifolia inflata* S6 responsible for this phenotype is under investigation. It is possible that some of the QTL involved in nectar production control nectary development, whereas some others are physiological QTL

affecting starch metabolism. While comparing F25 with the parental line *P. axillaris axillaris* N, no difference in number of nectar-releasing pores on the nectary surface could be observed. In contrast, nectaries of *P. integrifolia inflata* S6 are much smaller feature only half of the pores and are smaller in size (appendix). One possible reason of low nectar volumes of F25 might be that resources which are used by *P. axillaris axillaris* N to produce nectar are reallocated to produce scent in the low nectar line. Indeed, methylbenzoate in low nectar lines is emitted in a twofold, but nonsignificant concentration (chapter 2, appendix). Increased volatile emission might thus be a product of saving and reallocating glucose molecules. Raguso (2001) presents a scheme, in which glucose molecules can be converted to methylbenzoate after cycling through various metabolic pathways (figure 1).

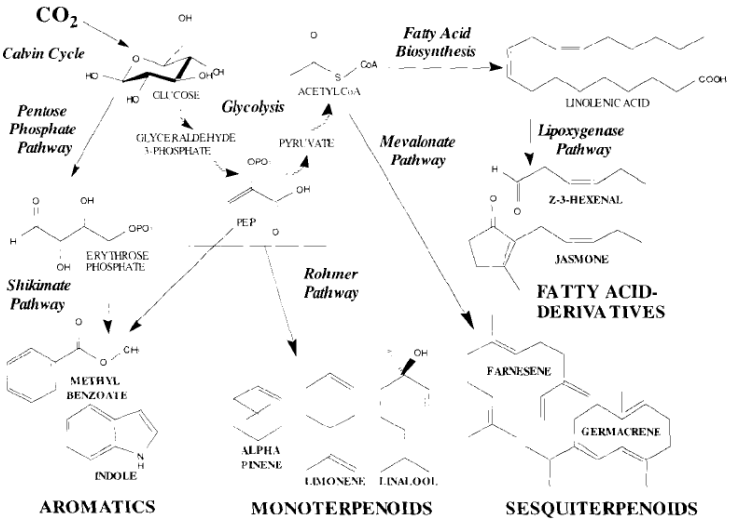

Figure 1: major biosynthetic routes that lead from glucose to volatile production (from Raguso 2001)

In contrast to previous studies done with transgenic plants (Ge et al. 2001), the introgression lines developed in this study allow performing behavioral assays for fitness analysis. This line enabled a proper cost-benefit analysis in plants with reduced nectar

volumes. This was not possible with transgenic lines derived by partially silencing the *NEC1* gene (Ge et al. 2001). The transgenic plants had a normal nectary phenotype but defective anthers and therefore a reduced male fecundity ("early open anther phenotype"). Therefore they could not be used to determine fitness. Unfortunately, nectar volumes were never measured in these plants. Furthermore, transgenic lines would not have been accepted in field experiments in Uruguay.

The introgression of *P. integrifolia inflata* S6 alleles into the low nectar line F25 were searched with a set of the 66 previously mapped single locus markers like SSR and CAPS (Bossolini et al., submitted). No marker loci retaining the *P. integrifolia inflata* S6 allele were identified. By backcrossing without selection we would have expected 12.5% of the genomic markers to be heterozygous, corresponding to approximately 8 markers. This indicates that the selection was very efficient in eliminating most of the *P. integrifolia inflata* S6 introgressions not involved with nectar volume determination. The selection index PASI was designed to identify those plants with the lowest nectar volumes yet the highest phenotypic similarity to *P. axillaris axillaris* N. Thus, it is not surprising that the similarity of the selected low nectar line to *P. axillaris axillaris* N is higher than expected by chance. The backcrossing and selection of a low nectar line by phenotypic characterization was effective in recovering *P. axillaris axillaris* N allele at all marker loci so far tested. However, it can not be excluded that the low nectar volumes in the F25 line are due to a *P.axillaris axillaris N* breeding-induced mutation or epigenetic inactivation. To clarify this, further genotyping of this line with multilocus markers like AFLP is currently conducted. It is unlikely that nectar volume is controlled by a single locus (Galliot et al. 2006), and thus it is still unclear whether F25 is retaining only one or

more QTL for nectar volume. The quantitative nature and the high environmental influence of this character hinder proper segregation analysis, and without closely linked markers it is extremely challenging to speculate on the number of QTL introgressed from *P. integrifolia inflata* S6.

Due to self-incompatibility and the resulting heterozygosity, there is a lot of variation in the genotypes of different *P. integrifolia inflata* S6 individuals, which can be seen in the additional bands on PM219 and 192 (appendix) that can presumably be assigned to the *P. integrifolia inflata* S6 plant originally used in the interspecific cross. However, with the original *P.integrifolia* S6 lacking we cannot confirm this assumption.

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Appendix

1. CAPS marker protocol

CAPS	Primer forward		Primer reverse		*Restr.enzyme*	*Buffer (NEB)*	Tm in °C
	Name in primer list	sequences	Name in primer list	sequence			
AN2	B54	ATGGTCACTTATAGCTGG	An2qR2ax	CAAGAAAAAGGATTCATTGCCG	No digestion	2	52
SAMS	B22	GACTTGCCCATGGCTCAGACCAG	B92	CTGCTACTTAACAGTTAACAG	Tsp509	2	47
ADH1	B137	GATTGATCCACAGGCACC	B138	CGTTAAGGCTCCATTAACAGC	No digestion	2	52
ADH2	B99	CGACAGGTACAGGCGAAACGACGATAGATTATG	2Adh2rev	CCACCATCAGTCATCTCAGC	Hae III	2	52
CHI A	CHI A F	ACACCAGTAAAAGTAGAGCAAAAA	CHI A R	ACAAGGGAATTCAGCACTAAAACA	HinfI	2	52
BSMT1	BSMT Fi	CAAATTTTCTCAAGTACCGTTCAG	B106	GTCTTAATTACAATATTTACC	Alu I	2	55
CH4 1	B59	GCGCATTGTTGTCCATGCTC	B83	GAGGTTGAAGCTGTTCAAGG	Dde I	3	55
F3H	B114	GCGGTTTGACATGTCTGGTGGC	B115	CCAATCTTGGACCACTTCACC	Xba I	2	54
HF1	B95	TCCCTCATTAATTAACCATATCTC	B96	CATGGATAGCTACCGAACG	Alu I	2	50
AN11	B116	ATGGAAAATTCAAGTCAAGAATCAC	B117	TTATACTTTAAGCAATTGCAACTT	Alu I	2	52

Table 1: CAPS markers and primer sequences used for genotyping. Note that AN2 and ADH1 are not CAPS markers, as they are not cleaved after amplification.

Mastermix: 2 µl PCR buffer
2 µl $MgCl_2$
0.4 µl dNTPs
0.4 µl RedTAQ DNA Polymerase
0.2 µl forward primer
0.2 µl reverse primer
14.8 µl H_20
20 µl MM(mastermix)
+ 1 µl DNA

PCR regime: Initial denaturation: 94°C 60 sec
94°C 30 sec
Tm 30 sec
72°C 60 sec
} 35 cycles
72°C 3 min

4°C hold

After the PCR is done, digestion (**2 hours**) with the restriction enzyme from the list.

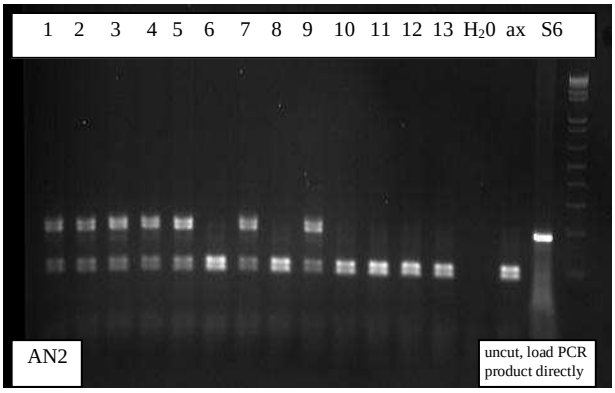
Digest: 0.5 µl buffer (NEB1-4)
0.5 µl restriction enzyme
4 µl H_20
5 µl MM
+10 µl PCR-product

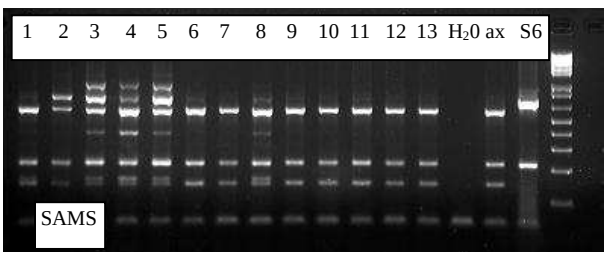
Digestion: 2-3 hours at 37°C
In the meantime: make a 2% agarose gel.
Load samples on agarose gel (no loading buffer, RedTaq already has a dye).
Photo with GelDoc

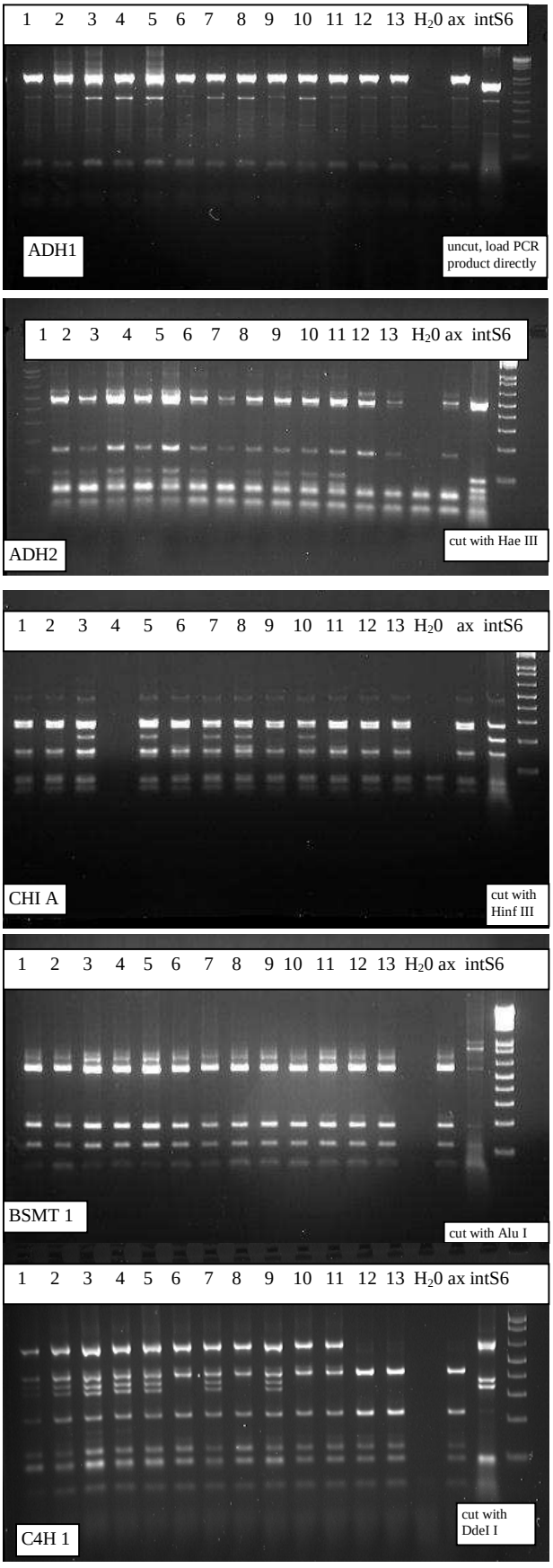
No. on gel	Plant
Ax	*P. axillaris axillaris* N
int S6	*P. integrifolia inflata* S6
1	16 (BC_1)
2	32 (BC_1)
3	99 (BC_1)
4	80 (BC_1)
5	103 (BC_1)
6	32-14 (BC_2)
7	80-20 (BC_2)
8	103-5 (BC_2)
9	A17 (BC_2F_1)
10	G15(BC_3)
11	A8 (BC_2F_1)
12	F27(BC_3)
13	F25 (BC_3)

Table 2: Sample arrangement on agarose gels

CAPS marker gels






1 2 3 4 5 6 7 8 9 10 11 12 13 H20 ax intS6
ADH1
uncut, load PCR product directly
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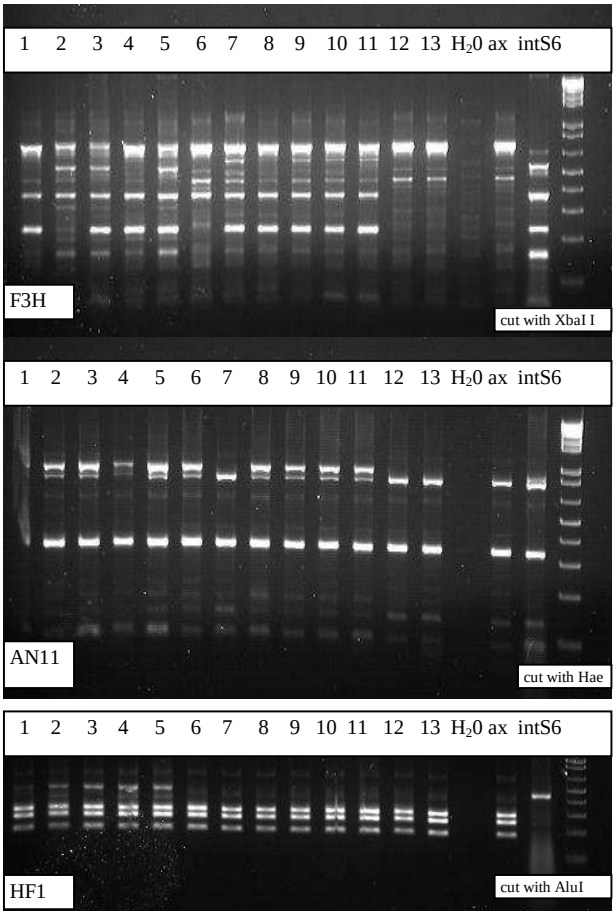
ADH2
cut with Hae III
1 2 3 4 5 6 7 8 9 10 11 12 13 H20 ax intS6
CHI A
cut with Hinf III
1 2 3 4 5 6 7 8 9 10 11 12 13 H20 ax intS6
BSMT 1
cut with Alu I
1 2 3 4 5 6 7 8 9 10 11 12 13 H20 ax intS6
C4H 1
cut with DdeI I



2. SSR marker protocol

Marker	Primer forward	Primer reverse
PM7	CGTTTTTCATTGCATTGTCG	CGTTTCCCTCCTTTGATCTG
PM8	TCTGCAAACTTCAAAGCCAA	ACATGCCATGCACTTTTGAG
PM10	CAAAATCCCGAGCCTCTACA	TTTCGTGCCAAAATGTACCTC
PM15	GTGGCTGGCAACATTGACTA	CACTTACCCCTCAGTCCTCG
PM17	TCCATCTCGTTTAGCAACCA	GGCTTCCAGCAAGAGAAGTG
PM19	ACCCTTGGAAAATGTCGTTG	TTCAAATTTCATCAGTGGCG
PM32	TTCTCTAAGAAGAAACAATAAAGCTCA	GGCTATGCCAGCTTTGGTAA
PM37	GGGGTGGGAATTCTAGTGGA	TGGATGAGCCATAATCTTTGC
PM40	AGCTTCCTTTTTGAGCCACA	TGGCTTAAGCAAGACAATGG
PM42	CGGCTCAAACACAATTTCCT	AATTCAACCGCCATGAAGTC
PM44	AGAATCCCCATATGCTCCG	AGCAGCACCAACAACACAAG
PM77	ACCACGAGAAGAAGGAAGCA	CGAACAACGAGTTAAACCCC
PM81	ACTGAAATCGTTGGGCGTT	AAAAGGAGTTGCATATCCTGATTA
PM94	CCGTGTTAGTATTGCCCAGG	CTCTAGATTGACCATAGC
PM98	ATGGAGGTAGCAAATGCAGG	CAACCAAATGCAGCTTCAGA

PM101	GAGAGAGAACCCTAACCC	GCAGAAGAAACAGAGATCC
PM103	GTGGATGACAAACTTGAGG	GACAGCAGTGGTGTTTGG
PM106	GTTCCTCCAGGCACTTCTGG	CAGAGAGGACACAACTCCTC
PM107	GTCAAAGGTTGCAATCTCT	TGTTGCTGATGAGCAGTAG
PM109	GGAGAAGTTACCAGGTGG	CCTCATGCTCCGCTACATG
PM110	GGTACAGGGCTAGCAGG	CTAGTTGGGTGTTCACAG
PM111	CACCATGAGGAACATCAAGC	GGAACTGGCTGAGGGAAACC
PM114	GGGTAAGGTCTGTGTACG	CCCTTAGCTGGTATTCGCAG
PM119	CCGACACATACCAATTCAC	CACCTAACGTACATTAGC
PM120	GGTTTAGATACTGAAGTTG	CCAGCATTACACCAACCTG
PM121	CCACTTACTGAATTCTGACATCC	GCAATGAGTTACCTACC
PM128	GGTCCTCGAAGGGAACTGC	CTGGTGTGCTACCTGGTGC
PM130	GCATTACGGCTCAACAC	CAACCCCATGAAGTCTC
PM131	CTCGTCTAGAAATCTCTCTG	CTGTCACCGCTCTCAACG
PM132	GCAGTAGGGCATTGCAG	CTGATTCCTCCTCCAGCTCGAG
PM134	CTCTCTCTAAACTAAACCCAC	GGAGAGTAACTTAGCTAGGG
PM137	CCACCTATCTACTCTTCC	CCGTTATGCCACCACACC
PM141	GAAGATTTGGTTCCGAG	GCATCATGGGCAAAGAGG
PM144	GCAGCCCTTCTTCACTG	CCATTGAATCCACAAGG
PM149	CCTAATCAAACACGTAACTC	GGATGATGACACGTGGATCG
PM150	CGTCGAATGCCTTAACTGC	GGAACAACACAGAAACTGTC
PM157	GTAGTAGTAGTAACCCCACC	CATCAGAAGCTTCTGGAG
PM158	GGAACATTCAAGGGGTGG	GGACAAGGACCAGGTCCAG
PM163	GCGATTGGCCATGGTAGC	CTCAAGATCAATAACACCG
PM164	GGGGATGGCTACAGCAGC	CTTGCAGCTCATGGCAAAGC
PM166	GGCACTTGATTGTCCTTGTG	CCATGAATCGAATGCAG
PM168	CCAGAACAGAGGGAACTTG	TCATCCTGCTCAACTGC
PM169	GCAGAGAAACTACACTAATAGGG	CCTGAGGAAGAGCAGCAGC
PM171	GGTGAGAGCATAGAGAATA	GAGACTTTCCATGCAGCCACG
PM177	CCCTTACTCTCTTCTTCACC	GAACTATGAACCATAGCTCTC
PM183	CCTATTTCAGTCCATGAGGC	GTTAGCTGTCTGCTGATCAC
PM184	GGACTTTTATCAACTACC	GCCTTGCCTTTATCGGAC
PM186	CCTTTACTAGTCTCAGAATTGC	GGATAATGATGATGACCC
PM188	CCCAACCATTGGCTACAGCC	GGACAACACAATACAATCTCTGC
PM189	GGATCTTGGTGATCGGACC	CAGGAACTTCAATCTTCACC
PM190	CGAGTTGATGGTGCAATTGTG	CTAGAAAGTTCCTCCGG
PM191	GGAGAAGATTGTTGGTAAC	GGGAAACGATCTCTTGCTG
PM192	GCTGCTTTAAGATTCAGAGGC	CTGAACTTTGCATTGGC
PM193	CGCAACATCACCACTATCAG	GCTGCCAAGTCCGACAATGG
PM195	GCCTTTCGCCGCTGTCACTG	GAGCAAATCGTGACCGTTGG
PM197	CCATAAGTGAAGGATCCTGC	CTGACAACTTACACAGGAACAC
PM198	GTTGGCAATTCTTGG	GGCAAACCATAATGATGAAGG
PM200	CCTGACCCTCCCAGAACC	GGTAACATCTCCCTCACTTCC
PM202	CCCTGTTTCTTCTTCAC	CATCCACCACTTGTTGTTGAG
PM219	GCTGTAACATGTAGCTGTG	GGCTGCCAATCCATGCAGTC
PTCOS13	CATGGCCTTGATGTCTCAGG	CCGCGAAGAAGTATGCAC

Mastermix:

14.7	µl H_20
2	µl PCR buffer
0.5	µl dNTP
0.14	µl forward primer (F)
0.4	µl reverse primer (R)
0.26	µl M13 tailed primer (700/800)
2	µl Taq polymerase

20 µl MM in every PCR tube
+2 µl DNA (KAB BC_1 1-200) (**1:15 diluted DNA)**

PCR regime: Initial denaturation: 94°C 4 min

95°C	30 sec	} 35 cycles
55°C	30 sec	
72°C	60 sec	
72°C	7 min	
4°C	hold	

Add 50 ul of Formamide to samples
Load 2 clicks with the multichannel loading pipette on Polyacrylamide gel
Add 1 ul size marker (700 oder 800)

Preparation of polyacrylamide gel on LICOR

1) clean **glass plates** with soap, rinse with normal **H_2O**, then with **bidest**, then **isopropanol**. Leave to dry for approx. 45 min.
   (every now and then soak plates in 0.1M NaOH for 30 min)
2) place **spacers** between the (dry) front glassplate (with the notch) and the back glassplate. Note that the **smoothed corners** are on the **bottom left**.
3) Fasten glassplates with **black screws**
4) for the Gel, mix
   20 µl Acrylamid (fridge)
   15 mg APS (crystalline) (10% solution)
   15 µl TEMED

Mix carefully and **rapidly** begin to pour into your glass plates. You can therefore place your plates angular on the plastic rack. **Try not to make any bubbles**. If you do, you can remove them with the fine wire (rapidly).

5) clamp the **comb upside down** in between the plates, fix with screws, let dry for approx **1 h**

In the meantime, produce 1 l of TBE buffer in a 1:4 solution

After one hour:

1) Remove casting plate by removing top screw carefully; wipe front plate clean if necessary, the notch as well. **Take out comb** and **clean the notch** with special paper (kimwipes).
2) **Place your plate** in the Licor-machine, fix the **buffer tanks** (up and bottom) and fill them with **TBE**. Place yellow card behind the two glass plates.
3) See if your gel is OK by filling some **Licor loading buffer** (blue, stock in freezer) in. Clean again with TBE (use plastic syringe to rinse).
4) Connect electrical connections,

PRERUN for 20 min

Focus error might occur – if your plates are clean you can re-start pre-run.

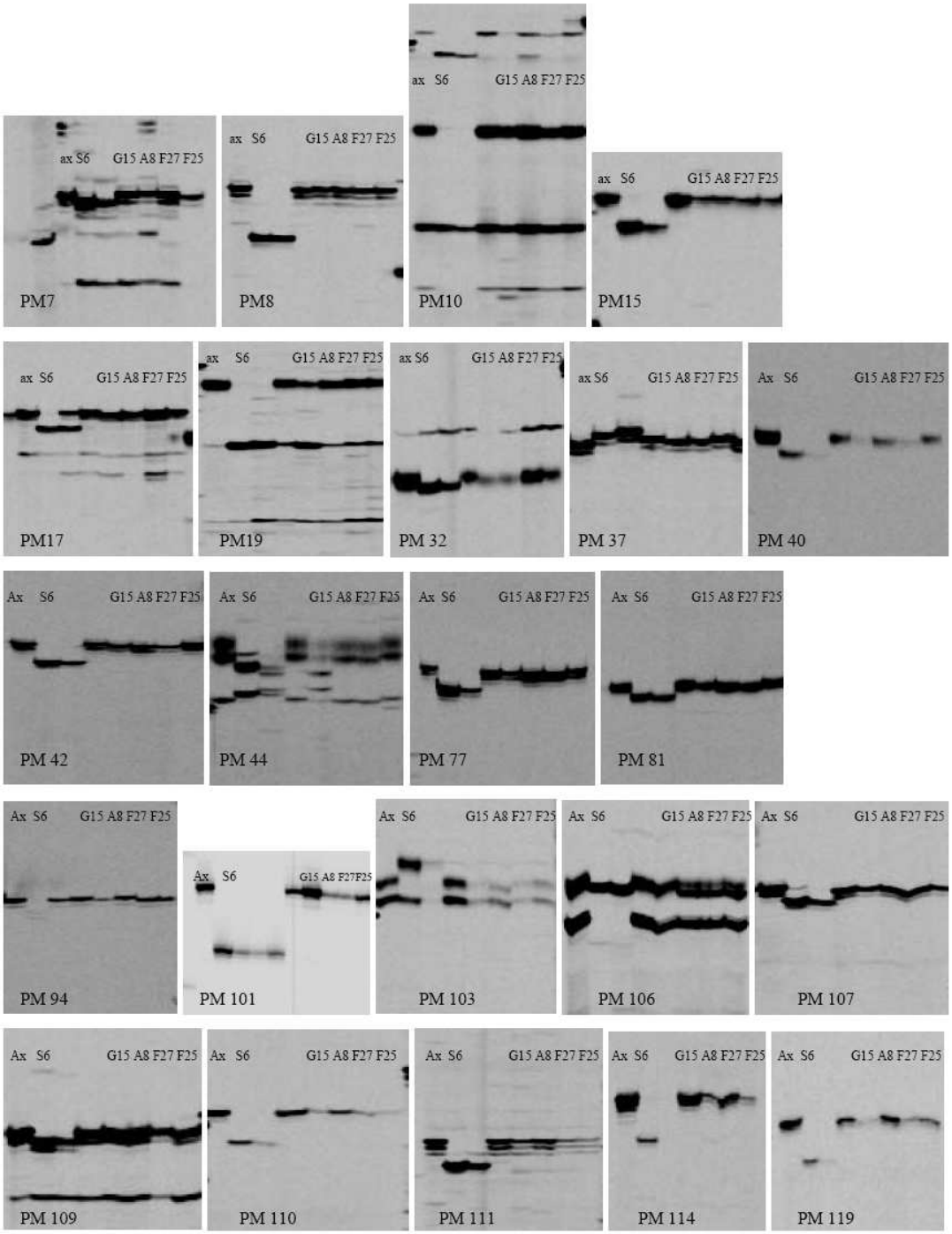
Loading the gel

Clean the well with TBE buffer (about three full syringes), place the comb in the notch

Load your samples with 2-click-syringe (ca. 0,25 µl), be aware that after sample 1 follows sample 4 etc.

Start immediately after loading.

SSR marker gels


ax S6 G15 A8 F27 F25
PM7
ax S6 G15 A8 F27 F25
PM8
ax S6 G15 A8 F27 F25
PM10
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PM15
ax S6 G15 A8 F27 F25
PM17
ax S6 G15 A8 F27 F25
PM19
ax S6 G15 A8 F27 F25
PM 32
ax S6 G15 A8 F27 F25
PM 37
Ax S6 G15 A8 F27 F25
PM 40
Ax S6 G15 A8 F27 F25
PM 42
Ax S6 G15 A8 F27 F25
PM 44
Ax S6 G15 A8 F27 F25
PM 77
Ax S6 G15 A8 F27 F25
PM 81
Ax S6 G15 A8 F27 F25
PM 94
Ax S6 G15 A8 F27F25
PM 101
Ax S6 G15 A8 F27 F25
PM 103
Ax S6 G15 A8 F27 F25
PM 106
Ax S6 G15 A8 F27 F25
PM 107
Ax S6 G15 A8 F27 F25
PM 109
Ax S6 G15 A8 F27 F25
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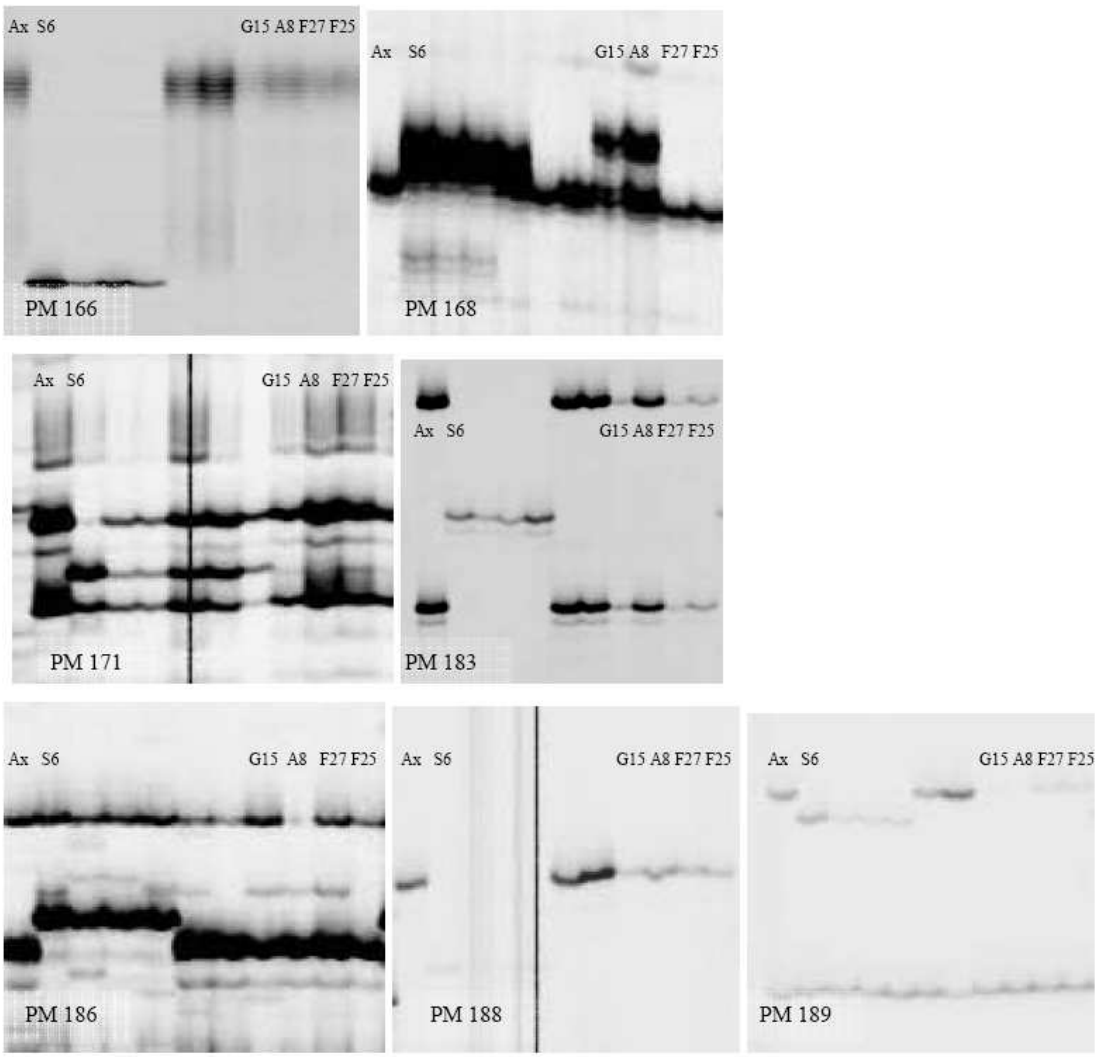
PM 111
Ax S6 G15 A8 F27 F25
PM 114
Ax S6 G15 A8 F27 F25
PM 119


Ax S6 G15 A8 F27 F25
PM 166
Ax S6 G15 A8 F27 F25
PM 168
Ax S6 G15 A8 F27 F25
PM 171
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PM 183
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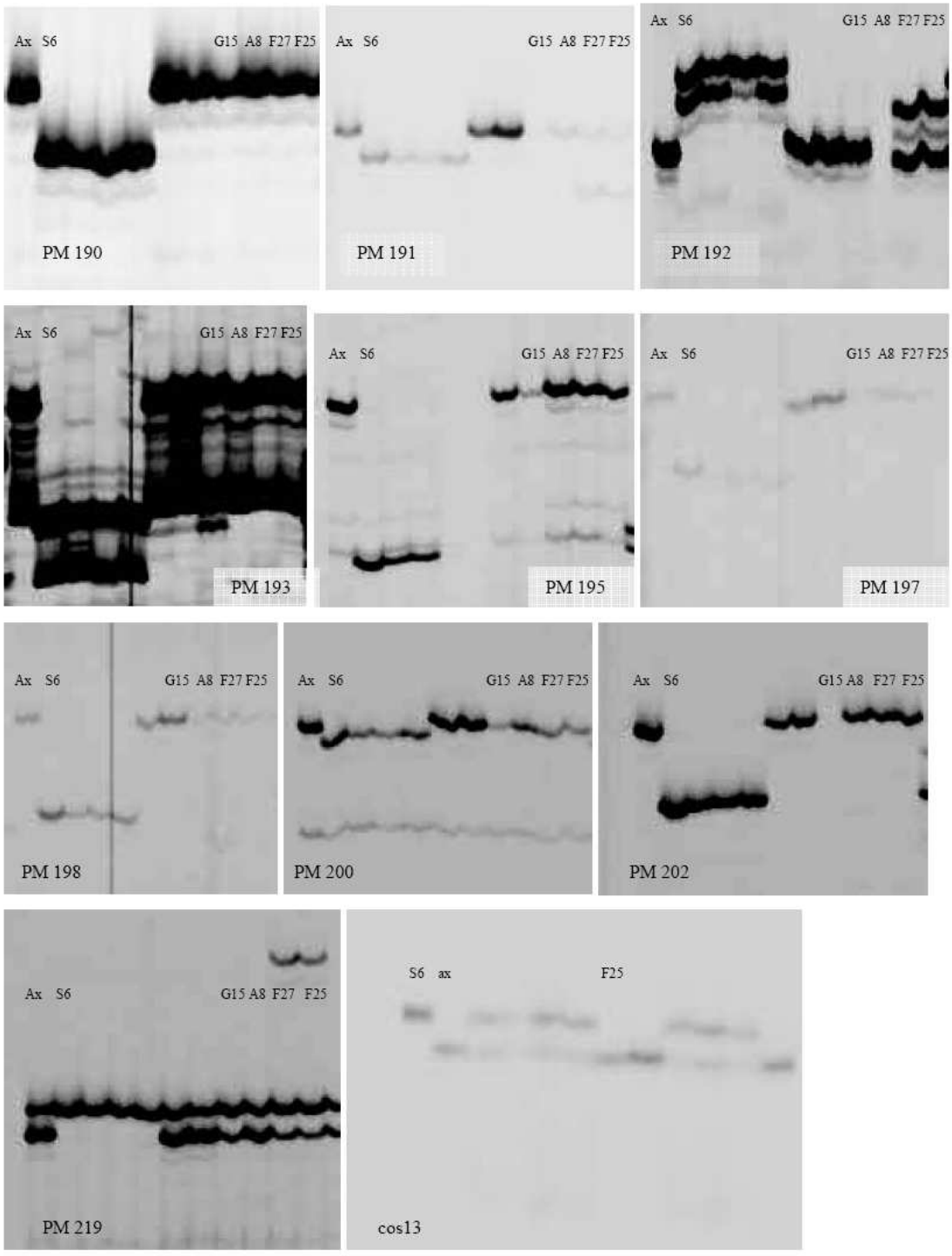
PM 188
Ax S6 G15 A8 F27 F25
PM 189


Ax S6 G15 A8 F27 F25
PM 190
Ax S6 G15 A8 F27 F25
PM 191
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PM 192
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Ax S6 G15 A8 F27 F25
PM 195
Ax S6 G15 A8 F27 F25
PM 197
Ax S6 G15 A8 F27 F25
PM 198
Ax S6 G15 A8 F27 F25
PM 200
Ax S6 G15 A8 F27 F25
PM 202
Ax S6 G15 A8 F27 F25
PM 219
S6 ax F25
cos13

3. Phenotypes of plants with reduced nectar volumes, including F25 (low nectar line)

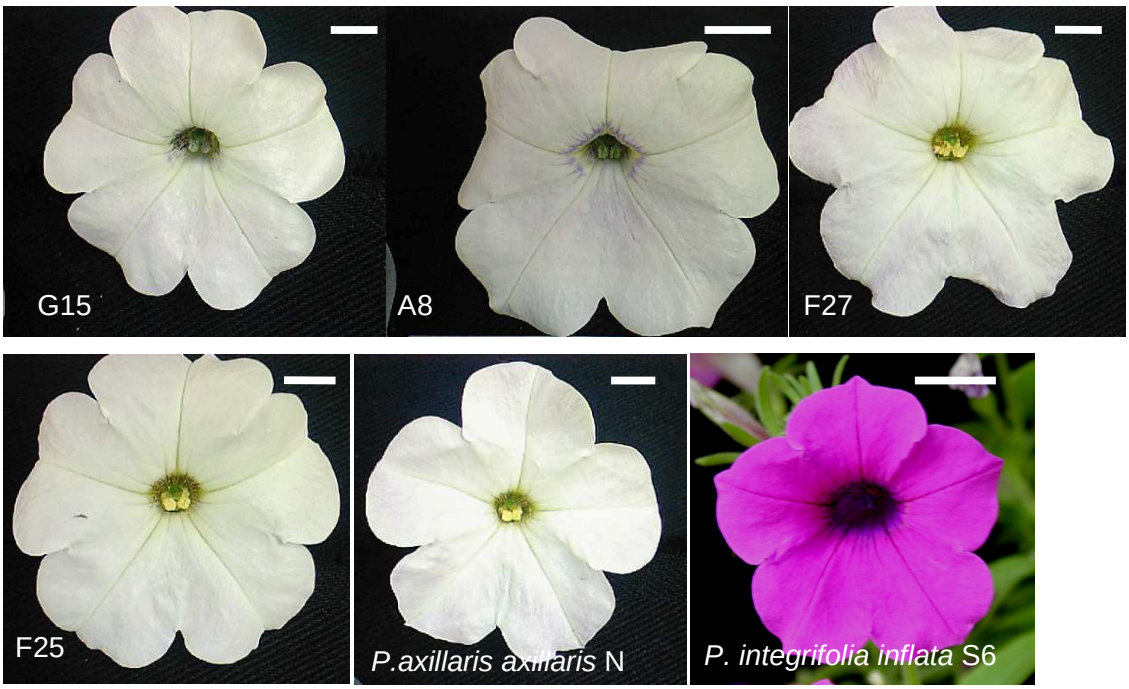

Figure 1: photographs of low nectar plants and parental plants *P. axillaris axillaris* N and *P. integrifolia inflata* S6. Size bars = 1cm

4. SEM images of nectaries

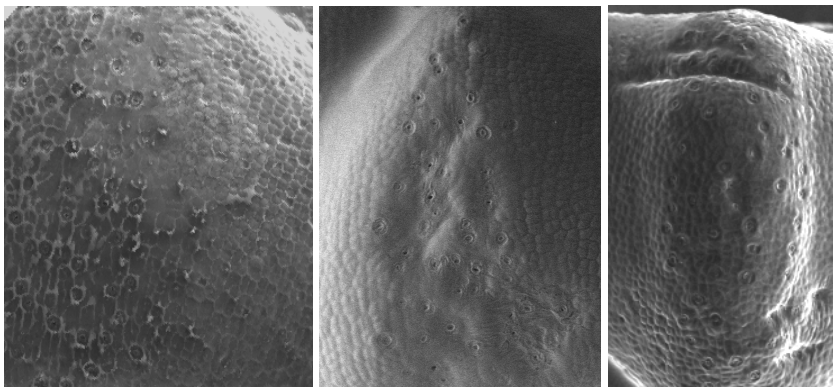

Figure 5: The number of pores on the surface of the nectary was counted on nectaries of F25, *P.axillaris* and *P.integrifolia* and F1. An image was taken with a scanning electron microscope (SEM, Hitachi S-3500N). Number of pores was counted directly from the image. It was not possible to retrieve the size of the images.

	P. axillaris	*P.integrifolia*	F25	F1
N pores	**46.8**	**25**	**52**	**38**
std dev	6.8	2.1	-	-

Table 2: Number of pores on nectary surface (modified stomata) in different plants

Chapter IV

Density-dependent foraging strategies of hawkmoths in two native *Petunia axillaris axillaris* populations with rewarding and non-rewarding individuals

Anna Brandenburg and Redouan Bshary

Unpublished data

Density-dependent foraging strategies of hawkmoths in two native *Petunia axillaris axillaris* populations with rewarding and non-rewarding individuals

Anna Brandenburg[1,2], and Redouan Bshary*[,2]

[1]Institute of Plant Science, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

[2]Institute of Biology, University of Neuchâtel, Emile Argand 13, CH-2009 Neuchâtel, Switzerland

*Corresponding author: anna.brandenburg@unine.ch

Summary

Plant density and distribution of nectar rewards are known to affect pollination. We compared hawkmoth behavior in two native populations of *Petunia axillaris* addressing the question whether pollinator foraging strategies change as a function of density and whether this behavioral response has any effect on conspecific cheaters. We designed a set-up with rewarding and manually depleted *P. axillaris* and observed the following pollinator foraging parameters: first choice and number of flowers visited. We found that in the dense population with abundant co-flowering plants, pollinators discriminated between rewarding and nectar-less plants and visited more flowers on rewarding plants. In the sparse population, pollinators did not discriminate between rewarding and nectar-less plants. We reason that in our system, an equivalent of the remote habitat hypothesis is at work: in a sparse population, cheating flowers benefit of the absence of inter- and intraspecific competitors. In a denser population, a pollinator's optimal foraging strategy involves more selectiveness, thus the abundance of co-flowering rewarding plants and species can cause competition for pollinators. In a pollinator-limited context like most hawkmoth-pollinated systems, pollinators adjust their foraging strategies to the density of food plants. We propose that nectar-provisioning of plants can be density-dependant.

Keywords: nectar, hawkmoth, density, foraging, Petunia

Introduction

Mutualisms are ubiquitous and important in most biological systems, yet their stability and persistence is puzzling (Herre et al 1999, Bronstein 2001, Sachs 2004). Mutualistic interactions often involve a costly investment of one partner (Bshary and Bronstein 2004). A key question is therefore what ecological conditions select for stable investment respectively for the breakdown of mutualism.

Plant-pollinator interactions offer a good example to study the conditions that affect the stability of mutualisms (Bronstein et al. 2006). Plants invest in nectar while most pollinators benefit the plants through pollen transfer as a by-product of self-serving foraging behavior. Nectar production seems to be costly (Southwick 1984, Pyke 1991), nevertheless most angiosperms produce a reward for pollinating insects. Therefore, we need to understand why there seems to be selection against nectar reduction in plants. Several aspects of pollinator foraging strategies may contribute to the promotion of nectar production: avoidance of non-rewarding plants (Gilbert et al. 1991), short drinking duration (Cresswell 1999), a reduced number of flowers per visit (Mitchell 1993, Hodges 1995) and low species constancy (Goulson 1999). The success of deceptive orchids however shows us that cheating can be an evolutionarily stable strategy (Maynard-Smith 1982, Jersakova et al 2006). These behavioral changes of pollinator foraging are named partner control mechanisms.

Here, we tested for the first time partner control mechanisms on rewardless *Petunia axillaris axillaris* in the field. The flowers display all characteristics of hawkmoth-pollination and receive regular visits by them (Ando 2001, Gübitz et al 2009). We compared the performance of pollinators on rewarding and non-rewarding *P. axillaris axillaris* in two natural populations in Uruguay, in order to identify which partner control mechanisms are exerted by naturally occurring hawkmoths. The two populations differed in size, density and co-occurring hawkmoth-pollinated plants. One aim of this study was to assess whether pollinator foraging strategies differ in the two different ecological settings or whether the observed effects are general.

We collected two measures of discriminative behavior, potential partner control mechanisms: first choice between rewarding and nectar-less *P.axillaris*, and the number of flowers visited per plant.

Material and methods

Field site and plant species

Field studies were conducted in January/February 2007 in Uruguay in two wild *Petunia* populations: the first near José Ignacio (JI) (34°45.739’’S 54°341.153’’WO), the second near Carmelo (C) (33°56’18.4’’S 58°22’13.3’’W), Uruguay. Population sizes were measured prior to experiments by counting the number of all detectable *Petunia axillaris* plants and their flowers at each study site. In JI, *Petunia axillaris* plants grew in a scattered manner along a 5 km road restricted to maximally 5 meters each side of the road

(total area: 50 000 m^2). In C, plants grew in a restricted area of 125m x 60 m (total area: 7500 m^2).

Plant density was determined by the occurrence of all plants/area in m^2 ("Population size" sensu strictu Kunin 1997). The maximal distance between two plants was 120 m in JI and 30 m in C. It is assumed that the foraging range of hawkmoths exceeds 400 m (Moré et al. 2005). We therefore assumed that all counted plants belonged to the same gene pool. Both sites were surrounded by habitat unsuitable for *Petunias*. Our experimental plants were *Petunia axillaris axillaris* N, a self-compatible line from the Botanical Garden of Rostock. Our lines are cross-compatible with the wild form and have a similar flower anatomy, characterized by white flowers with long floral tubes and abundant nectar. Plants were grown in plastic pots (ø 14 cm) under greenhouse conditions (Insituto Nacional de Semillas, Montevideo, Uruguay).

Experimental set up and pollinator observation

6 plants were arranged in a 3x2 m array, with 3"no nectar" and 3"with nectar" plants alternating in two rows. The plants were placed in vicinity (3m) of a naturally occurring *Petunia axillaris*, displaying 10-30 flowers. Number of flowers was equal in both "no nectar" and "with nectar" experimental plants; but different between nights. The range of open flowers on experimental plants was 1-8. In "no nectar" plants, nectar was extracted by piercing the tube at the bottom with a needle. The nectar was collected with a tissue once per hour. The "with nectar" plants were also pierced in the tube to control for wounding but above the nectar level to avoid any loss of nectar. Nectar removal did not cause any detectable differences in scent emission between treatments 30 minutes after

nectar extractions in a laboratory analysis using a Proton transfer reaction - mass spectrometer (High sensitivity PTR-MS, Ionicon®), a detector for continuous quantification of volatile organic compounds. Spatial arrangement of "no nectar" and with "nectar plants" was unaltered during the whole experiment. Pollinator visitation was observed from 01/10/2007-01/21/2007 in JI, and from 02/05/2007-02/15/2007 in C. Experiments were conducted from 2100 to 2400. Post-experimental capture studies in C included *Manduca diffisa*, *Manduca sexta*, *Eumorpha vitis*, *Eumorpha labruscae*, *Agrius cingulata*, *Eryinnyis ello* as floral visitors of *P.axillaris axillaris*. Hawkmoths were not marked so we cannot exclude that the same individuals visited our plants in the same / consecutive nights.

Results

Petunia population size and density at both sites

At Jose Ignacio (low density) the population comprised 235 plants with 252 open flowers. This corresponds to 0.0047 plants/m^2, or 0.005 flowers/m^2.There were no other co-flowering sphingophilous species in the habitat. At Carmelo (high density), the population consisted of 78 plants with 140 open flowers. Population size was about twice as high (0.01 plants/m^2) and flower density (0.02 flowers/m^2) 4 times as high as in the low density site. In addition to *Petunia axillaris axillaris*, other sphingophilous species were co-flowering: *Oenothera sp.*, *Nicotiana longiflora*, *Macrosiphonia longiflora* and *Datura stramonium*.

Pollinator behavior

	LOW DENSITY SITE JOSE IGNACIO		HIGH DENSITY SITE CARMELO	
BEHAVIOR	WITH NECTAR	NO NECTAR	WITH NECTAR	NO NECTAR
Total N of visited plants (both treatments)	53 (102)		42(96)	
Total N of visited plants "no" and "with" nectar	29(51)	24(51)	24(48)	18(48)
N visited flowers	40(29)	31(24)	43(24)	18(18)
N flowers per plant	114	114	155	155
first choice	9	8	13	3

Table 1:

Total N of visited plants depicts the total number of plants that were visited in all hawkmoth foraging bouts (in parentheses is the number of plants that were possible to visit).

Total N of visited plants is divided in **"No" and "with" nectar** in the 2nd row, with the number of flowers that were possible to visit in parentheses.

N visited flowers is the total number of flowers that hawkmoths visited in all foraging bouts. Only the first visit to each flower was counted. Revisited flowers are excluded from this table. In parentheses is the number of plants that displayed the visited flowers. **N visited flowers** was compared using Wilcoxon signed rank test (SPSS®).

N flowers per plant depicts the total number of flowers that were available for hawkmoth visitation on all plants.

First choice depicts which plant ("with" or "no nectar") hawkmoths selected when entering the experimental plot for the first time. Differences in first choice were analysed using χ^2 "Goodness of fit" test (VassarStats).

At both sites the number of plants per treatment visited did not differ significantly (Wilcoxon signed rank tests: low density site: N= 17, Z=-1.2, p=0.25, high density site: N=16, Z=-1.2. p=0.24). However, the sites appeared to promote differences in pollinator behavior with respect to first choice and number of flowers visited. While hawkmoths at the low density site did not show significant differences with respect to these two

parameters (preference: χ^2=0, n=17, df=1, p=1; n flowers visited: Wilcoxon signed rank test, N=14, Z=-0.84, p=0.4), hawkmoths at the high density site preferentially approached plants with nectar first (χ^2=5.06, n=16, df=1, p=0.02) and visited significantly more flowers on plants with nectar (Wilcoxon signed rank test, N=9, Z=-1.98, p=0.047). For all numbers, see table 1.

Discussion

Our results are in line with the general prediction of the remote habitat hypothesis that cheating plants do best under low densities (Lammi and Kuitunen 1995). The hypothesis is based on the prediction that optimal foraging decisions of pollinators depend on food availability, with pollinators becoming less discriminative when plant densities are low (Pyke 1984). Several studies on deceptive orchids have demonstrated that these plant species do better in low densities with few allospecific competitors (Lammi and Kuitunen 1995, Internicola et al. 2006). The reason seems to be that co-flowering plants offer pollinators the opportunity to switch quickly to another partner if the current one does not provide a sufficient reward, resulting in competition between plants over access to pollinators. Winning the competition can be best achieved by giving a better offer (Noë 2001) in a system where pollinators have control over the occurrence and the duration of interactions (Johnstone & Bshary 2008). A competitive situation thus leads to a decreased fitness of cheating plant due to improper pollen transfer (Duffy and Stout 2008, Flanagan et al. 2009, Kandori et al. 2009).

While the remote habitat hypothesis has been used to explain the evolution of deceptive orchids, our data suggest that its implications are much broader and apply as well to nectar-providing species. As pollinators did not show discriminative tendencies at the low density site, it seems likely that low densities generally provide a condition that selects on a reduction in nectar production.

Alternative explanations

Obviously, the two sites differ in more aspects than plant density and community composition which likely affects pollinator decision making: First of all, the sites are located 340 km apart, are in different habitats with different environmental conditions. Despite a low density, the Petunia population size and flower number is higher in Jose Ignacio. Furthermore, the pollinator community might be fundamentally different in both sites. It must be considered that species identity of pollinators and their learning capabilities might also play a role in discrimination behavior. Little is known about learning behavior or spatial orientation of sphingids (Daly and Smith 2000, Goyret and Raguso 2006), and even less is known from field studies. We were not able to determine which pollinator species was visiting the flowers as pointing a light source in the direction of the sphingids disturbed their foraging and hindered recording their genuine response. The difference in pollinator behavior might thus be a reflection of the pollinator guild composition or individual learning capacities rather than of the plant population density.

Conclusions and future outlook

Altogether, the density and composition of plant communities can have a crucial effect on pollinator behavior. In our example, we observed that pollinators behave more selectively in a dense food-plant aggregation while in a scarce population both non-rewarding and rewarding treatment were visited equally. One important future study is to manipulate plant densities and see whether this alters pollinator behavior in line with the predictions of the remote habitat hypothesis. The advantage of conducting density-dependant studies of hawkmoth foraging behavior in one population is that the above mentioned alternative explanations can be limited if not excluded.

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Chapter V

Learning behavior of hawkmoth *Manduca sexta* in a set-up with rewarding and non-rewarding *Petunia axillaris* plants

Anna Brandenburg, Cris Kuhlemeier and Redouan Bshary

Unpublished data

Learning behavior of hawkmoth *Manduca sexta* in a set-up with rewarding and non-rewarding *Petunia axillaris* plants

Anna Brandenburg[1,2], Cris Kuhlemeier[1] and Redouan Bshary[2]

[1]Institute of Plant Science, University of Bern, Altenbergrain 21, CH-3013 Bern, Switzerland

[2]Institute of Biology, University of Neuchâtel, Emile Argand 13, CH-2009 Neuchâtel, Switzerland

Summary

The persistence of mutualisms is puzzling, considering that cheating can be evolutionarily stable. Plant-pollinator interactions offer excellent study systems for mutualistic interactions. They are characterized by the existence of investment costs in terms of floral nectar. The reduction of nectar might be beneficial for plant fitness, e.g. by reallocating resources to other plant structures. However, only few angiosperms lack nectar and thus cheat on pollinators. A possible explanation for this scarcity may be that pollinator foraging behaviors select for nectariferous plants. Shorter drinking duration, interaction with the less flowers or even complete avoidance of plants with low/no nectar may reduce the fitness of cheating plants. It remains largely unresolved how and to which extent learning plays a role in the acquisition of these pollinator decision rules. Learning leaves a window for cheaters as they can exploit naïve pollinators. Here, we studied the importance of learning for the foraging strategies of the hawkmoth *Manduca sexta* interactions with nectariferous and nectarless *Petunia axillaris.* We found that moths did not discriminate between rewarding and nectar-less plants. In contrast, they significantly reduced the number of flowers visited and the drinking time per flower on nectarless plants. These two behaviors were exerted during the first encounter with nectarless plants and did not improve with experience. In conclusion, the foraging decision rules of hawkmoths that may reduce the fitness of cheating plants appear to be innate while learning is not of major importance.

Keywords: learning, *Manduca sexta*, *Petunia axillaris*, mutualism, nectar, cheating

Introduction

Mutualisms are interactions between non-related individuals which result in a net fitness increase of both partners. Mutualisms are thought to play an important role in nearly every ecosystem (Bronstein 1994). Their persistence and evolution however is puzzling, as they often involve a costly investment from at least one side that exploiters can take advantage of without reciprocating (Herre et al. 1999, Sachs et al. 2004). Exploiting species reap the benefits of the mutualism without paying the costs and thus gain a higher fitness advantage of the interaction (Bronstein 2001 a,b, Doebeli and Knowlton 1998, Hoeksema and Bruna 2000, Sachs et al. 2004, Doebeli and Hauert 2005). Exploiters or so called 'cheaters' may be phylogenetically unrelated opportunists from the community or they may evolve from within the mutualist lineage itself (Segraves et al. 2005). Under certain conditions, cheating strategies can be evolutionarily stable (Ferrière et al. 2002). It is thus surprising, that in some mutualisms, such as plant-pollinator interactions, cheating seems to be rare (Renner 2006). Mechanisms exerted by the mutualistic partner reassure the investment and therefore stabilize mutualisms, such as partner choice (a preferential selection of a suitable partner to interact with; Bull and Rice 1991) and sanctions (partners cut back on provisioning the traded good; Herre et al. 1999). The models typically assume a purely genetic basis of the strategies employed. In reality, however, information processing and learning may often be necessary to allow an individual to make appropriate decisions. The presence or absence of learning phases has major implications for the relative payoffs of cooperating versus cheating. Cheating will do best

in interactions with naïve partners while cooperating will do best in interactions with experienced partners or partners that follow genetic strategies optimized by natural selection.

Plant-pollinator mutualisms are suitable systems to explore the potential importance of learning on the successful employment of partner control mechanisms. Pollinators forage for nectar and pollen on flowering plants, thereby distributing the plant's pollen as a result of self-serving behavior. The plant invests in the production of nectar, which can have considerable negative effects on a plant's reproductive potential (Ordano and Ornelas 2005, Ornelas et al. 2007). While any means to reduce nectar production without compromising pollination should therefore be under positive selection, only about 4% of angiosperms have evolved into being cheaters (Renner 2006), most notably deceptive orchids (Dafni 1984, Schiestl 2005). One must therefore assume that a change in pollinator foraging behavior on rewardless plants counteracts in most cases the benefits achieved by nectar reduction, e.g. by transferring less pollen to conspecifics and therefore reducing the plant reproductive success. Three decision rules of pollinators have been identified in this context: avoidance of non-rewarding species (Gigord et al. 2002), reduction of drinking time on (Cresswell 1999, Warren and James 2008) and reduction of number of flowers visited on cheaters (Ohara and Highashi 1994, Smithson and Gigord 2001, 2003).

The role of learning (experienced-based modification of behavior) in pollinator foraging strategies has predominantly been studied in honeybees (Chittka and Thomson 2001). Foraging bees carry out a few orientation flights, during which they learn spatial locations of profitable food sources with the help of celestial cues, landmarks and path

integration (Dyer 1996, Menzel et al. 1996, 2006). During these learning flights, foragers establish a geometrical representation of the landmark layout, featuring the hive as central spot. This type of memory is termed the general landscape memory (Menzel 2001). Moreover, foragers learn to associate floral cues, such as odor, color and petal morphology with the quantity and quality of the reward offered by a flower. This information is used to make future decisions about revisiting or avoiding those species (Dukas and Real 1993, Ferdy et al. 1998, Gigord et al. 2002, Waddington 2001, Smithson and Gigord 2003). In the laboratory, classical conditioning protocols to establish associative learning have provided valuable information about the learning capacities, speed and accuracy of honeybees (Bittermann et al. 1983, Hammer and Menzel 1995). Bees are able to reliably associate a conditioned stimulus such as color (Menzel 1999, Lehrer 1999) and odor (Wright et al. 2009) with an unconditioned stimulus (sucrose), and retrieve this memory after a single learning trial for several days (Hammer and Menzel 1995). Associative learning studies using odor as conditioned stimulus were also successful in other pollinator species, such as the nocturnal hawkmoth *Manduca sexta* (Daly and Smith 2000).

These learning experiments indicate that diverse pollinators can learn to discriminate between colors and scents based on associative learning. However, these studies are not conclusive in respect to the role of learning in partner control mechanisms, i.e. the pollinator behaviors that maintain the mutualism.

Here we studied the importance of learning with respect to foraging decision rules that act as potential partner control mechanisms in the pollinator *Manduca sexta*. In previous studies, we found that hawkmoths visited less flowers and spent less time per flower on

Petunia axillaris axillaris N plants with reduced nectar investment compared to 'normal' plants (Brandenburg et al. unpublished, chapter 2). These results were obtained partly with naïve pollinators in the laboratory and with individuals of unknown experience in the field. The aims of the present study were to replicate the previous findings and to investigate whether performance would change with experience. We predicted that if the behavior was innate the discriminative behavior of moth concerning rewardless plants would be exerted from the very first foraging event and if learning plays a role, the foraging strategies of Manducas would improve over successive learning trials, e.g. significantly shortage of drinking time or reduction of flower numbers. In addition we wondered whether the moths can easily learn to avoid plants that consistently fail to provide nectar.

Material and Methods

All experiments were conducted in a greenhouse of the Institute of Plant Science, University of Bern, from August 2006 until October 2006

Pollinator species: *Manduca sexta*

Hawkmoths used in behavioral assays were *Manduca sexta* (Lepidoptera), the tobacco hornworm moth, a nocturnal sphingid frequently used in behavioral and neurobiological experiments (Hoballah et al. 2007, Riffell et al. 2009). *M. sexta* is native in America (D'Abrera 1986) and larvae are known as pests on solanaceous species such as potato and tomato (Lange and Bronson 1981). On the other hand, adult moths are efficient

pollinators of *Petunia axillaris*, *Datura wrightii* and *Nicotiana longiflora* (Grant 1983, Raguso and Willis 2005, Hoballah et al. 2005) since floral nectar is the exclusive food source of adult foragers.

Female pupae of *M. sexta* were obtained from NCSU Insectary (Raleigh, USA) and kept in BugDorm-3® insect tents at 24°C, with 60% air humidity and a 16/8 day/night cycle. Pupae were controlled daily for eclosion of adults that were subsequently used for the experiments. Adults emerged 1-5 days before the experiments and were starved for three days prior to use. Moths were completely naïve and were used unmated for experiments.

Plants species: *Petunia axillaris axillaris* N

The plant species used for experiments was *Petunia axillaris axillaris* N (later referred to as *P.axillaris*), is a self-compatible inbred line (Botanical Garden of Rostock), derived from a wild accession of *P.axillaris axillaris*. This line has been maintained by inbreeding for many generations in the laboratory of the Institute of Plant Science, University of Bern. Flowers of *P. axillaris* display all characteristics of a hawkmoth-pollination syndrome (according to Faegri and van der Pijl 1979): large white petals, sweet scent emitted at dusk, long floral tube filled with large amounts of nectar. *P. axillaris* is native to South America (Argentina, Southern Brazil and Uruguay), and hawkmoth visitation has been observed repeatedly in its natural habitats (Ando 2001, Hoballah et al. 2007).

For our experiments, plants were grown in peat-based soil, in 15 cm diameter plastic pots and kept under greenhouse conditions (supplementary light in winter months, minimum 14h light).

In our hawkmoth experiments, we used plants where nectar was manually removed ("no nectar") and plants that contained the full nectar reward ("with nectar"). To extract nectar from "no nectar" treatment group, the floral tube was pierced at the bottom of the floral tube, and exuding nectar was removed with a tissue. To avoid replenishment, nectar was removed hourly. To exclude that the tissue injury would elicit some kind of behavioral response in the pollinator, the control plants were also pierced in the floral tube, but above nectar levels.

Scent measurements were conducted with proton transfer reaction coupled with mass spectrometry (PTR-MS Ionicon®). Molecules are protonated from an interaction of the trace gas and protonated water (H_3O^+), resulting in an ionized molecule that can be measured by PTR-MS. No scent difference could be detected between "with" and "no nectar" treatment in flowers 30 minutes after cutting (data not shown).

Set-up of behavioral experiments with *Manduca sexta*

The experiments were conducted in a flight arena (144cm height, 248x368 cm surface area), situated in the middle of a greenhouse. Experiments started at around 1700 (winter) and 2030 (summer) and ended latest at 2300. Hawkmoths were kept in flight cages (BugDorm) before the onset of experiments. For pollinator observation, the flight arena was illuminated with a 15 V incandescent light bulb. Despite an innate attraction of hawkmoths to light, we could not determine an orientation towards the light source in previous and present experiments.

a. Learning procedure

One plant of each treatment group (“no nectar” and “with nectar”) was placed 1.7 m from one another in the flight arena and presented simultaneously to the pollinator. The positioning of “no nectar” and “with nectar” plants was kept identical throughout the experiment. Each moth was tested three times per night on three consecutive nights. The flight arena had three different entrance sites, which were chosen in a counterbalanced way between nights to exclude that the moths could develop a side bias. One insect was released into the flight arena at a time and the following behaviors were recorded: 1. **First choice** - noted as the plant (“no nectar”/”with nectar”) that hawkmoths first probed on. 2. **Number of flowers visited** - total number of flowers on each plant that hawkmoths drank from. 3. The **drinking time** was recorded from the insertion of the proboscis until its retraction. For the last two behaviors, only the first drinking event on each flower was noted. Flowers were supposedly emptied after the first drinking, and a further probing on the same flower would contort the results if counted for the category “with nectar”. The drinking duration was measured with a chronometer. All behaviors were recorded with a Dictaphone and analyzed the following day. After a pollinator had visited both plants, the plants were exchanged for a new repeat. We set a maximal time interval of 300s for the pollinators to interact with the plants in each trial, after which plants were exchanged. Moths that failed to interact with either plant on the very first trial were not used further. The procedure of exchanging plants was done three times thus each individual hawkmoth was exposed to three pairs of nectar-less and rewarding plants in succession. The plant exchange process was realized within a few seconds, during

which the moth remained in the flight arena. Moths were removed after the three trials and returned to their cages until the next night.

Data analysis

1. global analysis

In first global analyses we calculated one value per individual for all three behavioral responses to see whether there are significant differences between foraging behavior on rewarding and non-rewarding plants. **First choices** in all learning trials and nights of individual hawkmoths were summed (one pool “no nectar”, one “with nectar”), while the median values were used to compare n flowers visited and drinking duration between ‘no nectar’ and ‘with nectar‘ plants. For the first choice we included all trials in which a hawkmoth had visited at least one plant while for the other two variables we only used data for a trial if the moth had visited both plants.

2. First trials

For the variables that would yield overall significant differences in moth behavior towards plants ‘with nectar’ compared to plants ‘without nectar’ in the global analyses, we also assessed whether this difference was already manifested in the very first trial.

3. Learning

We tested the three variables for potential improvements in discrimination both within one night and between nights. Within each of the three nights, the data per individual moth per trial were used for Friedman tests. For the analyses of first approach we simply

used the original data. For n flowers visited, we calculated for each hawkmoth quotient of the number of flowers visited on “with nectar” plants divided by the number of flowers visited on “no nectar” plants. Similarly, for drinking duration, we divided median drinking time per flower on ‘with nectar’ plants by median drinking time on ‘no nectar’ plants. Between nights, we used per individual moth the sum of first approaches per night and the median values for n flowers and drinking duration per night on plants ‘with nectar’ and plants ‘without nectar’ to calculate the quotients for Friedman tests.

To investigate whether with advanced experience hawkmoths improve flower handling, we tested whether the drinking time on flowers would shorten in the course of the experiments. We conducted separate analyses for plants ‘with nectar’ and plants ‘without nectar’, comparing median values per individual per night.
Wilcoxon signed rank test and Friedman test were analyzed using SPSS for Windows, version 17.0.

Results

First Choice

There was no significant difference in first choice behavior of each moth over the course of the experiment (Wilcoxon signed rank test, N=21, Z=-0.591, p=0.555). An a priori discrimination is not detectable. Discrimination behavior prior to probing did not improve within each night (Friedman Test; night 1: N=21, χ^2=0.875, p=0.646, night 2: N=20,

χ^2=0.933, p=0.627, night 3: N=16, χ^2=0.545, p=0.761) and neither between nights (N=21, χ^2=4.361, p=0.113).

Number of flowers visited

A global analysis of median number of flowers visited for each hawkmoth revealed that moths visited significantly more flowers on plants with nectar than on plants without nectar (Wilcoxon signed rank test, N=21, Z=-4.017, p<0.001). Moths demonstrated discrimination with respect to number of flowers visited during the very first trial (Wilcoxon signed rank test, N=18, Z=-2.578, p=0.01).

The proportion of number of flowers visited on “with nectar”/”no nectar” plants for each individual hawkmoth over the course of each night did not change significantly. (Friedman test, night 1: N=17, df=2, p=0.844, night 2: N=12, df=2, χ^2=0.52, p=0.772, night 3: N=12, df=2, χ^2=3.622, p=0.146; difference in number of hawkmoths (N) is caused by the removal of hawkmoths from the analyses that visited only one of the plant in the trials). Furthermore, the proportion of number of flowers visited on nectarless plants and plants with nectar did not change between nights (Friedman Test, N=12, df=2, χ^2=1.55, p=0.461).

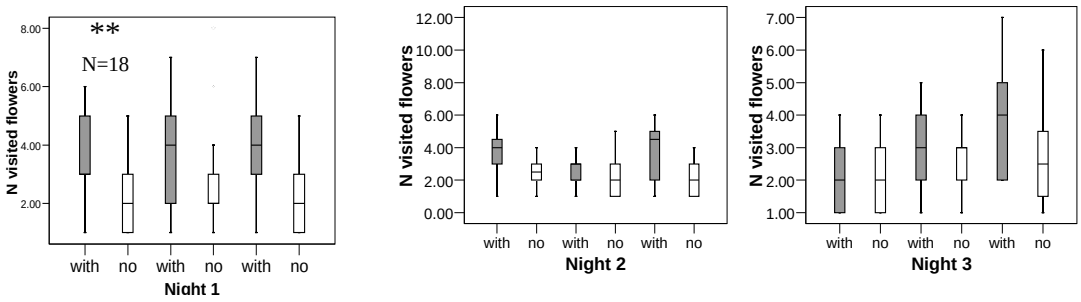

Figure 1: median number of flowers visited per flower per foraging bout for each trial (E1-3) in the three consecutive experimental nights (night 1-3)

Drinking time per flower

A global analysis of median drinking time per flower for each hawkmoth revealed that moths drank significantly longer on flowers with nectar ("with nectar" plants) than on flowers without nectar ("no nectar" plants; Wilcoxon signed rank test, N=21, Z=-3.921, $p<0.001$). Moths demonstrated discrimination with respect to drinking time per flower during the very first trial (Wilcoxon signed rank test, N=18, Z=-2.201, p=0.03).

The drinking duration between rewarding and non-rewarding plants did not significantly change within each nights (Friedman test, night 1: N=17, df=2, χ^2=1.6, p=0.449, night 2: N=12, df=2, χ^2=0.571, p=0.751, night 3: N=12, df=2, χ^2=2.182, p=0.336). Analyzing the difference in drinking duration between rewarding and non-rewarding plants between nights, there was no significant change in behavior of hawkmoths (Friedman-test, N=12, df=2, χ^2= 0.667, p=0.717).

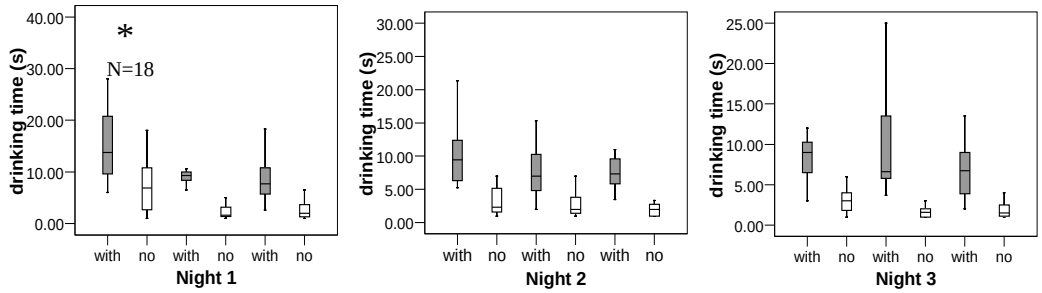

Figure 2: median drinking time for each trial (E1-3) in the three consecutive experimental nights (night 1-3)

It is noteworthy, that the drinking duration on rewarding plants does significantly decline over the three nights (Friedman-test: N=17, df=2, χ^2=7.636 p=0.022). The drinking time per flower on rewarding plants never declines below "no nectar" plants. The drinking

duration on plants with no nectar does not change over the three nights (Friedman-test, N=12, df=2, χ^2=1.911 p=0.385).

Discussion

The aim of this study was to determine the importance of learning for foraging decision rules exerted by *M. sexta* that might favor a cooperative behavior of *Petunia axillaris axillaris* N (maintain nectar production). Three foraging decision rules of pollinators namely discrimination of cheaters prior to probing, reduction of flower number visited on nonrewarding plants and reduction in drinking time per flower on rewardless plants had been identified that might select for reward provision in plant-pollinator interactions (Smithson and Gigord 2003). Of these three potential control mechanisms, the last two have been identified in hawkmoth-*Petunia* interactions (chapter 2 and 4). Here we could reproduce results obtained in previous experiments: Drinking was significantly reduced on low nectar lines, and number of flowers visited on rewardless plants reduced. Also in accordance with earlier results was that one behavioral response, namely prior discrimination of nectarless plants could not be observed. The inability to discriminate empty from rewarding flower seems to be a common pattern of foraging insects (Thakar et al. 2003). Nectar is mostly concealed within the plant and visually not accessible for pollinators, the content cannot be evaluated prior to probing. Deceptive orchids exploit this circumstance by luring naïve pollinators driven by an innate preference to attractive floral displays or mimicked mating signals. Pollinators of deceptive orchids are usually hymenopterans (Dafni 1984, Schiestl 2005), an order, where learning is well-described

(Chittka and Thomson 2001). The time frame where outcrossing nectarless orchids can reproduce is therefore limited to the state of naivety of pollen vectors and significantly decreased by progressed pollinator learning (Ferdy et al. 1998, Gigord et al. 2002). Learning to avoid cheaters is constrained if “mimicks” (cheaters) resemble closely the phenotype of “models” (nectariferous plants) (Dyer and Chittka 2004, Internicola et al. 2007). In our case, nectar has been removed manually from one plant and the scent profile remains similar after nectar extraction (unpublished data). A phenotypical discrimination of the two plants is therefore not possible. Furthermore, nectar content is visually not accessible as it is concealed at the bottom of a long floral tube. We assume that under the given conditions, avoidance learning by hawkmoths of *Petunia* flowers where nectar has been removed manually is constrained.

However, we are aware that our learning set-up might not have been adequate and masked hawkmoth learning abilities: first, the number of learning trials might have been too low for hawkmoths to memorize rewarding and nectarless plants. Classical conditioning protocols conducted with bees lead to the establishment of a associative memory after the very first trial, and after three flower visits, this memory lasts as long as a lifetime (Hammer and Menzel 1995). Training protocols with free flying hymenopteran foragers usually involve 70-150 visits to flowers during one day (Cnaani et al. 2006, Internicola et al. 2009), corresponding to 3-5 foraging bouts. Avoidance learning has been demonstrated to be successful after the very first visit to a rewardless species (Dukas and Real 1993). In this case, the experiments were conducted with wild caught bumblebees with unknown experience. Avoidance learning has furthermore been

observed in hummingbirds, which remember the flower after having depleted it and subsequently avoid it (Healy and Hurly 2001).

The situation seems to be more complex for lepidopteran species: Classical conditioning experiments conducted with *Heliothis virescens* (Skiri et al. 2005) and *Manduca sexta* (Daly et al. 2001) using scent as conditioned stimulus, showed that 8 learning trials were needed to elicit a positive response to the odor. In experiments with free-flying *Macroglossum stellatarum*, 20 learning trials were required to establish associative learning of differently colored artifical flowers (Kelber 1996, Kelber and Pfaff 1997), but fewer trials (1-10) to learn spectral colors (Kelber and Henique 1999). Generally, hawkmoths seem to have strong innate preferences that can overshadow a learning effect (Kelber 2002, Balkenius et al. 2008). Due to the inconsistencies of learning protocols and experimental outcomes in hawkmoth learning assays, the design of our learning set-up was challenging and might have been not appropriate for avoidance learning.

Nevertheless, the results of this study and other experiments show, that discrimination of cheaters prior to probing is not learnt and cannot act as partner control mechanism in the *Manduca-Petunia* system.

The two other decision rules, namely reduction of drinking time and number of flowers visited are part of the foraging strategies exerted by hawkmoth. These two behaviors are exerted from the very first trial and do not improve with successive experience. There is no evidence for the importance of learning with respect to these potential partner control mechanisms. It is more likely, that the behavioral response to an unrewarding plant has a genetic component. Hawkmoths might adjust their drinking time and the number of flowers visited on the plant, based on the reward ingested in the first flower encountered

on the plant. If the reward is above a certain threshold the pollinator will continue feeding on the same plant whereas empty flowers will be rejected. A possible explanation for this foraging strategy is that it is a response to the reward distribution in natural plant populations. Nectar is never uniformly distributed under natural conditions. Individual flowers have large intraspecific variations in nectar volume due to evaporation, preceding depletion, genetic variation, access to nutrients and water and other environmental factors (Mitchell 2004). It is generally assumed, that learning is advantageous in variable environments (Dukas 2008). However, if the environment is too variable up to a point where it becomes unpredictable, the genetic component or innate behaviors should be favored (Stephens 1993). The "hard-wired" foraging strategies of *Manduca sexta* might thus be deducible to the random and unpredictable distribution of nectar in natural plant populations. We assume that learning is more important in other aspects of the hawkmoths' life, such as host plant choice (Cunningham and West 2008), associative learning (e.g. in the beginning of the foraging career) or in handling time of flowers (Goyret and Raguso 2006). It is noteworthy, that in our experiments, there is a decline in drinking (=handling) time on rewarding flowers during the course of the experiment. It has been demonstrated, that experienced pollinators can reduce their handling time substantially after only a few probing events which greatly improves their foraging efficiency and intake rate (Laverty and Plowright, 1988, Chittka et al. 1999, Goulson 1999) including *Manduca sexta* (Goyret and Raguso 2006). Hawkmoths thus have the ability to improve their motor skills on rewarding *Petunia* flowers. If pollen uptake was a factor of probing time, this pollinator behavior would actually be problematic for the plant. With increased experience, the pollinators would decrease pollen uptake. In our

case, the drinking time on rewarding flowers was reduced in the course of the experiment, but never dropped below the time spent drinking on non-rewarding flowers.

Conclusion and outlook

Hawkmoths did not discriminate between rewarding and nectarless plants before probing. The drinking time and number of flowers visited was significantly reduced on empty flowers from the very first encounter to the last. These foraging decision rules are not learnt by hawkmoths and do not improve with learning. We suggest that behavioral responses to rewardless plants are genetically predetermined and are not acquired by learning.

Learning might be of importance in other respects of hawkmoths' behavior, for example in associative olfactory learning (Daly and Smith 2000), handling skills (Goyret and Raguso 2006) or in finding suitable host-plants for oviposition (Cunningham and West 2008). Understanding the interaction between innate behavior and learning may turn out to be important in understanding the conditions which favor the evolution of cheating in plants.

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General Discussion

General discussion

We have investigated foraging behaviors of hawkmoths confronted with *P. axillaris* with reduced or no nectar rewards, both under laboratory settings with naïve and experienced *Manduca sexta* and in the field with naturally occurring hawkmoths.

In the laboratory experiments, we have identified that 1. hawkmoths do not discriminate between rewarding and non-rewarding flowers before probing, 2. hawkmoths reduce their drinking duration on non-rewarding and less rewarding flowers and 3. contrasting results regarding hawkmoth visitation of a reduced flower number in low or no nectar plants: there was a significant lower overall visitation of number of flowers in one experiment (chapter 5) and no reduction of number of flowers in another experiment (chapter 2).

In the field, the behavioral response seemed to be influenced by the surrounding density of co-flowering foraging petunias: 1. hawkmoths did not discriminate before probing in the low-density site, but in a high density site they visited significantly more rewarding petunias in first choice experiments than nectar less ones. However discrimination is incomplete, as other non-rewarding flowers were visited after their first choice, 2. the number of flowers visited on nonrewarding plants was significantly reduced in the high-density site, but not in the low density site, 3. drinking duration was too short to measure (<1 s).

To summarize this, discrimination prior to probing does not seem to be a partner control mechanism of hawkmoths. This outcome is in contrast to other systems such as deceptive orchids, where pollinators (mostly Hymenoptera) can learn to discriminate between

rewarding and unrewarding species and avoid deceptive species subsequently (Ferdy et al. 1998, Smithson and Macnair 1997). Presumably, this mechanism is not prevalent in hawkmoths, as the one behavioral response, namely drinking time reduction might be sufficient to discriminate against cheaters and reduce their spread in the population. In addition to that, nectar depletion, drought and evaporation might reduce nectar volumes of hawkmoth-pollinated plants in the field and to our knowledge, there are no hawkmoth-pollinated species known that fail completely to produce nectar, as opposed to the orchids. However, this variability of nectar distribution is impossible to learn.

Concerning the reduction of drinking time, we were able to show that seed set of plants with reduced nectar volume was increased when pollinated by hand and decreased when pollinated by a moth. Unlike in deceptive orchids, where the mere visit to the flower is sufficient to pick up the maximum pollen (which is comprised in sticky pollinia) and one more visit to deposit the pollen, it might rather be the drinking time in our hawkmoth pollinated system that determines the pollen uptake. The only other study that we found that correlated drinking time and seed set was carried out with *Silene maritima* and the pollinating hoverfly *Eristalis pertinax*: in line with our results seed set was reduced with shorter drinking time (Warren and James 2008). The reduction of drinking time thus counterbalances the benefits of cheating and drinking duration might be a partner control mechanism that discriminates against cheaters, whereas discrimination before probing is not.

Reproduction of cheaters in deceptive orchids is thus limited by the learning capacities of their pollinators, and in our system it is limited by the innately exerted behaviors such as

drinking duration. An important additional experiment that needs to be conducted concerning drinking duration is to measure the pollen uptake of *Manduca sexta*.
The number of flowers visited is puzzling in respect to whether it can act as a partner control mechanism. Generally, it was demonstrated that large floral displays attract more pollinators (Bosch and Waser 2001) but also increase inbreeding depression by higher visitation rates in one plant (Charlesworth and Charlesworth 1987). As our *Petunia axillaris* plants are also self-compatible, future experiments might give similar results.

Conclusions and outlook

A challenging next step will be to assess how the number of visited flowers might influence male and female reproductive success of cheaters and *P.axillaris*. If the reduction of number of flowers visited leads to a reduction of capsule maturation, this can be another partner control mechanism of pollinators preventing the spread of cheaters. Additionally, pollen export as a measure of male reproductive success can be assessed. These behavioral assays can be conducted under “laboratory” conditions in a large flight hall. Additionally, as field experiments have indicated that the number of flowers visited might also be a function of plant density, the plants can be arranged at different densities in this flight hall.
In order to complete the picture about fitness consequences (or advantages) that cheaters might face independent of pollinator behavior, we would like to continue to study other fitness parameters of plants with reduced nectar production such as plant height, biomass, number of flowers produced per lifetime and number of flowers blooming simultaneously, furthermore pollen production.

Finally, we would like to continue field studies with the low nectar line F25 in *P.axillaris* habitats in South America. Pollinator number, identity and learning abilities as well as plant density, population size and composition are crucial components that shape the net fitness outcome of a flowering plant.

Altogether, breeding and selection of a *Petunia* line with reduced nectar investment opened a gateway to investigate a large number of interesting questions in plant-pollinator interactions and to explore the genetic background of nectar production.

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