Aspects of the Sensory Physiology of the Pollination of *Petunia axillaris* and Mating in Moth Pests

Thèse présentée à la Faculté des Sciences
Institut de Biologie
Université de Neuchâtel

Pour l'obtention du grade de docteur ès Sciences

Par

Alexandre Gurba

Acceptée sur proposition du jury:
Dr Patrick Guerin, Université de Neuchâtel, directeur
Prof. Cris Kuhlemeier, Université de Berne, rapporteur
Dr Christophe Praz, Université de Neuchâtel, rapporteur
Dr Patrik Kehrli, Agroscope fédéral – Changins, rapporteur

Soutenue le 10 septembre 2013

Université de Neuchâtel
2013

UNIVERSITÉ DE
NEUCHÂTEL

Faculté des sciences
Secrétariat-décanat de Faculté
Rue Emile-Argand 11
2000 Neuchâtel - Suisse
Tél: + 41 (0)32 718 2100
E-mail: secretariat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Monsieur Alexandre GURBA

Titre:

"Aspects of the Sensory Physiology of the Pollination of *Petunia axillaris* and Mating in Moth Pests"

sur le rapport des membres du jury composé comme suit:

- Prof. Patrick Guerin (Université de Neuchâtel), directeur de thèse
- Dr Christophe Praz (Université de Neuchâtel)
- Dr Patrik Kehrli (Station de recherche Agroscope-Changins-Wädenswil ACW)
- Prof. Cris Kuhlemeier (Université de Berne)

Neuchâtel, le 17 octobre 2013

Le Doyen, Prof. P. Kropf

SUMMARY

Compounds produced by plants have an important impact on many aspects of the biology of insects from whom plants are a particularly important source of food. As an adult, insects use chemical messages to identify and locate a suitable food source. The specificity of the host plant relationship is also crucial for healthy development of larvae. The choice of oviposition site is determinant for the survival of offsprings and is largely selected by the specific array of host plants stimuli. It has also been shown that the sexual behaviour of insects is influenced by host plant odours by increasing the sensitivity of males to sex pheromones of females.

The genus *Petunia* is particularly interesting to study the role of floral traits in speciation because all the known species are artificially crossable. However, hybridization in the field is rare as the reproductive isolation is provided by the selective attraction of pollinators. Three distinct pollination syndromes have emerged in *Petunia,* corresponding to as many guilds of pollinators: bees, hummingbirds or hawkmoths. *P. axillaris* attracts hawkmoths such as *Manduca sexta*, a Sphingidae, by releasing a strong sweet odour. In contrast, *P. exserta* which is pollinated by hummingbirds is devoid of scent. Crossings between these two species were exploited here to study the genetic basis of the evolution of fragrance in *Petunia* and its importance in foraging preferences of *M. sexta*. We characterized the scent of *P. axillaris* and confirmed that *P. exserta* produces no odour. We show that the antennal olfactory receptor cells of *M. sexta* is very sensitive to the compounds released by *P. axillaris*, especially methyl benzoate, benzyl alcohol and methyl salicylate. Finally, we observed through choice tests in a wind tunnel that plants producing odours were always preferred, regardless of the colour of the flower. We conclude that the production of fragrance is an important floral trait in this plant-insect relationship and that this feature alone can modify the foraging preference of *M. sexta*. The implications of these findings on speciation processes in *Petunia* are discussed.

In insects specific attraction by odours is largely modulated by the presence of food. We were interested in the perception of different essential energy nutrients including nectar sugars by *M. sexta*. We have characterized the taste sensilla on the proboscis and measured the neural response of the gustatory receptor cells of styloconic and basiconic sensilla to glucose, fructose and sucrose. For both sensillar types, the response to fructose is slightly stronger than to sucrose while glucose was not detected at the dose tested. Meanwhile, the feeding preference of the moth was assessed through binary choices experiments. It turns out that the *M. sexta* prefers to feed on sucrose or fructose compared to glucose. In addition, it can detect low concentrations of sucrose and prefers to feed on the most concentrated solutions. Finally, it seems that the volume of sucrose solution imbibed is determined by its concentration. Behavioural preferences by *M. sexta* for these sugars correlate well with their mouthpart sensory cell responses.

The European grapevine moth, *Lobesia botrana,* and the codling moth, *Cydia pomonella*, are both very important worldwide insect pests. Without adequate control, fruit damage can be important and the arising financial losses substantial. Mating disruption is a specific control method using the female sex pheromone to attract males to lures and thus reduce their chances of mating. The use of host plant volatiles to improve the attractiveness of males to sex pheromones presents an interesting possibility to improve the mating disruption control method. We measured the attraction of male *L. botrana* to different doses of heptane and octane in combination with the underdosed sex pheromone. While the attractiveness of the mixture increases with dose for both products, a maximum number of males arrive at the source with a release rate of 10 ng/min of the alkanes released with the pheromone. This level of attraction is comparable with that obtained with the sex pheromones released directly from calling females. While the alkanes are inactive on their own, the pheromone diluted directly in heptane induces not only contact to the source no different to the control, but recruits more moths in the early stages of flight. Another plant compound, 2-phenylethanol also increases the attraction of the underdosed *L. botrana* sex pheromone to males when released at 100 pg/min. In addition, when one of these plant compounds is added to the pheromone, the time needed to reach the source is halved in *L. botrana*. The findings with these plant compounds were confirmed in *C. pomonella*. The wide range of concentrations over which these alkanes are active in both species provides a promising mean to improve control of field populations using mating disruption.

Throughout this thesis we studied plant-insect interactions from different angles. We have shown how a plant can manipulate a pollinator to achieve pollination, alternatively, how a moth selects plant stimuli to cover its energy needs. We have also shown that hitherto unstudied plant volatiles improved the chances of male moths to encounter females.

Keywords: Plant-insect interactions, sensory physiology, *Petunia axillaris*, *Petunia exserta*, plant volatiles, neurophysiology, olfaction, taste, sugar preference, mating behaviour, *Lobesia botrana*, *Cydia pomonella*, pheromone, wind tunnel, alkanes.

RÉSUMÉ

Les composés produits par les plantes ont un impact important dans plusieurs aspects de la biologie de nombreux insectes et les plantes sont une source de nourriture particulièrement importante. A l'état adulte, les insectes utilisent ces messages chimiques pour identifier et localiser une source de nourriture qui leur convient. La spécificité de la plante est aussi cruciale pour le bon développement des larves peu mobiles. Le choix du lieu de ponte est donc important pour la survie de la progéniture et est également grandement déterminé par l'odeur spécifique des plantes hôtes. Il a aussi été prouvé que le comportement sexuel des insectes est influencé par les odeurs de plante hôte en augmentant la sensibilité des mâles aux phéromones sexuelles.

Petunia est un genre de plante particulièrement intéressant pour étudier le rôle des caractères floraux dans la spéciation, car toutes les espèces connues sont capables de se croiser artificiellement. Toutefois, l'hybridation sur le terrain reste rare. L'isolation reproductive est alors assurée par l'attraction sélective des pollinisateurs. Il existe chez *Petunia* trois syndromes de pollinisation distincts correspondant à autant de guildes de pollinisateurs : abeilles, sphingidés ou colibris. *P. axillaris* attire *Manduca sexta*, un sphingidé, en produisant une puissante odeur. En revanche, *P. exserta* qui est pollinisé par des colibris ne relâche aucune odeur. Le croisement de ces deux espèces permet d'étudier la base génétique de l'évolution du parfum chez *Petunia* et son importance dans la préférence de butinage de *M. sexta*. Nous avons caractérisé le parfum de *P. axillaris* et confirmé que *P. exserta* ne produisait pas d'odeur. Nous avons montré que *M. sexta* est très sensible à ces composés, particulièrement au methyl benzoate, benzyl alcohol et méthyl salicylate de *P. axillaris*. Finalement, nous avons observé par le comportement en chambre de vol que les plantes produisant des odeurs étaient toujours préférées, quel que soit la couleur de la fleur. Nous en avons déduit que la production de parfum et un trait floral déterminant dans la relation plante-insecte et que ce trait peut à lui seul changer la préférence de butinage de *M. sexta*. Les conséquences de ces résultats sur la spéciation chez *Petunia* sont discutées.

Chez les insectes l'attraction spécifique par certaines odeurs est en grande partie modulée par la présence de nourriture. Nous nous sommes intéressés à la perception de *M. sexta* aux différents nutriments énergétiques essentiels que sont les sucres. Après avoir caractérisé les sensilles gustatives de son proboscis, nous avons mesuré la réponse neuronale des sensilles styloconiques et basiconiques au glucose, fructose et sucrose. Pour les deux types sensillaires, la réponse au fructose est légèrement plus intense qu'au sucrose alors que celle au glucose est imperceptible à la dose testée. Parallèlement, la préférence de nourrissage du papillon a été évaluée en choix binaires. Il en découle que les papillons préfèrent se nourrir de sucrose ou de fructose par rapport au glucose. De plus il parvient à détecter de faible concentration de sucrose et préfère se nourrir sur les solutions les plus concentrées. Finalement, il semble que le volume de solution de sucrose soit déterminé par sa concentration. La préférence comportementale pour ces sucres est bien corrélée avec leur perception neuronale.

Le ver de la grappe, *Lobesia botrana*, et le ver de la pomme, *Cydia pomonella*, sont tous deux de très importants insectes ravageurs des cultures à l'échelle de la planète. Sans mesures de contrôle adéquates, les pertes financières sur les cultures peuvent être considérables. La confusion sexuelle est une méthode spécifique de lutte utilisant la phéromone sexuelle pour attirer les mâles vers des leurres et ainsi diminuer les probabilités d'accouplement. Utiliser des produits de plantes hôtes pour améliorer l'attraction des mâles par les phéromones sexuelles présente une possibilité intéressante. Nous avons mesuré l'attraction des mâles de *L. botrana* à différent doses d'heptane ou d'octane en combinaison avec une concentration sous-dosée de phéromone. Alors que l'attractivité du mélange augmente avec la dose pour les deux produits, nous obtenons une proportion maximale de papillons arrivant à la source avec 10 ng/min d'alcane relâché avec la phéromone, un niveau d'attraction comparable avec celui obtenu avec des phéromones directement relâché avec des femelles. Alors que ces alcanes sont inactifs seuls, la phéromone directement dilué dans l'heptane induit non seulement autant de contact avec la source qu'avec un solvant classique, mais recrute plus de papillons dans les premières phases d'attraction. Un autre composé de plante, le 2-phénylethanol augmente également l'attraction de la phéromone lorsqu'il est relâché à 100 pg/min. De plus, lorsque l'un de ces composés de plante est ajouté à la phéromone, le temps que met le papillon pour atteindre la source est réduit de moitié. Tous ces résultats sont confirmés également chez *C. pomonella*. Le large spectre de concentrations où ces alcanes sont actifs chez ces deux espèces offre un outil prometteur pour améliorer le contrôle des populations sur le terrain en utilisant la confusion sexuelle.

Au travers de cette thèse, nous avons étudié les interactions plantes-insectes sous plusieurs facettes. Nous avons montré comment une plante arrive à manipuler un pollinisateur pour parvenir à se reproduire, ou comment un papillon utilise les stimuli chimiques d'une plante pour se nourrir. Nous avons également démontré que certaines odeurs de plantes amélioraient les chances des papillons mâles à trouver une femelle.

Mots-clés : Interaction plante-insecte, physiologie sensorielle, *Petunia axillaris*, *Petunia exserta*, odeurs de plantes, neurophysiologie, olfaction, perception du gout, préférences de butinage, comportement de reproduction, *Lobesia botrana*, *Cydia pomonella*, phéromone, chambre de vol, alcanes.

CONTENTS

ACKNOWLEDGEMENTS

This research project would not have been possible without the support of many people.

First of all, I thank my supervisor Dr Patrick Guerin for his guidance and support throughout my thesis work and for teaching me to work more rigorously and accurately. Thank you for your comprehension for my needs to leave sometimes earlier for sports trainings.

- Thanks to Prof. Cris Kuelemeier for his precious input and support during this project. It was always a pleasure to visit you in Bern for stimulating and instructive discussions.
- Thanks to Dr Ulrich Klahre for his close collaboration and for all the sweet smelling flowers. It was a great opportunity for me to work with such a passionate researcher.
- Many thanks to my colleagues Sébastien Kessler, Charles Chappuis, Caroline Joris, Jerôme Frei, Thomas Kröber, Jonas Durant and Maxime Jaquet. Thank you guys for all the good moments spent with you and all the interesting discussions about science.
- Thanks to all the people from the Institute of Plant Science, University of Bern, especially Alexandre Dell’Olivo, Anna Brandenburg, Katrin Hermann, Moritz Saxenhofer and Antonina Internicola for your warm welcome.
- I also want to thank my thesis committee, Dr Patrik Kehrli and Dr Christophe Praz. I am grateful for your interest in my research.

- Thanks to Martine Bourquin, Susana da Costa and Alessandro Bassetti for the efforts they made for rearing of my moths and for their tasty cakes. The high quality of the insect rearings was the cornerstone of my successful behavioural experiments.

- Thanks to Michèle Vlimant for her precious experience in microscopy. Thank you for your help in the characterisation of the sensory organs of my insects and for your everyday good humour.

- Thanks to Radu Slobodeanu at the Institute of Mathematics for his help in finding the most relevant statistical tests.

- Thanks to Prof. Joachim Schachtner and especially to Martina Kern from the University of Marburg, (Germany) for the generous supply in *M. sexta*.

- Thanks to Armelle Vallat for her help and her broad knowledge in natural product analytics.

- Thanks to the technical service of the Faculty of Science,University of Neuchâtel, especially Daniel Varidel and Christian Heche. Thanks for having helped me in realizing my experimental setups.

- Thanks to the building maintenance staff of the Faculty of Science, especially Luis Amavel, Fabio Beltrame and Jean-Pierre Duvoisin for their technical support in installing and maintaining the climate chambers.

- Thanks to the National Centre of Competence in Research (NCCR) Plant Survival, a research programme of the Swiss National Science Foundation, Dr Patrick Guerin and Prof. Felix Kessler who were involved in the funding of this thesis research.

- Last but not least, I like to thank my family for supporting me, for their comprehension and encouragement in my educational pursuits, to my friends for providing me with healthy dose of distraction and to my sport coaches and partners for always having believed in me despite my irregular presence at training sessions.

Chapter I.

General introduction

The present thesis focuses on the chemical ecology of insects. I am interested in how insects use plants to survive, alternatively how plants use insects to survive, by looking at sensory cues produced by plants and utilized by insects. I have measured the sensitivity of insect neuronal systems to stimuli derived from plants to reveal signals that may contribute to induce an adequate behavioural response. I have focused my efforts on the sensory modalities underlying the two most important behaviours of an insect's life: foraging for food and seeking a mate for reproduction.

Insect sensory ecology, the case of *Manduca sexta*

The tobacco hornworm, *Manduca sexta* Linnaeus (Lepidoptera, Sphingidae)*,* has been widely studied from the point of view of its sensory ecology and physiology. This moth is known to be attracted by a wide spectrum of olfactory and visual stimuli. The nocturnal activity of the moth dictates that it uses olfactory cues mainly to detect and orient towards its source of food. Night-blooming flowers generally emit a strong fragrance. Several studies have shown the sensitivity of *M. sexta* to over 20 volatile plant compounds from solanacaeous plants using gas chromatography linked to electroantennogram recording (GC-EAG;Fraser et al. (2003), Hoballah et al. (2005) and Stuurman et al. (2004)).

Non-floral odours are known to be attractive, but do not increase attraction of *M. sexta* when added to floral scent (Raguso and Willis, 2005). Raguso and Willis (2002; 2005) demonstrated the attractiveness of *M. sexta* to an odour source and to an unscented visual stimulus. However, despite the ability of the moths to locate the source of the stimulus, both olfactory and visual information are required to elicit a feeding response. Despite low light intensity during their period of activity, hawkmoths are able to distinguish colours where bees and humans are colour blind (Kelber et al., 2003). Flowers represent strong visual stimuli that attract *M. sexta* on their own and artificial unscented flowers are preferred over an olfactory stimulus (Goyret et al., 2007). Colour of the flower limb has an important impact on the feeding preference of *M. sexta* as it prefers to feed on white petunias when white and purple plant siblings are in competition (Hoballah et al., 2007). Moreover, limb size affects its foraging behaviour as bigger limbs induced more feeding bouts in *Petunia* (Venail et al., 2010) and more dissected corolla limbs were also preferred on *Nicotiana* (Kaczorowski et al., 2012). However, Goyret and Raguso (2006) showed a negative correlation between corolla limb size of artificial flowers and handling efficiency of *M. sexta*. Moreover, the mechanosensory input provided by radial grooves on the limbs of artificial flowers, to mimic veins enhanced the efficiency of the moth to feed and visual stimuli helped in proboscis placement while tactile stimuli controlled proboscis movements (Goyret, 2010).

Many night-blooming flowers show intensive nectar and fragrance production coupled to spectacular bud growth preceding anthesis (Raguso and Willis, 2003). Such metabolic activity should increase floral CO_2 production. This was confirmed by Guerenstein et al. (2004) on *Datura wrightii*. *M. sexta* is capable to detect CO_2 via labial pit organ receptors (Kent et al., 1986) and naïve moths prefer to feed on scented artificial flowers liberating higher levels of CO_2 than on those releasing CO_2 near ambient levels (Thom et al., 2004), suggesting that CO_2 plays a role as a guide to nectar. These results were confirmed by Goyret et al. (2008a), but CO_2 did not increase the feeding behaviour, suggesting that CO_2 is used rather as a distance attractant than as a stimulus guiding the proboscis to aim at nectar. Water vapour is another floral cue released within 30 minutes after anthesis. In the fragrant evening primrose, *Oenothera cespitosa,* the increase in relative humidity can reach 4% above the corolla and is directly linked to nectar volume. This slight increase of relative humidity is sufficient to induce more approaches and probing of the white-lined sphinx *Hyles lineata* (Raguso et al., 2005; von Arx, 2013; von Arx et al., 2012a).

M. sexta is a generalist forager. Its high energy needs push it to find a meal on numerous plant species producing large amounts of nectar. This plant attribute is often accompanied by other floral traits such as UV non-reflecting white corollas, powerful sweet scent and a long, narrow tube such as in *Nicotiana sp*., *D. wrightii* and *P. axillaris*. However, the combination and the quality of these floral traits vary between species. *M. sexta* has therefore to handle with these floral characteristics that all code for a reliable energy resource. It shows a high level of neuronal plasticity and an ability to learn fast giving it the advantage to switch to the most abundant or most rewarding plant species (Alarcon et al., 2008; Goyret et al., 2008b; Riffell et al., 2008).

Flower fragrance

Plant floral scent is an ancient medium for communication between plants and pollinators (Pellmyr and Thien, 1986). Flower fragrance is used by insects for orientation from a distance, approach, landing, feeding, oviposition and associative learning. Odour is also used as a strategy to invoke discrimination and preferences by pollinators for similar plant species, especially among sympatric species, and so serves to drive reproductive isolation (Dodson et al., 1969). Floral scents are mixtures of small organic volatile compounds derived from glucose (Paré and Tumlinson, 1997; Raguso, 2003). The four major chemical classes are biosynthesised in plants by different pathways. Aromatics compounds such as indole and benzenoids are produced the by pentose-phosphate and shikimate pathways. The Rohmer biosynthetic pathway gives rise to monoterpenoids such as pinenes, linalool and limonene. Sesquiterpenoids issue from the mevalonate pathway and linolenic acid gives rise to fatty acid-derivatives such as ketones and aldehydes through of the lipoxygenase pathway (Raguso, 2003). In addition, fragrance depends on environmental conditions linked to the nature of the different compounds and the circadian rhythm of the plant (Hoballah et al., 2005). *Petunia axillaris* releases a complex fragrance of various benzenoid compounds such as benzyl alcohol, benzyl benzoate, methyl benzoate, benzaldehyde and other minor compounds such as eugenol, isoeugenol and vanillin (Hoballah et al., 2005; Stuurman et al., 2004). Most of these volatiles are synthetized by the shikimate pathway through the metabolism of phenylalanine.

Pollination syndrome

The term pollination syndrome refers to convergent evolution between a plant and its pollen vector. Its results from a set of floral traits such as colour, shape, size, contrast, odour, nectar type and amount, and the timing of flowering that are geared to a particular group of pollinators (Faegri and Van der Pijl, 1979). Flower specialisation increases the probability that the pollinator transfers pollen from one plant to a conspecific and finally enhances plant fitness, but reproductive success is dependent on a narrower spectrum of pollinators. The concept of pollination syndrome is criticized on the grounds that most flowers attract a large spectrum of pollinators and that flowers often diverge without excluding one type of pollinator in favour of another. Pollination syndromes can be thought of as extremes of a continuum of greater or lesser specialization or generalization to particular functional groups of pollinators that exert similar selective pressures, but the frequency with which flowers actually conform to the expectations of the pollination syndrome is relatively rare. Despite these criticisms, the concept of pollination syndromes helps us in our understanding of the mechanisms underlying the dramatic diversification in Angiosperms and reproductive isolation.

Petunia, a study model for pollination

The genus *Petunia* (Solanales, Solanaceae) is proposed as a model to investigate some mechanisms in a pollination syndrome. It groups at least 17 species originating in South America with the majority of them spreading into the south and southeast of Brazil (Stehmann and Semir, 2005) and several demonstrate broadened examples of pollination syndromes involving melitophilous, sphingophilous and ornithophilous species. This genus has a long history of artificial selection, and crossings and hybrids of *P. axillaris* and *P. integrifolia* are sold worldwide in garden centres as ornamental plants (*P. hybrida*). Species among this genus can produce fertile crosses. As the reproductive barrier seems to be weak (Watanabe et al., 1996), reproductive isolation among these species is more related to the selective visitation by the different animal taxa rather than to genetic incompatibilities. Absence of hybrids of *P. axillaris* (hawkmoths) and *P. integrifolia* (bees) in sympatric populations argues in favour of a pollination syndrome as a means to maintaining isolation between these species in the wild (Ando et al., 2001).

P. integrifolia presents a melitophilous pollination syndrome with poorly scented purple flowers and its wide tube provides easy access to bees at low levels of nectar. *P. axillaris* is a species derived from *P. integrifolia*. In *P. axillaris* the gene AN2 coding for the anthocyanin, a factor acting on the purplish colour of the corolla, is deficient and flowers are white. Hoballah et al. (2007) showed that the change of colour in *P. integrifolia* produces a shift in the pollinator's foraging preference demonstrating the contribution of colour to the reproductive isolation of the two species. Olfactory cues have also diverged between the two species. Hoballah et al. (2005) described the difference of the fragrance between the two species and the nocturnal periodicity of scent emissions by *P. axillaris*. While *P. integrifolia* releases only traces of benzaldehyde, *P. axillaris* produces copious amounts of benzenoids, terpenoids and phenylpropanoids, including benzaldehyde, methylbenzoate and benzyl alcohol, mainly through the metabolism of phenylalanine. With its attractive colour and fragrance *P. axillaris* rewards its pollinators with a large amount of nectar harboured in a long narrow tube. Floral traits of this plant are more related to a sphingophilous pollination syndrome. *P. exserta* (Stehmann) is an extremely rare and endemic species (Lorenz-Lemke et al., 2006; Stehmann, 1987) pollinated mainly by humming birds. The difference in reproductive strategy between *P. axillaris* and *P. exserta* is reflected by the different floral traits. Unlike *P. axillaris*, *P. exserta* produces unscented red flowers with stigmas extending from the tube-shaped corolla. However, *P. exserta* seems not to be a close relative of other red-flower hybrids but appears to be more closely related to *P. axillaris* (Ando et al., 2005; Kulcheski et al., 2006). Furthermore, the composition of its pigments is not similar to the ones of other red/purple species. This means that evolution permitted a population of *Petunia* to develop from a purple flower to a white flower (*P. axillaris*) and then back again to a red species (*P. exserta*). This speciation appears to have happened recently and reproductive isolation is not yet complete.

By taking advantage of the weak genetic isolation between closely related *Petunia* that express a well-defined pollination syndrome (Gübitz et al., 2009), the genetic basis underlying these syndromes was largely elucidated (Galliot et al., 2006b; Stuurman et al., 2004). The quantitative loci involved in the regulation of flower size (Galliot et al., 2006a;

Venail et al., 2010), nectar volume (Brandenburg et al., 2012a; Galliot et al., 2006a), flower pigmentation (Hoballah et al., 2007), tube length (Venail et al., 2010) and flower fragrance (Klahre et al., 2011) have been characterised within the genome of *Petunia*. Such tools may permit to isolate floral traits of *Petunia* for the evaluation of their role in foraging behaviour of typical pollinators.

Nectar reward

Nectar is the principal source of energy for most of butterflies and nocturnal moth. As pollinators are seeking such a resource in flowers, the latter have to produce nectar that fulfil the requested energy needs of the insects to induce forager loyalty and, consequently, increase the probability that pollen will be transferred onto conspecific plants for reproduction. However, nectar production is very energy-demanding and affects seed production (Brandenburg et al., 2009). Such a nutritive source does not only attract pollinators. Microbes (Herrera et al., 2009; Herrera et al., 2008) and robbers can use this reward without transferring pollen. Pollinators may also deposit pollen on the wrong plant. To counterbalance these disadvantages, exclusive relationships between the plant and one or few pollinators have emerged.

Nectar offers to pollinators a rich source of various sugars such as sucrose, glucose and fructose. Other constituents such as proteins, phenols, lipids and amino acids are found in much lower proportions. The production and concentration of nectar constituents can be subject to fluctuations, even within individual flowers. These compounds, especially essential amino acids (Arg, His, Ileu, Leu, Lys, Met, Phe, Thr, Try and Val) should be crtitical for long-living pollinators such as *Manduca sexta*. Mevi-Schutz and Erhardt (2005) showed that the quantity and quality of amino acids in the diet may enhance the longevity and fecundity in butterflies. However, the adult hawkmoth *Amphion floridensis* is able to synthesize nonessential ovarian amino acids from sugar and essential amino acids used for egg production are entirely provided from the larval diet (O'Brien et al., 2002).

Nectar is considered as one of the floral traits involved in pollination syndromes because of it selectivity for a pollinator type and this trait should have coevolved with its pollinator to optimise the reproductive success of the plant (Krömer et al., 2008). However, from the flower perspective it is not advantageous to be emptied of nectar by the first visiting pollinator. To be able to offer reward to more visitors and so increase the chances of disseminating its pollen, nectar may hold deterrent secondary metabolites such as alkaloids to diminish drinking time by the pollinators and repel robbers. A corollary of this is that pollinators have to visit more flowers to imbibe enough nutrients and so increase pollen dissemination. In jasmine, *Gelsemium sempervirens*, the presence of gelsemine in nectar is responsible for a decrease in both visitation frequency and duration of visits while it increases the number of visited flowers (Irwin and Adler, 2008). *Nicotiana attenuata* produces nicotine in its nectar that acts as a deterrent to decreases the drinking time of hawkmoths, hummingbirds (both pollinators) and ants (robbers) (Kessler and Baldwin, 2006).

As nectar composition differs between plants and plants pollinated by hawkmoths produce nectar of comparable sugar composition, then the preferences of *M. sexta* for the different sugars present should reflect the nectar composition of such plants. Moreover, the sensory input of sugar perception from mouthpart organs should support this assumption.

Because of the difficulty to procure regularly *M. sexta* in suitable quantity to continue my experiments either from our own insect rearing or from other laboratories, we decided to point the second part of my thesis to the improvement of the mating disruption blends of compounds for two pest moths: the European grapevine moth, *Lobesia botrana*, and the codling moth, *Cydia pomonella*. This part of my thesis represents a continuation of research performed on this topic by two PhD students and two Postdoc in this laboratory (Becher and Guerin, 2009; Schmera and Guerin, 2012; Schmidt-Buesser et al., 2009; Schmidt-Busser et al., 2011; von Arx et al., 2011; von Arx et al., 2012b).

The European grapevine moth *Lobesia botrana*

The grapevine moth, *Lobesia botrana* (Denis and Schiffermüller; Lepidoptera, Tortricidae) is an important pest in vineyards as it spreads across Asia, Africa, Europe and recently into North and South America. It is responsible for massive damage on grapes and the subsequent economic loss is considerable in the absence of control measures. The larvae feed on flower buds and the grape, and the wounds made on fruits favour subsequent infestation by fungi and bacteria such as the grey mould, *Botrytis cinerea*. The grapevine moth is a polyphagous insect and can grow on over 20 plant species including the vine, *Vitis vinifera*, flax-leaved daphne, *Daphne gnidium*, rosemary, *Rosmarinus officinalis* and raspberries, *Rubus sp.* (see Thiery and Moreau, 2005). In Switzerland, the moth is known since 1880 in the Valais and its first infestation in vineyards was recorded in 1910 (Bovey et al., 1972). At our latitudes moths achieve two generations annually. Pupae overwinter under the bark of the vine and emerge at the end of April. Adults are active at dusk. Mature females release sex pheromone to attract males for mating. The sex pheromone is a mixture of several sex pheromone, including E7,Z9-dodecadienyl acetate, E7,Z9-dodecadien-1-ol and Z9-dedecenyl acetate at a ratio of 100 : 20 : 5 (Arn et al., 1988), which are required to elicit a robust attraction of the males. The eggs (40 – 50 per female) are laid on flower buds and pedicels of the host plants. Larvae feed mainly on flower buds and pedicels over the five instar stages and pupate under the bark of the stem. The second generation of adults usually emerges at mid-July and females lay eggs on immature fruits. Larvae then feed on grapes that give rise to high levels of damage on the entire bunch (Bovey et al., 1972).

The codling moth *Cydia pomonella*

The codling moth, *Cydia pomonella* (Linnaeus; Lepidoptera, Tortricidae), is one of the most harmful pests of orchards all over the world. Already known in Antiquity, the codling moth probably originates from the Mediterranean region. The codling moth is a polyphagous Lepidopteran that can survive on multiple host plants such as apple, *Malus domestica*, pear, *Pyrus communis*, walnut, *Juglans nigra* and different species of *Prunus sp.* including apricot, plum and peach. Larvae hibernate in a cocoon on the host plant and pupate in April. Adults are essentially crepuscular. As in *L. botrana,* the research for a mate is facilitated by the release of species specific sex pheromone by the calling females. This pheromone is composed almost entirely of E8,E10-dodecadien-1-ol or codlemone (Roelofs et al., 1971). Each gravid female can lay up to 80 eggs on immature fruits. Larvae then dig galleries into the fruit flesh. When environmental conditions are favourable, the larvae can give rise to a second generation of adults. Otherwise, they leave the fruit and wrap themselves in a thick silk cocoon to overwinter (Bovey et al., 1972).

Male moth responses to semiochemicals and methods for pest control

Over the last 50 years the agricultural model has substantially evolved from an agriculture of self-sufficiency towards a strategy of mass production. In 2012, the world vineyards reached a total surface area of over 7.5 mha representing a market of over 28 billion $ (Castellucci, 2013). In 2011, the Food and Agriculture Organisation of the United Nations estimated the world apple market at 31.8 billion $ with a total surface area of 4.76 million Ha. Intensive agriculture implies blanket monocultures that favour outbreaks of phytophagous insect pests, fungi and diseases causing heavy damage on crops. The systematic use of pesticides is widespread to limit production loss. However, nowadays organisms show resistance to most of the treatments and the lethal dose applied increases (Reyes et al., 2007; Varela et al., 1993). Meanwhile, the consumer demands healthier products. Other alternatives for controlling pests have emerged for a more sustainable production. Some of these consist of exploiting the chemical ecology of insect pests. The use of semiochemicals to monitor pest insect populations as a component of integrated control has increased over the last 30 years. The most successful method using semiochemicals for insect pest control is the release of synthetic sex pheromones from dispensers to prevent mating in an insect species, or at least, to delay it. This strategy, called mating disruption, is based on the response of males to sex pheromones released by females. The principle consists of diminishing the probability of a male finding a female for mating by disrupting the mating behaviour of males using artificial pheromone plumes released in the crop. The principle mechanism involved in mating disruption is assumed to be ‘false-trail-following’ as described in (Sanders, 1997). This method offers many advantages compared to the insecticides such as the non-toxicity of the compounds released that permit to target only the targeted species rather than affecting both pest and beneficial insects on the crop. However, the latter advantage becomes a disadvantage when multiple pests infest the crop. Moreover, the method does not affect gravid females that might migrate into the crop.

Thus, the efficiency of the method greatly depends on the crop area treated and on the environmental conditions. Despite the high specificity of sex pheromones and its conservation at the species level, cases of mating disruption resistant strains have been reported in Japan (Tabata et al., 2007). When compared with pesticides, mating disruption is 2-3.5 times more expensive (Degen et al., 2005), largely due to the costs the synthesis of the pheromone products.

Green-leaf volatiles are important olfactory cues used by female insects for the recognition of host plants for oviposition (Honda, 1995). Additionally, host plants act as rendezvous sites where mating occurs (Visser, 1986) such that host plant odours may also attract males as it facilitates the location of relevant mating sites. In the cabbage looper, *Trichoplusia ni*, mated and unmated females as well as males were attracted to their host plant volatiles in a wind tunnel (Landolt, 1989) and von Arx et al. (2011) showed attraction of *L. botrana* males to single host plant volatiles. Host plant volatiles may therefore influence the responses of males to their sex pheromones. Many studies have reported synergism between host plant odours and sex pheromones in the attraction of males (Landolt and Phillips, 1997; Schmera and Guerin, 2012; Schmidt-Buesser et al., 2009; von Arx et al., 2012b). Moreover, the sensitivity of olfactory neurones specific to sex pheromones on the antenna of the corn earworm, *Helicoverpa zea*, males to sex pheromone is increased when host plants volatiles are present, but the host plant volatiles do not evoke any response from the same neurones on their own (Ochieng et al., 2002). The most striking example is the host plant compound pear ester, ethyl-E2,Z4-decadienoate, that attracts both male and female codling moths (Light et al., 2001). Moreover, it increased the response of males to codlemone in wind tunnel tests (Schmera and Guerin, 2012) and enhances the efficiency of codling moth mating disruption in the field (Knight et al., 2005; Light et al., 2001).

Objectives and outline of the thesis

In *Petunia*, pollination syndromes serve as the main factor that maintains reproductive isolation in sympatric plant communities. Therefore, they are reliable traits for defining a species. In Chapter II (which is partly included in Klahre *et* al. 2011), I address the question as to whether scent production is sufficient to induce a foraging preference of a typical pollinator, *M. sexta*, between two closely related *Petunia* lines through the selective response of the olfactory sensory system of *M. sexta*. Such a shift would indicate that scent production contributes largely to the reproductive isolation of fragrant *Petunia* species such as *P. axillaris*. Otherwise, reproductive isolation of scented *Petunia* would rely on either multimodal sensory input or other floral traits.

While olfactory and visual cues influence the foraging behaviour of *M. sexta*, the ultimate goal of such behaviour is to find adequate energy sources. Nectar should provide a reliable energy source that can be efficiently metabolised and used for flight. In Chapter III, I have tested the perception by *M. sexta* of the three main nectar sugars, glucose, fructose and sucrose, and have investigated whether the quality and quantity of these nutrients is sufficient to drive a preference.

L. botrana and *C. pomonella* are both major crop pests that have spread worldwide. Pest control tools for these moth pests are frequently inadequate. In the last decades much effort has been made to improve control methods using sex pheromones in mating disruption programmes by including host plant volatiles in pheromonal formulations. In Chapter IV, I studied the effects of a new class of plant volatiles to increase the attractiveness of the female sex pheromone of the European grapevine moth and the codling moth to conspecific males.

Chapter V provides a general discussion of the findings presented in this thesis.

Chapter II.

Manipulation of Fragrance to Understand a Pollination Syndrome

The results presented in this chapter were already partially published in Klahre et al. (2011).

Abstract

Floral traits such as petal colour, scent, morphology and nectar content constitute sensory cues that attract foraging pollinators. Particular associations of these traits can lead to specific insect-plant associations that can lead to reproductive isolation. *Petunia* is an attractive model to study the contribution of floral traits in the definition of such interactions. *P. axillaris* releases a strong sweet scent to attract *Manduca sexta*, a night active hawkmoth. On the contrary, *P. exserta,* which is pollinated by humming birds, is scentless. The crossing of both species permits to produce lines expressing mixed floral cues. Such lines are used to study the importance of floral scent in the foraging behaviour of *M. sexta* and the genetic basis underlying its production. We characterised fragrance production in *P. axillaris* and confirmed the absence of fragrance in *P. exserta.* Among the volatiles released by *P. axillaris* at least three of them, benzyl alcohol, methyl benzoate and methyl salicylate elicit robust electroantennogram responses with a threshold at approximately 5 pg. In a wind tunnel choice bioassay we demonstrated that *M. sexta* prefers to forage on scented flowers, regardless of their colour. When exposed with a conflicting cue situation of colour versus scent, *M. sexta* shows no preference, indicating that colour and scent are equivalent cues. Our experiment shows that scent is a determinant floral trait that

defines the insect-plant interaction in *Petunia*. As the genetic basis of scent production remains relatively simple, the rapid gain or loss of such a major attribute through hybridization can lead to reproductive isolation.

Introduction

Plants provide multiple sensory cues to advertise the presence of a nutritive reward to pollinator insects (Raguso, 2004). Floral visitors can use these cues to detect and discriminate suitable flowers within a plant community. Pollination syndromes (Faegri and Van der Pijl, 1979), i.e. suite of floral traits dedicated to attract a restricted number of pollinator species, have emerged as they offer to the pollinator signals from plants where nectar is chemically profitable or physically available and this pollinator loyalty to the plant prevent pollen outspreading on other plant species. This interaction results in a co-evolution between the two partners and in a mutualism. Fortuitous variations in one or few floral traits within a population can alter the attraction of the preferred pollinators in favour of another guild of pollinators and can lead to divergent adaptations of the plant and ultimately to speciation (Ando et al., 2001; Bradshaw and Schemske, 2003). Whereas the effect of flower colour (Jones and Reithel, 2001; Waser and Price, 1981) and morphology (Dell'Olivo et al., 2011; Kaczorowski et al., 2012) on foraging insects has been well studied, only few reports exist on the role of scent on the foraging preference of pollinators.

The genus *Petunia* (Solanaceae) is an attractive plant model to study the genetic basis underlying the expression of floral traits and their inference on pollinators (Galliot et al., 2006b) as it features three distinctive pollination syndromes (Figure II.1; Gübitz et al. (2009)). Most of the species of the genus such as *P. integrifolia* are bee pollinated and show poorly scented pinkish flowers with a wide, short tube that holds low amounts of nectar. *P. axillaris* shows a typical sphingophilous pollination syndrome with a strongly scented white flowers mounted on a long, narrow tube with a high nectar volume. A third pollination syndrome in *Petunia* has been recently found in *P. exserta* which expresses a scentless red flower with a long narrow tube with a similar nectar volume as *P. axillaris*. Even though all these species are artificially crossable, no hybrids between *P. integrifolia* and *P. axillaris* have been reported in the field. Hybrids between *P. exserta* and *P. axillaris* have been observed

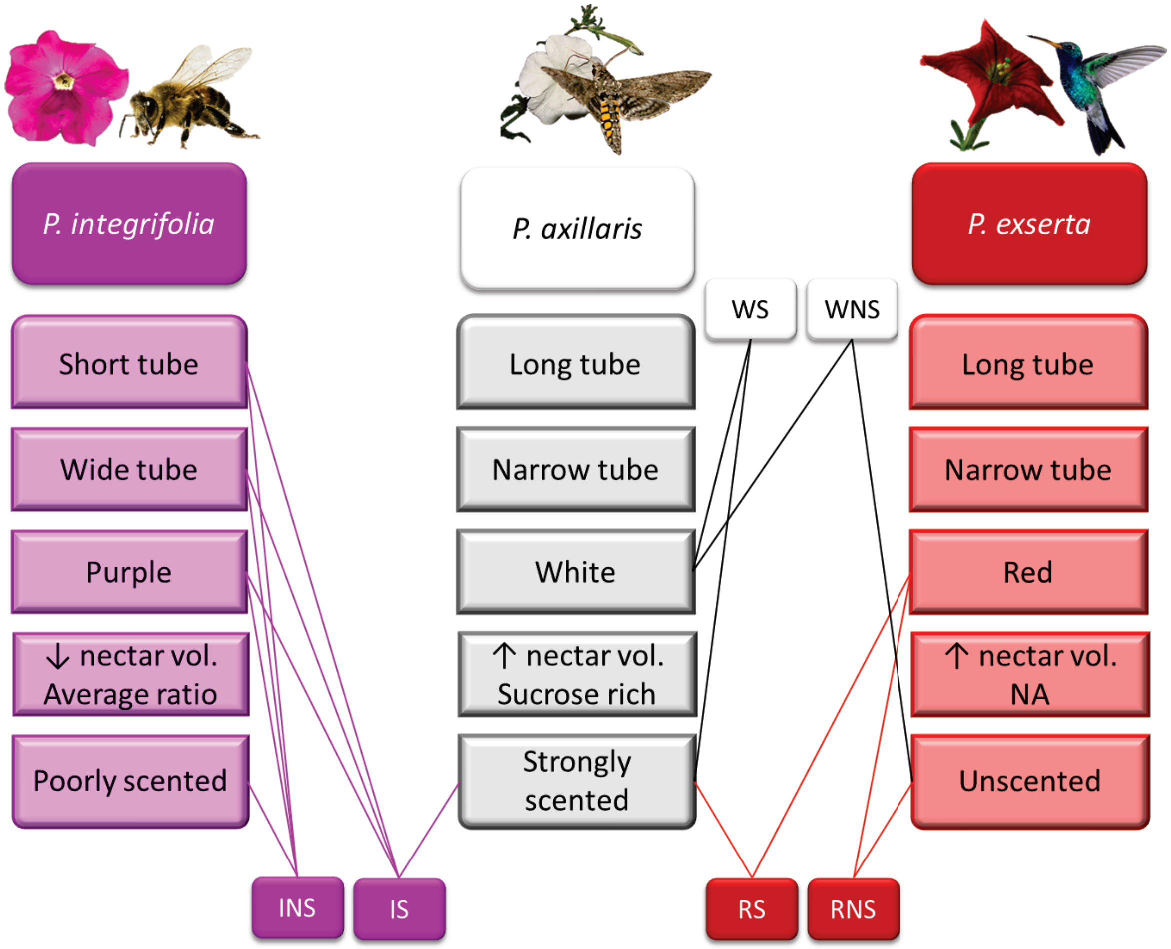


Figure II.1 Floral traits that define the three pollination syndromes in *Petunia*. Intermediate plants are the introgression lines used in this study (IS & INS: scented & unscented *P. integrifolia*-like flowers, see Annexe 1; RS & RNS: scented & unscented *P. exserta*-like flowers; WS & WNS: scented and unscented *P. axillaris*-like flowers). Connectors link the introgression lines to discriminative floral traits.

due to humming bird cross-pollination (Lorenz-Lemke et al., 2006). This model offers a unique opportunity to dissect the genetics governing a pollination syndrome with the aim to establish the contribution of each floral trait to the foraging preference of insects. Are they purely redundant or do they act synergistically? The expression of flower pigmentation (Hoballah et al., 2007), morphology (Dell'Olivo et al., 2011) and nectar production (Brandenburg et al., 2012a) have already been decrypted in Petunia. Scent production dynamics, biosynthetic pathways as well as the genes involved in scent regulation have been well studied in *Petunia* (Boatright et al., 2004; Dudareva and Pitchersky, 2006; Schuurink et al., 2006; Spitzer-Rimon et al., 2010; Verdonk et al., 2005). Methyl benzoate and benzaldehyde are produced by all accessions of *P. axillaris* in copious quantity, and other benzenoid volatiles are found only in some accessions (Hoballah et al., 2005; Kondo et al., 2006; Verdonk et al., 2003). Some of these compounds elicit electroantennogram (EAG) responses from the antenna of *Manduca sexta*, a natural pollinator of *P. axillaris* (Fraser et al., 2003; Hoballah et al., 2005).

In the present study, we ask the question whether scent production can influence the foraging preference of *M. sexta* in the pollination system of *Petunia* by using near isogenic lines from repetitive back-crosses between *P. axillaris* and *P. exserta* that only differ in scent production resulting. The fragrance profile of these plants was characterised and some compounds that may be pertinent in the chemical ecology of *M. sexta* were identified.

Materials and Methods

Insects

M. sexta pupae were obtained from the Laboratory of Animal Physiology (Philipps University, Marburg, Germany) and held in an environmental cabinet where light was provided by six fluorescent tubes (Philips TDL, 36 W, >1 kHz) with a 16:8 light:dark cycle at 26°C and 65% relative humidity (RH). Pupae were sexed prior to emergence and placed in rearing cages (BugDorm-4180F, MegaView Science Co., Ltd., Taichung 40762, Taiwan) containing a wet cotton tissue. In the behavioural assays we used 4 to 6 day old, unmated and unfed naive adults. Unmated and unfed moths of 2-4 days were used for electrophysiological experiments.

Plants

The plant species used in this study were the non-scented red *P. exserta*, and two accessions of *P. axillaris* (*P. axillaris* N and *P. axillaris* S7) which only slightly differed in corolla shape and UV reflectance. *P. axillaris* S7 and *P. exserta* were crossed in order to produce hybrids that inherit specific traits. To produce unscented *P. axillaris*-like lines, F_2 were back-crossed with P. axillaris S7 and then selfed 3 times. At each step, scent production was measured using the PTR-MS (HS-PTR-QMS, Ionicon, Innsbruck, Austria) and genotyped to select plants that had inherited the fewest gene fragments of *P. exserta*. The selected plants were back-crossed and selfed. The resulting line produced white unscented (WNS) plants and white scented plants (WS). The WS plants were heterozygous on chromosome II and the WNS plants were *P. exserta* homozygous on chromosome II at 5 sites.

Scented *P. exserta*-like lines were produced by 4 back-crosses of the F2 plants. After each step, plants were genotyped and the ones showing the fewest gene fragments inherited by *P. axillaris* were selected. The resulting lines produced red scented (RS) and red unscented (RNS) plants. RS plants were heterozygous at 8 sites on chromosome II and at 4 sites on chromosome VII. RNS plants were heterozygous at 4 sites on chromosome VII.

Lines from *P. axillaris* and *P. exserta* were bred in a growth chamber at the Institute of Plant Science (University of Bern, Switzerland) as described in Klahre et al. (2011). Plants were then moved to the University of Neuchâtel and kept with a 15:9h light:dark cycle in a climate chamber (CMP 4030, Conviron, Winnipeg, Canada) at 22:17°C and 65:75% R.H.

Measurement of flower traits

Colour

The spectral reflectance of the corolla of each plant line was measured using a spectrophotometer (Check Pro, Datacolor, Lawrenceville, USA) on a sampling surface of 0.5 cm^2. The corolla was cut and spread out on white paper. Each sample reading is the mean of 4 measurements taken at different places on the corolla and in different orientations.

Scent

Single flowers at the 2nd day of anthesis were enclosed on the plant in a ~500 mL food storage polyester foil (Toppits, Melitta GmbH, Egerkingen, Switzerland) tied around the flower stem at the beginning of the scotophase to form a bag. Pure air was vented at 2 L/min through the bag and 1L/min was pulled for 120 min through 25mg 80-100 mesh Porapak Super Q (Altech Assoc., Deerfield, Ill., USA) held in a glass column (76.2 mm long, 6.35 mm o.d. borosilicate volatile collection trap, Analytical Research System, Gainesville, Florida, USA) previously conditioned under N_2 at 200°C for 90min. The remaining air in the bag was evacuated through a hole to maintain a higher pressure in the collection bag passing over the surrounding. In this manner entry of ambient air via the enclosure on the plant stem which could not be completely enclosed, was avoided.

Flower volatile effluent was desorbed from the adsorbent column with dichloromethane (DCM) and the first 10 µL collected. This aliquot contained over 95% of all volatiles trapped with the exception of benzaldehyde (60%).

One µL of fragrance samples was injected on-column and analysed by gas chromatography (GC; 5300 chromatograph, Carlo Erba Instruments, Italy) mounted with a ZB-5 (30 m, 0.32 mm i.d., film thickness 25 µm, Zebron, Newport Beach, California, USA) apolar column coupled with a flame ionisation detector (FID) with H2 as carrier gas (1.57mL/min). Column conditions were 40°C for 5min then programmed at 3°C/min to 230°C and held for 5 min. Retention times of individual fragrance constituents present in P. axillaris (Hoballah et al., 2005) were compared to those of standards (Table II.1) and confirmed by GC linked mass spectrometry (GC-MS; Trace GC-PolarsiQ, Finnigan, USA) on a ZB-5MS apolar column (30m, 0.25 i.d., 35 µm thick, Zebron, Newport Beach, California, USA).

Electrophysiology

Electroantennogram setup

The antenna of *M. sexta* anesthetised with CO_2 was excised at the base and placed on a filter paper saturated with 0.1 M KCl to keep it at high humidity. Its base was then mounted on a glass capillary electrode (2 mm o.d.) filled with 0.1M KCl and transferred to a pure air flow (25 ± 2°C, 90-100% RH) delivered at 1m/s via a water-jacketed glass tube (i.d. 6mm). This served as the reference electrode. A 4 cm long Teflon tube with a 4 mm lateral hole was placed as an extension of the glass tube releasing the air to insure that the entire antenna bathed in the air flow (Figure II.2). The antenna was introduced into the extension through the lateral hole. The tip of the antenna was cut and connected to the recording electrode of the same diameter filled with 0.1M KCl and connected to a pre-amplifier with an Ag-AgCl wire (Figure II.2). The electroantennogram (EAG) signal was fed into an AC/DC amplifier (100x) via the high impedance preamplifier (10x) and recorded at a rate of 48 Hz on the hard disk of a PC via a 16-bit analogue-digital IDAC card (Syntech, The Netherlands) using GC-EAG software (version 4.6, Syntech, The Netherlands).

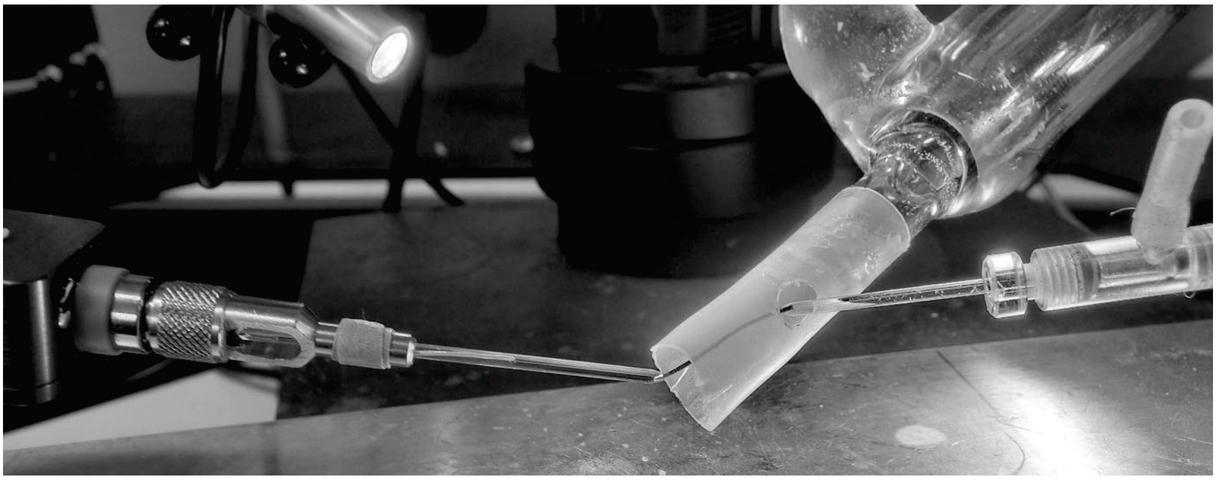


Figure II.2 *M. sexta* antenna mounted between 2 glass capillary electrodes (measuring electrode connected to the preamplifier on the left) and placed inside the Teflon extension of the water-jacked glass tube to expose the entire antenna to the humid air flow.

Stimulation

Different Petunia floral compounds (Table II.1) at 10-50-100-500pg, 1-5-10-50-100ng in dichloromethane (DCM, Suprasolv purity) or Petunia fragrance extracts were injected on-column into a gas-chromatograph (5300 chromatograph, Carlo Erba Instruments, Italy) equipped with a FFAP polar column (30 m, 0.25 mm o.d., film thickness 0.25 µm, BGB Analytik, Rheinfelden, Germany) and heated (40°C for 3min, at 20°C/min to 230°C and held at 230°C for 10min). Half of the separated compounds was directed from the column effluent to the FID and the other half was delivered into the air flow over the preparation via a heated transline (heated at 230°C) between the oven and the air flow to avoid condensation of eluting compounds. The positive control (10 µg Z3-hexen-1-ol on a filter paper) was delivered in odour puffs to the preparation as described in Guerenstein and Guerin (2001) at the start and the end of each experimental period to permit accounting for the decline in the sensitivity of the antennal preparation with time. One to five males and two-six females were tested.

Table II.1 Compound used for the GC-EAG analyses and for comparison of the retention times with the headspace collections.

NAME	FORMULA	MOLECULAR WEIGHT (g/mol)	RETENTION TIME (S)*	PURITY	COMPANY
Dichloromethane	CH_2Cl_2	84.93	90	Suprasolv	Merck, Germany
Z3-hexen-1-ol	$C_6H_{12}O$	100.16	443	>98 %	Fluka, Switzerland
Benzaldehyde	C_7H_6O	106.12	516	>99 %	Merck, Germany
Benzyl alcohol	C_7H_8O	108.14	664	99 %	Fluka, Switzerland
1-octen-3-ol	$C_8H_{16}O$	128.21	476	>98 %	Fluka, Switzerland
Methyl benzoate	$C_8H_8O_2$	136.15	561	>98 %	Fluka, Switzerland
Methyl salicylate	$C_8H_8O_3$	152.15	627	>99 %	Fluka, Switzerland
Eugenol	$C_{10}H_{12}O_2$	164.20	769	99 %	Fluka, Switzerland

*using the gas chromatographic programme described in the method for the EAG

Wind Tunnel

The climatized wind tunnel (working area: 250 x 100 x 100 cm) is made of non-reflecting glass. A centrifugal ventilator moves the humid- and temperature-controlled air (85% ± 1%RH, 26 ± 0.1°C) across the tunnel at 56 cm/s through active charcoal cartridges placed at either end of the working area. For our experiments, a laminar air flow was produced through a white nylon laminar flow screen (50 mm mesh) and two aluminium honey comb screens (6 cm thick, 1 cm mesh) covered with white mosquito netting (nylon, 1mm mesh) at the upwind end of the wind tunnel and a second white nylon laminar flow screen (50 mm mesh) at the downwind end of the wind tunnel. Overhead illumination was provided by high-frequency fluorescent lighting 186 cm above the tunnel floor (36 W, >1 kHz, with eight 120 cm long Philips TL-D tubes) running the length of the tunnel in two groups of four tubes 27 cm apart in a housing that was 120 cm wide (with fans to eliminate heat).

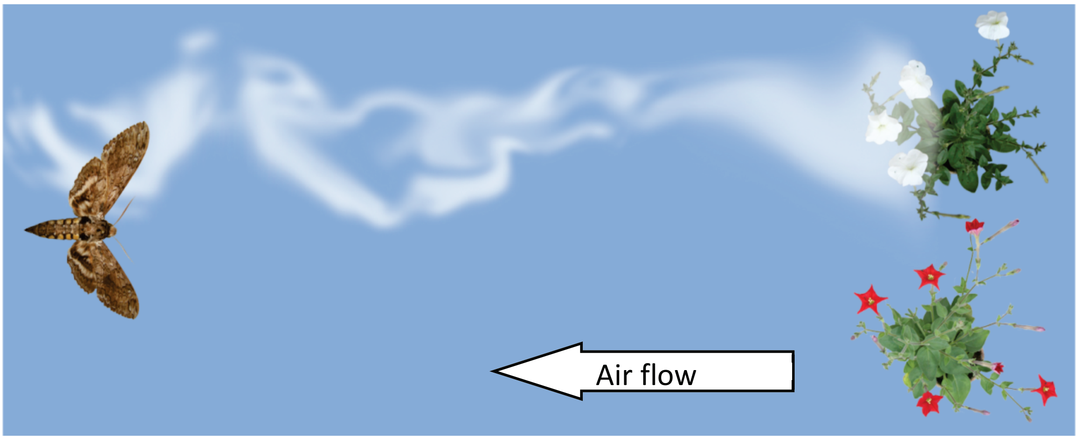


Figure II.3 Experiments made in a wind tunnel investigating preferences of the moth *M. sexta* for scented flowers. Moths were released at the downwind end of the tunnel and two plants of differing scent production were presented to them at the upwind end. Diagrammatic representation of the wind tunnel setup, where pure, standardized air (see methods) entered from the right.

Brown paper was placed on the top of the wind tunnel to decrease light intensity to 2–4 lux. The floor of the wind tunnel was covered with a medium density fibreboard (4 mm thick, light brown), and the white side walls had vertical light blue stripes 7 cm wide and 7 cm apart. Two plants similar in shape with the same number of flowers stood 40 cm from each other, 30 cm from the upwind end of the wind tunnel. Plant height was adjusted to place the flowers at 50 cm from the floor. Plant positions were systematically switched to avoid experimental bias. Both insects and plants were tested at the beginning of the scotophase. Insects were placed in the darkened wind tunnel room 20 min prior to experiments. Single quiescent naive moths were placed on a 50 cm high platform at the downwind end of the tunnel. When moths were not active (wing fanning) after 1 min they were gently activated by brushing the tip of their abdomen with a gloved finger. First choices for flowers, proboscis extension, and feeding were observed over 5 min (Figure II.3) for the following treatments: *P. axillaris* vs *P. exserta*, *P. axillaris* vs *P. exserta* hidden by a screen, WS vs WNS, RS vs RNS, RS vs *P. exserta*, RS vs WNS (15-37 individuals tested per treatment).

Statistical analysis

EAG responses to compounds were analysed using linear regressions fitted on the log-transformed responses tested at the log-transformed doses for each product and sex and were compared using an analysis of variance (ANOVA).

Differences between plants in terms of first choice and proboscis extension made by *M. sexta* were compared by using the exact binomial test. All the analyses were performed using the statistical package R (version 2.15.1).

Results

Flower traits

Plants were selected across generations according to their flower phenotype. To insure the consistency of flower traits of the replicate in the resulting crossings, we measured the colour of the petal, the production of scent and the corolla surface (Klahre et al., 2011).

Colour

The spectral reflectance of the white phenotypes is consistent in the visible light spectrum and all flowers show a similar pattern above 450 nm with a plateau at 80 % of reflected light. However, the parent plant absorbs all light between 360 and 400 nm whereas the introgression lines partially reflect light at these wavelengths (Figure II.4A). The red phenotypes are more similar and absorb most of the light below 550 nm. Above that the reflectance increases to reach a peak at 650 nm (Figure II.4B). The red parent reflects slightly less at the higher wavelengths than its introgression lines. The apparent colour of the top side of the corolla within each phenotype is comparable, except the red parent which is slightly darker than its introgression lines (Figure II.4C). The dark greenish colour of the leaves contrasts with the white phenotypes more than with the red ones.

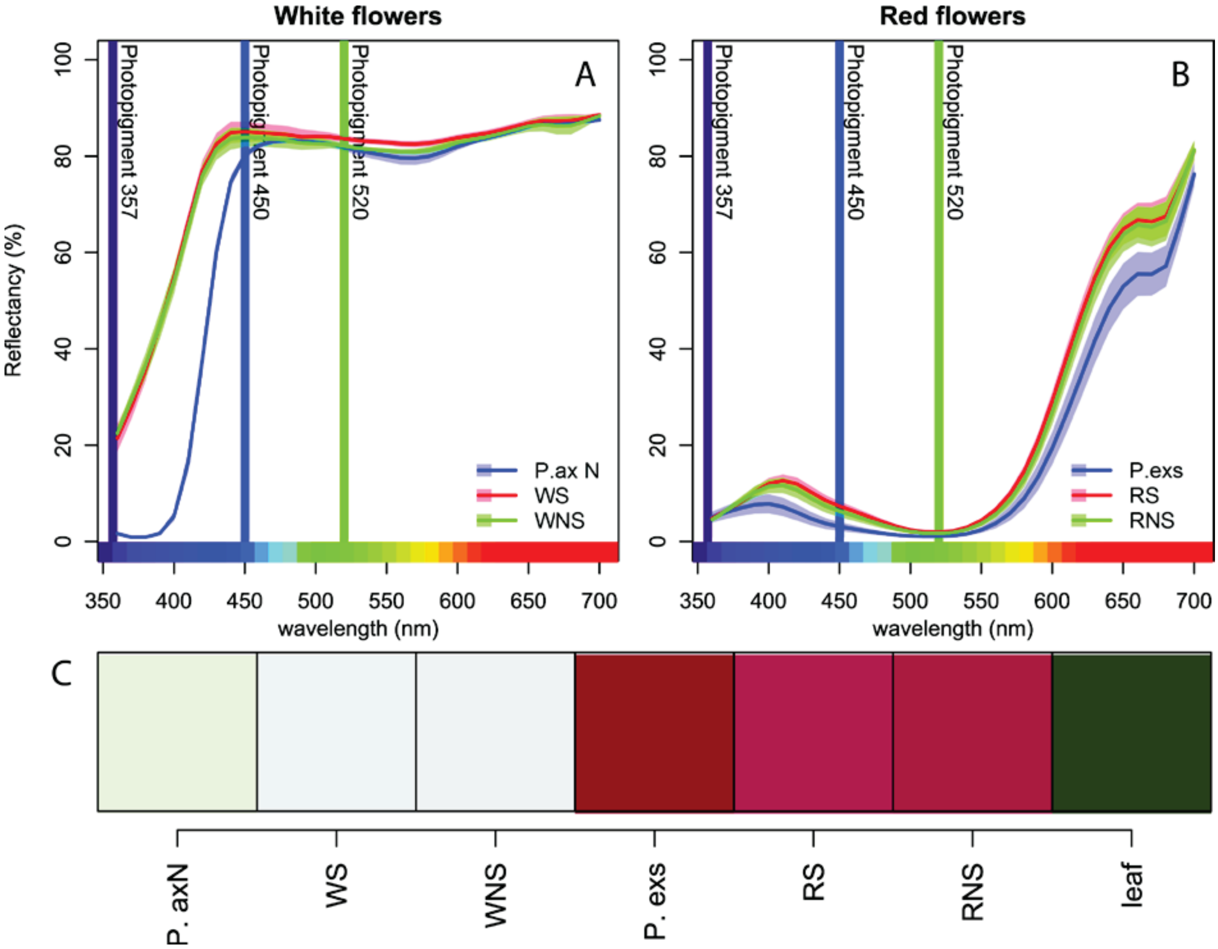


Figure II.4 Spectral reflectance of the corolla of *P. axillaris, P. exserta* and their respective introgression lines. Comparison of the colour of the top side of the corolla of white phenotypes (A) and the red phenotypes (B). The apparent colour of the upper side of the corolla and leaf is reconstructed in C for all phenotypes. Shades around the mean lines in A and B represent the 1st and 3rd quartiles of the dispersion of the 4 samples measured. The vertical bars in A and B indicate the maximum absorbance of the 3 photopigment types present in the compound eye of *M. sexta* (Cutler et al., 1995)

Fragrance production

Fragrance measurements were routinely performed using GC-FID analysis to build scent production profiles for each flower type (Figure II.5). As expected, volatiles from the white parents *P. axillaris ax*N (Figure II.5A$_{1,2}$) and *P. axillaris par*S7 (Figure II.5B$_{1,2}$) were released at high amounts. Methylbenzoate, benzaldehyde, and benzylbenzoate were the predominant compounds. *P. axillaris ax*N produced a similar quantity of methylbenzoate to *P. axillaris par*S7 but 4 times less benzaldehyde and 2 times less benzylbenzoate. Surprisingly, a low amount of benzyl alcohol was detected in *P. axillaris par*S7 but no trace was found in *P. axillaris ax*N. It was difficult to separate benzyl alcohol from 4-hydroxybenzaldehyde as the retention times for these compounds were close on the chromatographic phase employed. Differences in phenylpropanoid production were observed between these two plants. *P. axillaris ax*N produced traces of eugenol and relatively high amounts of isoeugenol while only a small amount of eugenol was found in *P. axillaris par*S7. Other compounds such as benzyl salicylate, methyl salicylate, benzyl acetate and a terpinene isomer were detected in small amounts only in *P. axillaris par*S7. The red parent, *P. exserta*, produced no detectable amount of scent (Figure II.5$E_{1,2}$).

To produce lines with diverging phenotypes only in scent production, interspecific crosses between *P. exserta* and *P. axillaris ax*N*/parS7* were made. The first generation F1 produced homogenous phenotypes with pink corollas. All the compounds detected in their respective parents were found at similar or lower amounts in these plants, suggesting than the loci responsible for scent are dominant. Successive backcrosses and selfings of these lines were performed to finally produce white scented (WS, Figure II.5$C_{1,2}$) and white unscented (WNS, Figure II.5$D_{1,2}$), red scented (RS, Figure II.5$F_{1,2}$), red unscented (RNS, Figure II.5$G_{1,2}$) introgression lines. The phenotype of these plants was similar to their parents except for scent production. The RS plants produced scent of the same quality as *P. axillaris axN* but in lower amounts. Scent quality for the WS was similar to *P. axillaris parS7* except for benzylbenzoate which was produced at much lower amounts. As expected for *P. exserta,* the RNS and WNS lines did not produce fragrance at relevant level (Figure II.5D,E, & G). Several compounds could not be identified by GC-MS because of the low amounts present. Moreover, these compounds did not consistently occur except for 2-hydroxybenzaldehyde.

The total amount of scent released was very different between plants. White flowers produced the highest amount of fragrance. *P. axillaris parS7* was the most productive and P. *axillaris axN* released about 22% less compounds. Only the production of methyl benzoate was comparable between these plants. The white and red scented introgression lines released much less fragrance with, respectively, 34% and 60% less than *P. axillaris parS7* (Figure II.5H).

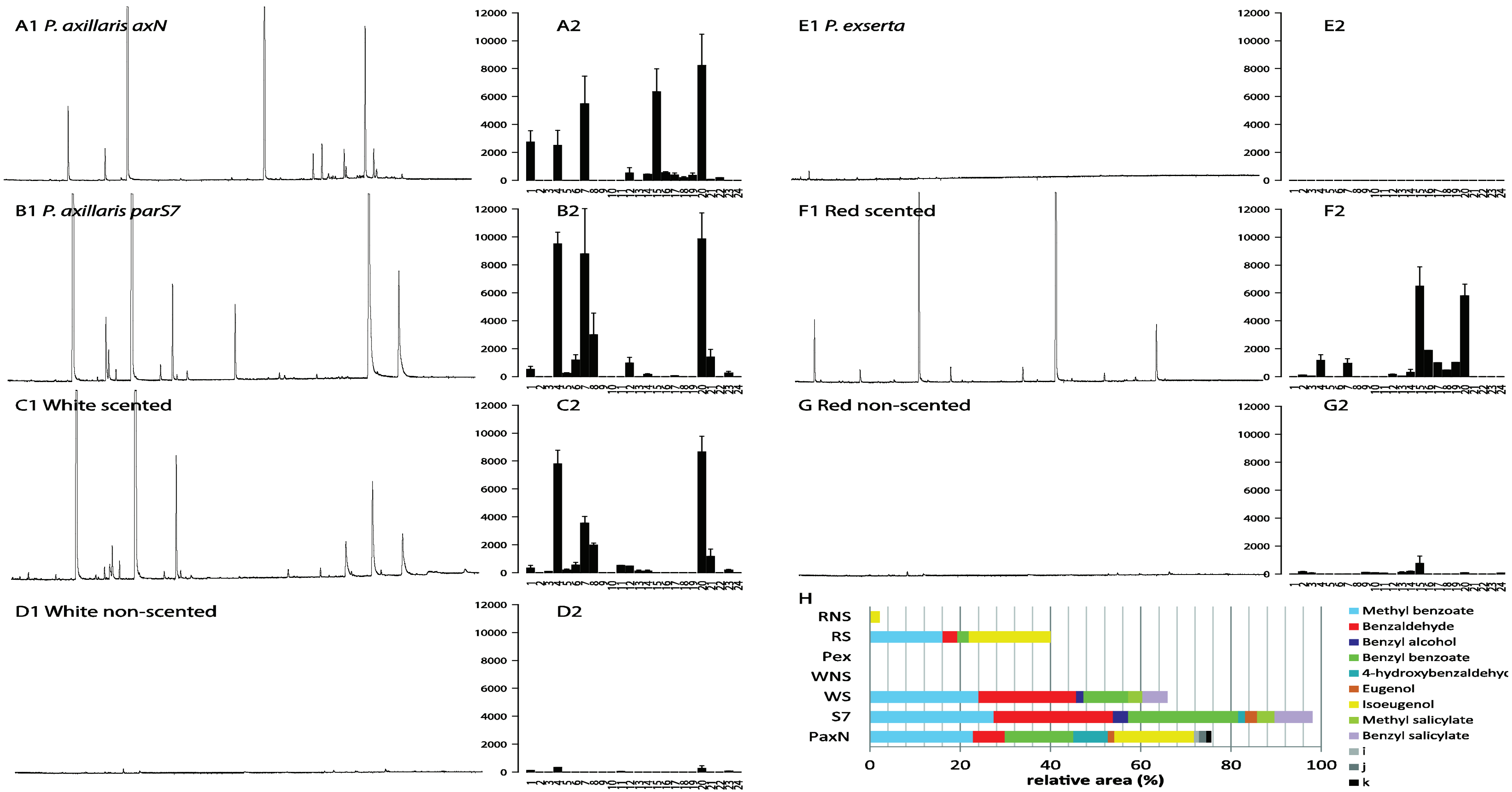


Figure II.5 Relative scent production measured by GC-FID of *P. axillaris* axN (A_1; N=4), *P. axillaris* parS7 (B_1; N=3) and *P. exserta* (E_1; N=3) and their scented and unscented introgression lines WS (C_1; N=4), WNS (D_1; N=4), RS (F_1; N=4), RNS (G_1; N=5). GC traces were cut at 560s and 3700s to remove solvent peak and irrelevant information. Blanks for the headspace collections showed no trace of any compounds (not shown). Peak area for each compound was measured in each extract (A_2-G_2) for 1: 2-hydroxybenzaldehyde, 2: unknown, 3: unknown, 4: benzaldehyde, 5: benzyl acetate, 6: benzyl alcohol, 7: benzyl benzoate, 8: benzyl salicylate, 9-11: unknown, 12: eugenol, 13-14: unknown, 15: isoeugenol, 16-19: unknowns, 20: methyl benzoate, 21: methyl salicylate, 22: unknown, 23: a terpinene product and 24: vanillin. The relative amounts of compounds released by each plant at least 2 times and at significant levels (>1% of the total amounts) were compared (H) with 100% as the total quantity of compounds collected on the most productive plant line (*P. ax. par.* S7).

All the headspace extracts showed a strong consistency in scent quality (Table II.2). Whereas only 4-hydroxybenzaldehyde, benzaldehyde, isoeugenol and methyl benzoate were found in all volatile extracts of *P. axillaris axN*, more products were collected in all extracts of *P. axillaris parS7* such as benzyl acetate, benzyl alcohol, benzyl benzoate, benzyl salicylate, methyl salicylate, an isomer of terpinene product and eugenol. The white scented introgression line consistently released the same products as its parent *P. axillaris parS7*. The red scented introgression line consistently released only benzaldehyde, methyl benzoate and eugenol. Very low amounts of compounds were regularly recorded from the scentless plants, such as methyl benzoate and a terpinene product (WNS) or "i" in RNS.

Table II.2 Compound occurrence in scent extracts of *P. axillaris* N, *P. axillaris* parS7, *P. exserta* and their introgression lines. Compounds that were present in all the extracts are in green and compounds that were found in less than half of the extracts are in red.

	2-hydroxybenzaldehyde	a	b	Benzaldehyde	Benzyl acetate	Benzyl alcohol	Benzyl benzoate	Benzyl salicylate	c	d	e	Eugenol	h	i	Isoeugenol	j	k	l	m	Methyl benzoate	Methyl salicylate	n	Terpinene	Vanillin	**Extract Nb.**
PaxN	4	0	0	4	0	0	3	0	0	0	0	2	0	2	4	2	2	2	2	4	1	1	0	0	**4**
PaxS7	3	0	0	3	3	3	3	3	0	0	0	3	0	2	0	0	1	0	0	3	3	0	3	0	**3**
WS	3	0	1	4	4	4	2	2	0	0	1	1	2	2	0	0	0	0	0	4	4	0	4	0	**4**
WNS	1	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	2	0	0	2	0	**2**
Pexs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**3**
RS	0	1	2	4	0	0	3	0	0	0	0	4	1	3	3	1	1	1	1	4	0	0	0	0	**4**
RNS	0	2	2	0	0	0	0	0	1	1	1	0	2	3	2	0	0	0	0	1	0	0	0	2	**3**

Electroantennogram responses to host plant volatiles

Plant volatiles that occurred consistently in the headspace collections (i.e. benzaldehyde, benzyl alcohol, methyl benzoate, methyl salicylate and eugenol; Table II.2) and 1-octen-3-ol were tested by GC-EAG at 9 different doses on both male and female antennae of *M. sexta*. The antennae responded to all products in a dose dependent manner (Figure II.6 and Table II.3). Females were more sensitive to low doses (below nanogram levels) due to a less noisy baseline in the EAG traces. However, the responses of both sexes showed the same general pattern and were not significantly different for benzaldehyde, methyl benzoate and benzyl alcohol (ANOVA, $P < 0.05$). The slight differences between the sexes for the other products are mainly due to the low number of valid EAG responses at the lowest doses tested. The EAG profiles revealed two kinds of response patterns. Antennae were highly sensitive to benzyl alcohol, methyl benzoate and methyl salicylate, not significantly different from each other (ANOVA, $P > 0.05$) with detectable responses at 5 pg, i.e. near to the response threshold. The d10 (the dose necessary to induce 10 % of the response of the positive control) was reached at 0.34 ± 0.05 ng for these products and the d25 at 9.29 ± 1.1 ng. At the high dose of 50 ng the antennae responded at levels up to 55.6 ± 6.5 %, 48.5 ± 4.7 % and 46.5 ± 3.8 % of the positive control for methyl benzoate, methyl salicylate and benzyl alcohol, respectively. The antennae were much less sensitive to eugenol, benzaldehyde and 1-octen-3-ol (ANOVA, $P < 0.001$). The responses of the antennae to these products were not significantly different between each other (ANOVA, $P > 0.05$). The threshold for these latter products is estimated between 50 pg and 250 pg. At the dose of 9.95 ± 3.35 ng these products induce 10 % of the response of the positive control. At the high dose of 50 ng, EAG responses were recorded at 30.1 ± 4.9 % for eugenol, 28.4 ± 2.2 % for benzaldehyde and 41.4 ± 3.9 % for 1-octen-3-ol of the response to the positive control.

Table II.3 Log-dose log-response models of *M. sexta* EAG response (data represented in Figure II.6). The dose (in ng) that is necessary to induce 10 % and 25 % of the response of the antenna to the positive control is represented by the d10 and d25 values, respectively. Data followed by “!” are outside the range of the models.

Product	**Sex**	**Model**	**R^2**	**d10**	**d25**
1-octen-3-ol	Male	$y = 0.4x + 1.59$	0.550	6.05	60.3(!)
	Female	$y = 0.67x + 1.32$	0.921	4.31	16.89
Benzaldehyde	Male	$y = 0.68x + 0.47$	0.803	15.15	58.87(!)
	Female	$y = 0.42x + 1.47$	0.515	7.27	63.91(!)
Methyl benzoate	Male	$y = 0.25x + 2.59$	0.748	0.32	11.88
	Female	$y = 0.28x + 2.63$	0.879	0.3	8.18
Methyl salicylate	Male	$y = 0.24x + 2.6$	0.679	0.29	12.63
	Female	$y = 0.26x + 2.78$	0.846	0.16	5.48
Benzyl alcohol	Male	$y = 0.31x + 2.5$	0.720	0.54	9.99
	Female	$y = 0.32x + 2.57$	0.807	0.42	7.59
Eugenol	Male	$y = 0.31x + 1.33$	0.324	24.18	487.36(!)
	Female	$y = 0.4x + 1.91$	0.779	2.72	27.4

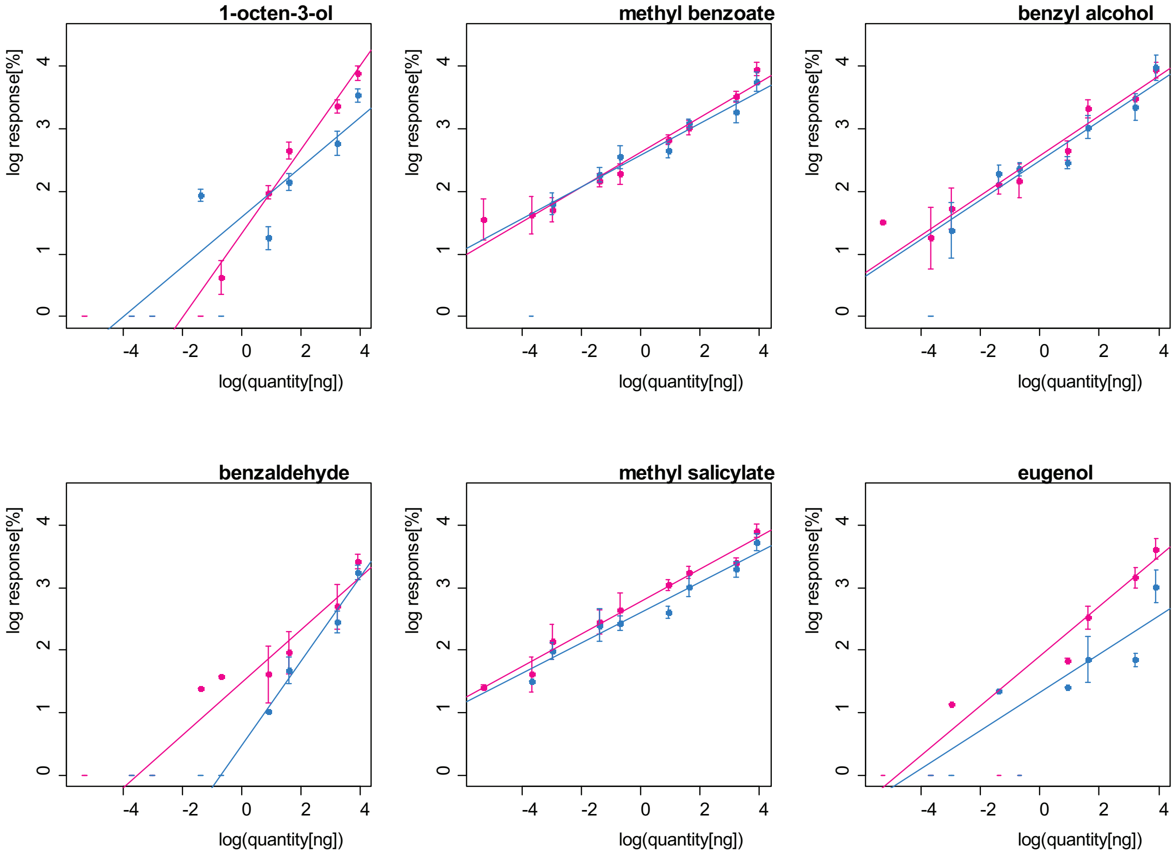


Figure II.6 Mean ± s.e.m. of the electroantennogram responses of *M. sexta* (n = 1-5 male (blue) and 2-5 female (purple) antennae) to defined doses (5 pg, 25 pg, 50 pg, 250 pg, 500 pg, 2.5 ng, 5 ng, 25 ng and 50 ng delivered to the antenna) of 1-octen-3-ol and to *Petunia* volatiles as delivered from a gas chromatograph (GC-EAG). EAG responses were normalised by using a puff of pure air passing over 10 µg Z3-hexen-1-ol in a stimulus syringe at the beginning and the end of each GC run. Linear regressions were fitted to the log-transformed responses of the tested log transformed doses (see Table II.3 for more details).

Effect of scent and colour on the visiting preference

To clarify the effects of colour and scent on pollination frequency in the context of intact plants we performed wind tunnel experiments with naive *Manduca sexta* (Figure II.7). When the two wild species, *P. exserta* and *P. axillaris,* were placed 40 cm apart in a simple dual choice experiment that allowed the moths to see the plants, only few moths visited *P. exserta*, as expected (exact binomial test, $P < 0.05$; Figure II.7). After they took off the moths first initiated casting across the wind tunnel before flying upwind. In this setup the hawkmoths may be guided more by the colour than by the scent. To test for only the scent component, plants of the same species were positioned upwind behind a screen. The moths

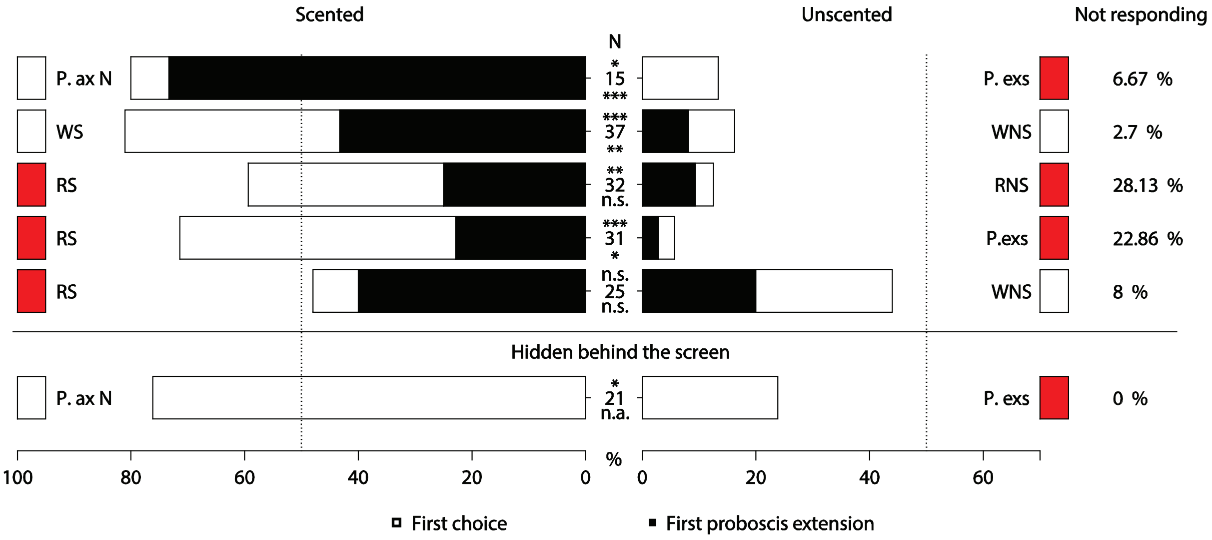


Figure II.7 Choice experiments investigating preferences by the moth *M. sexta* for scented flowers. For each experiment, the proportion of the moths that made their first choice (white bars) and first proboscis extension (black bars) on scented plants (left) is plotted against unscented plant (right bars). N is the number of moths tested in each experiment and asterisks above and below N represent, respectively, probability levels of first choice and first proboscis extension by *M. sexta* on plants (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; exact binomial test). Moths that made no choice (not responding) either did not reach the plants or flew to the downwind end of the wind tunnel or to its floor.

reached the upwind end of the wind tunnel while flying in the plume of odour of *P. axillaris* and then initiated local casting just downwind of *P. axillaris* at the same height as the scented flowers and 56% of them made contact on the screen with their forelegs. However, they flew slower when no visual cues were present and they were more likely to fly back downwind and initiate a new upwind flight in the plume. Only few moths visited *P. exserta* first and none of them persisted in flight to this species as they rapidly shifted to the scented side. As a control, we tested behaviour in the absence of both visual and olfactory stimuli. When *P. exserta* and the RNS introgression line were placed behind the screen, only few moths were activated and none showed upwind flight (16 moths tested).

To disentangle visual from volatile stimuli, we used the introgression lines that are indistinguishable by eye (Figure II.4). Plants were placed at the upwind end of the wind tunnel in front of the screen. When non-scented and scented *P. axillaris*-like plants were tested (WS versus WNS), moths clearly preferred the scented plants (exact binomial test, $P < 0.001$; Figure II.7). When non-scented and scented *P. exserta*-like plants (RNS versus RS) were tested the moths were also attracted by the scented line (exact binomial test, $P < 0.01$;

Figure II.7). Similar results were obtained when scented RS plants were compared to the parent *P. exserta*. However, even though the moths invariably visited the scented flowers at first in these experiments, they often visited the non-scented plant subsequently.

When we exposed the moths to conflicting cues, that is, a non-scented white *P. axillaris*-like plant (WNS) and a scented red *P. exserta*-like plant (RS), the moths flew in the plume of odour but on arriving at the plants they usually made lateral casting from one plant to the other before making any choice. The moths visited both plants equally as a first choice (exact binomial test, $P = 1$; Figure II.7). This means that there was no distinct choice of colour over scent by the moths for the first choice but they ended up feeding preferentially on the scented flower (40% RS, 20% WNS). In all the choice experiments where flower visitation was allowed, 85% of the moths visited the plants, but only 52% of these moths initiated proboscis extension. Nevertheless, the 53 that fed did so preferentially on the scented flowers (82%; exact binomial test, $P < 0.001$).

These experiments clearly show that volatile production affects not only the pollinator's choice over distance, i.e. to find a population of plants, but also determines which individual is pollinated first. We observed that moths tended to visit plants without scent production as a second choice once in the vicinity of the plant.

Discussion

In this work we studied the role of scent in the foraging preference of a night-active Lepidopteran pollinator using two closely related plant species, the white *P. axillaris* which is strongly fragrant and the red-coloured *P. exserta* which is devoid of scent. We showed that the scent production mechanism in *P. exserta* is completely silenced but can be restored by replacing only two QTLs from *P. axillaris* on chromosomes II and VII. The alteration of this floral trait shifts the foraging preference of *M. sexta* to the fragrant flowers, be they red or white. The odour bouquet of *P. axillaris* is mainly composed of benzenoid compounds and EAG recordings from *M. sexta* show that the moth has highly sensitive antennal receptor cells to at least 3 of these compounds.

Genetic basis underlying scent production

The headspace collection of flower scent volatiles showed that *P. axillaris* produces substantial amounts of scent at the beginning of the scotophase. On the contrary, no trace of scent released by *P. exserta* could be detected. When QTL on chromosome II originating from *P. exserta* was inserted into *P. axillaris* (WNS), scent production was completely silenced indicating that this locus is absolutely necessary for scent production. However, the introgression of the two QTLs on chromosomes II and VII are required to restore scent biosynthesis in the *P. exserta* introgression (RS). This suggests that the QTL on chromosome VII has a quantitative effect on scent production. The total amount of scent released by the modified *P. exserta* is lower than in *P. axillaris*, indicating that other minor QTLs may also play a role in the regulation of scent production. Klahre et al. (2011) found another QTL on chromosome III of *P. axillaris* involved in scent production, but introgression of *P. exserta* with chromosomes II and III of *P. axillaris* did not produce any scent, leading to the conclusion that this QTL plays only a minor role. Genes involved in the 2 major QTLs have not yet been completely determined but strong clues suggest the gene ODORANT1 as a good candidate on chromosome VII (Klahre et al., 2011). This gene is involved in the regulation of the shikimate pathway responsible for the synthesis of phenylalanine, the precursor of the benzenoid compounds released by the flower petals (Boatright et al., 2004). It has been demonstrated in *P. hybrida* that scent production is reduced when ODORANT1 is silenced (Verdonk et al., 2005). Moreover, this gene also regulates the production of benzoic acid, the precursor of methyl benzoate (Boatright et al., 2004), along with the biosynthesis of phenylpropanoids and vanillin. However, the alteration of the shikimate pathway did not affect the production of flavonols and anthocyanins, the photopigments involved in the coloration of the petals that occurs downstream the biosynthesis of phenylalanine (Verdonk et al., 2005). Alternatively, Maeda et al. (2010) uncovered another gene involved in phenylalanine biosynthesis, ADT, coding for a key enzyme in the regulation of the arogenate pathway in *P. Hybrida*.

The fragrance of *P. axillaris* contains several compounds, mainly benzenoids, released in copious amounts. Among these products, methyl benzoate and benzaldhyde are the most conserved as they are measured in reliable quantities in all the scented plant types. These compounds were also found in other wild *P. axillaris* populations (Hoballah et al., 2005) at comparable levels. Other compounds such as benzyl alcohol and methyl salicylate were only detected in the white scented plants, suggesting that genes expressing specific enzymes in the regulation of these products (i.e. S-adenosyl-L-Met:benzoic acid/salicylic acid for benzyl alcohol and salicylic acid carboxyl methyltransferase for methyl salicylate) (Boatright et al., 2004) were not associated with the *P. axillaris* QTLs transfered in the red background. Alternatively, as silenced ODORANT1 *P. hybrida* produce lower amounts of all compounds and *P. axillaris* naturally produced benzyl alcohol and methyl salicaylate in low amounts, their release level may have been below the detection threshold of the method used in the red scented line. The production of phenylpropanoids diverted between the two *P. axillaris* accessions. As *P. axillaris* N produced both eugenol and isoeugenol, *P. axillaris parodii* lacked isoeugenol. The absence of isoeugenol production in the latter is due to a mutation in the gene IGS coding for isoeugenol synthase (Koeduka et al., 2009).

Fragrance bouquet of *P. axillaris* is closely related to the olfactory performance of *M. sexta*

It is already known that night-blooming plants release a substantial quantity of odour to recruit susceptible pollinators over a long disance (Brantjes, 1978). Sphingophilous plants usually show similar pollination syndromes with white flowers with long tube releasing strong sweet scent at night such as *Datura wrightii* (Raguso et al., 2003a), *Nicotiana sp.* (Raguso et al., 2003b), *Brugmansia sp.* (Kite and Leon, 1995) and *P. axillaris* (Gübitz et al., 2009; Stuurman et al., 2004). As the scent structure of these plants involves benzenoids (Knudsen and Tollsten, 1993), espescially in *P. axillaris* (Hoballah et al., 2005) they may represent a honest food signal for pollinators. To ensure repeatable and consistant recordings of the antennal response of *M. sexta*, we adapted the standard EAG method because of the large size of the antenna. We inserted the whole antenna into the tube vehiculating the odour flux to insure that the whole antenna is optimally stimulated by the tested compounds. This methods permitted to reduce the variability and increased by x10^4 the sensitivity compared to the preliminary experiments and other studies (Fraser et al., 2003; Hoballah et al., 2005). EAG recordings revealed that *M. sexta* is very sensitive to benzyl alcohol, methyl benzoate and methyl salicylate with a threshold below 5 pg as delivered from the GC, indicating that this moth displays numerous receptor cells finely tuned for sensing these compounds (see Annexe 1). As both sexes responded at the same level to methyl benzoate and benzyl alcohol these compounds seem to be related to a nutrient source rather than to oviposition or mating signals. Methyl benzoate is thought to play a central role in pollinator attraction as it displays a rythmic emission pattern that corresponds to the activity period of *M. sexta* (Hoballah et al., 2005; Kolosova et al., 2001). Moreover, Kessler et al. (2013a) showed that transgenic varieties of *P. hybrida* releasing methyl benzoate were more attractive for florivore insects. Hoballah et al. (2005) recorded strong EAG responses of high doses of benzyl alcohol, methyl benzoate and benzaldehyde by *M. sexta,* and benzyl alcohol and methyl salicylate evoked EAG response thresholds at 100 ng as delivered from the GC on the same species (Fraser et al., 2003). Additionaly, Shields and Hildebrand (2001) characterized the response of 35 olfactory neurons of female *M. sexta* type-A trichoid olfactory receptor cells and showed several receptor cells specific to benzenoids. By contrast, *M. sexta* is not as sensitive to eugenol and benzaldehyde. Similar EAG responses pattern were recorded to these compounds at 100 µg as delivered from a filter paper in a syringe by Hoballah et al. (2005). Eugenol, isoeugenol and benzyl benzoate act as deterrents to protect the plant against herbivores and nectar robbers in *Petunia* sp. (Kessler et al., 2013a) but only poor EAG responses were recorded for these compounds, even at a high dose (data not shown for isoeugenol and benzylbenzoate). EAG recordings from *Hyles lineata*, another hawkmoth pollinator, showed similar patterns of responses to methyl benzoate, benzyl alcohol, methyl salicylate and eugenol as in *M. sexta* (Raguso et al., 1996).

Manduca sexta **prefers to forage on scented flowers**

Resource location in *M. sexta* relies on several plant traits such as flower colour (Goyret et al., 2008b; Hoballah et al., 2007; White et al., 1994), nectar quantity (Brandenburg and Bshary, 2011; Brandenburg et al., 2012a), flower morphology (Kaczorowski et al., 2012) and scent (Raguso and Willis, 2002). We were interested to know if *M. sexta* uses volatile emissions to discriminate between closely placed plants that differ only in scent. We showed that the hawkmoth clearly preferred to forage on scented plants over the scentless ones regardless of the colour background (white, red or pink, see Annexe 2), even when they were placed very close to each other (see Annexe 4). Moreover, in a situation where the plants were hidden, the moths were attracted to the scented ones. Obviously, scent is an important floral trait that can bias plant attraction and the foraging preference of the pollinator between two sibling plants. This shift in the behaviour may be sufficient to lead to selection towards scented individuals by *M. sexta*. In the context of red flowers, fewer moths were interested in the plants. The red corolla does not fit with the sphingophilous pollination syndrome and the visual performance of *M. sexta* is not optimal at high light wavelengths (Cutler et al., 1995; White et al., 2003). Consequently, these flowers do not stand out of the background, especially at night. On the contrary, the white flowers types reflect light at wavelengths that perfectly match the absorbance maxima of the blue and green photoreceptors of the compound eyes of *M. sexta* (Cutler *et al.*, 1995) and are absorbent in the ultraviolet range. White et al. (1994) showed that feeding behaviour by *M. sexta* is visually triggered by stimuli that reflect blue and/or green light, but is altered by ultraviolet wavelengths.

Many studies have underlined the effect of scent variations on the relationship between plants and pollinators. The behaviour of pollinator guilds can be modulated by changes in odour ratios in different populations of eveningsnow, *Linanthus dichotomus* (Chess et al., 2008). Fine changes in the quality of a flower odour bouquet can also cause a preference towards one type of fragrance. In the common strawberry, *Fragaria virginiana*, there is a difference in the scent quality between hermaphrodites and females that mediates a foraging preference by the pollinators towards the former (Ashman et al., 2005). In the dame’s rocket, *Hesperis matronalis*, individuals that produce more scent at night have an increased fitness through a higher seed set (Majetic et al., 2009), suggesting that night-active pollinators visited more frequently plants with a higher fragrance level.

When *M. sexta* was confronted with a scented red flower versus an unscented white flower in this study, they could not make a clear choice, as unexpected attractive floral traits were combined on both treatments. The outcome of this conflicting situation suggests that both colour and odour are almost equally important in the selection of a feeding site. Raguso and Willis (2002) showed that a single floral cue (colour or scent) is sufficient to induce attraction by naïve *M. sexta* to artificial flowers but the combination of both is mandatory to elicit proboscis extension. These findings support our observations as less insects were attracted and were less likely to extend their proboscis on plants expressing only one attractive cue, i.e. scent (RS versus RNS), than on plants that displayed both scent and colour (P. axN versus P. exs). Additionally, Goyret et al. (2007) showed that when olfactory and visual attractive stimuli were spatially decoupled, the foraging response to an artificial flower by the moth was diminished.

In this study we showed that a slight change in the genetics of *Petunia* can drastically alter the feeding habit of its pollinator. The alteration of this trait can induce selection by hawkmoth pollinators toward scented plants in a population were hybridisation occurs fortuitously. Additionally, the genetic basis underlying petal pigmentation in *Petunia* is governed by 6 QTLs (Wijsman, 1983). Most of the colour trait is regulated by a single gene and its alteration causes a major shift in the pollination biology of the plant (Hoballah et al., 2007). These findings support the hypothesis that only few genes with strong effects are involved in the adaptation of plants to a new pollinator. The phylogeny of *Petunia* shows that colour and scent alteration occurred several times to adapt to the different pollination strategies. Colour loss in *P. integrifolia* occurred several times to give rise to a *P. axillaris* phenotype (Quattrocchio et al., 1999) and the anthocyan pigmentation genes of *P. exserta* are closely related to those of *P. axillaris* indicating that *P. axillaris* recovered colour to give rise to the *P. exserta* phenotype (Gübitz et al., 2009). Similarly, scent production was strongly transformed between *P. integrifolia* and *P. axillaris* on the one hand and between *P. axillaris* and *P. exserta* on the other. As colour alteration in *P. integrifolia* seems to have appeared late in the adaptation process to *P. axillaris* (Hoballah et al., 2007; Quattrocchio et al., 1999) and the genetic architecture of the regulation of scent in *Petunia* remains rather simple, the latter trait should permit the initiation of the speciation process by a hawkmoth pollinator. However, only naïve, laboratory-reared insects were tested in this study and trained *M. sexta* are known to be able associate an odour with a reward (Daly and Smith, 2000). More experiments in the field with our unscented lines in wild hawkmoth populations may be necessary to consolidate our findings.

Chapter III.

Sugar Selection in Adults *Manduca sexta*: an Electrophysiological and Behavioural Study

Abstract

Carbohydrates in nectar provide an important energetic resource for foraging insects, yet little is known regarding the quality of food they prefer to feed on. Examination of the proboscis of *Manduca sexta* reveals presence of styloconic and basiconic terminal-pore gustatory sensilla at its tip. Receptor cells in these sensilla respond to solutions of sucrose, fructose and glucose with strongest responses to fructose and sucrose but with no response to glucose. Two-choice experiments were conducted in a wind tunnel with artificial flowers bearing different types and quantities of sugars. Fructose and sucrose were preferred over glucose solutions by *M. sexta* and volume of sucrose imbibed solutions was linked to concentration. The behavioural choices recorded correlate well with the neurophysiological responses of the proboscis sensory cells and with the sugar content of plants pollinated by this hawkmoths.

Introduction

The sensory physiology of food detection and the resulting feeding behaviour in Lepidoptera has been extensively studied in larvae (Glendinning et al., 2007; Schoonhoven, 1969). However, there are fewer reports on food selection and its underlying sensory input in adults. Even though some Lepidoptera can reproduce without feeding, intake of nectar increases fecundity (Mevi-Schutz and Erhardt, 2005) and extends life span. Carbohydrate intake is particularly necessary in hawkmoths as the energy costs of hovering flight is substantial, especially during the first 30 min of flight where metabolism of carbohydrates is preferred over lipid metabolism (Ziegler and Schulz, 1986). Moreover, carbohydrate concentration may influence volume intake as diluted solutions have water in excess, a low energetic value and increased metabolic costs due to a higher osmotic load.

The tobacco hawkmoth, *Manduca sexta,* is able to fly over long distances to feed and can consume over 900 µL of sugar solution in one feeding bout (personal observations). It feeds on night blooming flowers such as the sacred Datura, *Datura wrightii*, tobacco *Nicotiana sp.* and *Petunia axillaris*. These plants display a well-defined hawkmoth-pollination syndrome with flowers showing long tubes and white corollas releasing a substantial amount of sweet scent (Gübitz et al., 2009; Raguso et al., 2003b). The nectar of these plants is rich in sucrose but poor in fructose and glucose (Hoballah et al., 2007; Kaczorowski et al., 2005; Raguso et al., 2003a), something that may also contribute to the pollination syndrome. Nectar volume may influence foraging behaviour of *M. sexta*. Brandenburg et al. (2012b) showed that reward-reduced *P. axillaris* were less likely to be visited by *M. sexta*. However, the influence of the carbohydrate quality of nectar on food selection in pollinator moths is unknown.

In this study, we show how the hawkmoth can discriminate between sugars and their dilutions presented in artificial flowers in a wind tunnel. In addition we show how receptor cells in styloconic and basiconic sensilla on the hawkmoth proboscis respond selectively to sugars commonly found in nectar.

Materials and Methods

Insects

M. sexta pupae were obtained from the Laboratory of Animal Physiology (Philipps University, Marburg, Germany) and held in an environmental cabinet where light was provided by six fluorescent tubes (Philips TDL, 36 W, >1 kHz) with a 16:8 light:dark cycle at

26°C and 65% relative humidity. Pupae were sexed prior to emergence and placed in rearing cages (BugDorm-4180F, MegaView Science Co., Ltd., Taichung 40762, Taiwan) containing wet cotton tissue. For behavioural assays we used 5 to 8 day-old, unmated and unfed males. Unmated and unfed female moths of 3-5 days were used for electrophysiological experiments as younger individuals salivate extensively during manipulations.

Scanning electron microscopy

Proboscises of female *M. sexta* were dissected and stored in ethanol 70 % for 7 days. Samples were then washed in graded concentrations of acetone (70 % to 100 %) to remove water and ethanol and dried at the CO_2 critical point. Preparations were mounted on stubs and coated with a 23 nm gold layer in a Sputter coater (Baltec SCD 005; Oerlikon Balzers, Lichtenstein). Preparations were examined at 10 kV using a Philips XL 30 scanning electron microscope.

Single sensillum recordings

An anesthetized female *M. sexta* with CO_2 was inserted into a 5 mL plastic syringe (BD Plastipak™, Spain) whose tip was cut and the female was pushed through it to place the eyes outside of the syringe. The syringe was then fixed on a plate of Plexiglas® with the moth ventral side down. The proboscis was gently unrolled and held by using adhesive tape. A 2 mm o.d. borosilicate reference electrode filled with 100 mM KCl was inserted in the left eye. The preparation was placed on a microscope (BX51WXIF, Olympus Schweiz, Volketswil, Switzerland) at a magnification of 1000.

Single sensillum recordings from styloconic sensilla (n = 6 sensilla from different moths) and basiconic (n = 3-4 from different moths) on the dorsal side of the proboscis tip (see Figure III.1) were performed as in (Kessler et al., 2013b) by using the following treatments: 10 mM KCL and 292 mM glucose, 292 mM fructose and 147 mM sucrose, each in 10 mM KCl. Recordings were analysed with Dataview (Heitler, 2012). Concentrations of test products were adjusted to equalize the number of molecules per unit volume. Each recording of action potential started at 30 ms after stimulation to remove the noise generated by contact of the electrode with the cuticle and recording continued for 2 s. Spike detection was performed by applying a threshold at 6 times the robust standard deviation of all points recorded in each trace.

Behavioural assay

Binary choice experiments were conducted in the same wind tunnel and environmental conditions as described in Klahre et al. (2011). This consisted of presenting to a free flying *M. sexta* two artificial flowers filled with 500 µL of one of the following test solutions: distilled water, 10 % sucrose, 2 % sucrose, 1 % sucrose, 2 % glucose or 2 % fructose. The artificial flower was made of a 7 cm diameter white filter paper disk mounted on a 1 mL Eppendorf tube. To measure the quantity of sugar solution taken up by the moth, tubes were weighed when empty, when filled with 500 µL of solution and after the behavioural experiment. Two flowers were placed on a stand at a height of 50 cm, 19 cm apart and at an angle of 60°. Prior to experiments, 10 µL of a 1:20 dilution of bergamot essential oil (Bergamote bio, Farfalla Essentials AG, Uster, Switzerland) in dichloromethane was applied to the filter paper of each artificial flower to render them attractive (Goyret et al., 2007).

Artificial flowers were placed 30 cm from the upwind end of the wind tunnel. A single moth was placed on a 5x15 cm platform situated 15 cm of the downwind end of the wind tunnel, at a height of 50 cm. When the moth did not activate within 30 s it was gently prodded with a finger. Moths that were too weak to fly or that did not respond to the artificial flowers were discarded. The experiment ended after 5 min. Experiment were repeated between 4-20 times, at low numbers for strongly appetitive treatments and at higher numbers of repetitions for treatments with lower levels of sugars.

Statistical analysis

All statistical analyses were performed with R statistical package (R Core Team, 2012). To establish if differences in action potential generation between treatments were significant a generalised linear model was fitted followed by a Tukey contrast post-hoc test. A Kruskal-Wallis test was used to analyse the electrophysiological response patterns over time to sugars. A pairwise Wilcoxon test was performed on the volumes imbibed by the moth on the two flowers to compare moth preferences within experiments.

Results

Characterisation of the sensilla of the proboscis of *M. sexta*

The proboscis of *M. sexta* is covered with at least three types of sensilla. The most abundant are styloconic sensilla sparsely spread in a row along the lateral side of each maxilla (Figure III.1A). Only eight styloconic sensilla spaced 672 ± 101 µm apart were observed on the last 5 cm of each maxilla, except at the tip where two are placed on the ventral dorsal sides at 80 and 197 µm from the tip (Figure III.1B). Styloconic sensilla project from a depression of 36 µm in diameter and are formed by a peg 15 µm long and 11 µm in diameter capped by a smaller peg of 4.8 long and 2.7 µm in diameter (Figure III.1C). Three basiconic sensilla occur on the last 300 µm of the tip of each maxilla. They are formed by a small peg 4.7 µm long and 2.9 µm in diameter situated in a depression of 14.5 µm in diameter (Figure III.1D). Two aporous chetifrom sensilla occur in the last 200 µm of the tip of each maxilla and are hair like sensilla 23 µm long and 2.6 µm in diameter at the base set in a depression 14.5 µm in diameter.

Response of gustatory sensilla on the proboscis to different sugar solutions

Single sensillum recordings were made from on the dorsal styloconic sensillum and the 2nd basiconic sensillum on the tip of the proboscis of *M. sexta* (Figure III.1B). Neurons in the styloconic sensillum were poorly activated by 10 mM KCl with a mean number of spikes per 2s (MNS) of 9.6 ± 4.72 (mean ± SEM) and by 292 mM glucose in 10 mM KCl (MNS = 2.4 ± 0.67). When the sensillum was stimulated with 147 mM sucrose in 10 mM KCl the MNS significantly increased to 46.2 ± 5.51. Fructose at 292 mM in 10 mM KCl increased the response of responding neurones to a MNS of 64.6 ± 6.43, significantly higher than for sucrose (Figure III.2A & C-F). The responses of the basiconic sensillum neurones to sugars displayed the same pattern as for the styloconic sensillum neurones in that they responded only slightly to 10 mM KCl (MNS = 8.5 ± 4.73) and to 292 mM glucose in KCl 10 mM (MNS =

0.25 ± 1.25). However, the responses increased significantly to MNS of 64 ± 2.65 when stimulated with 147 mM sucrose in 10 mM KCl and to MNS of 89.7 ± 21.4 with 292 mM fructose in 10 mM KCl. The response for fructose is significantly higher than for sucrose (Figure III.2B & G-J). The overall responses of the basiconic sensilla were ca. 33% higher than those of the styloconic sensilla, except for KCl, where the firing rates were similar.

Stimulation of the sensilla with fructose or sucrose systematically induced movements of the proboscis that lasted a few tens of seconds after stimulation. Moreover, one to two day-old adult moths abundantly salivated when stimulated with these sugars, making further recording impossible. This was never observed on stimulation with either glucose or KCl. Recordings on the chetifrom sensillum never induced a response to any treatment.

The response pattern of the sensilla to sucrose is strongly tonic, with no adaptation within the two seconds of stimulation (Figure III.3; Kruskal-Wallis, $df = 1$, $P_{styloconic} = 0.46$, $P_{basiconic} = 0.22$). However, a slight phasic response pattern with a decrease in frequency was observed within 200 ms of stimulation with fructose. This decrease was significant for the styloconic sensilla neurones (Kruskal-Wallis, $df = 1$, $P < 0.01$, Figure III.3C), but not for those of the basiconic sensilla (Kruskal-Wallis, $df = 1$, $P = 0.09$, Figure III.3D).

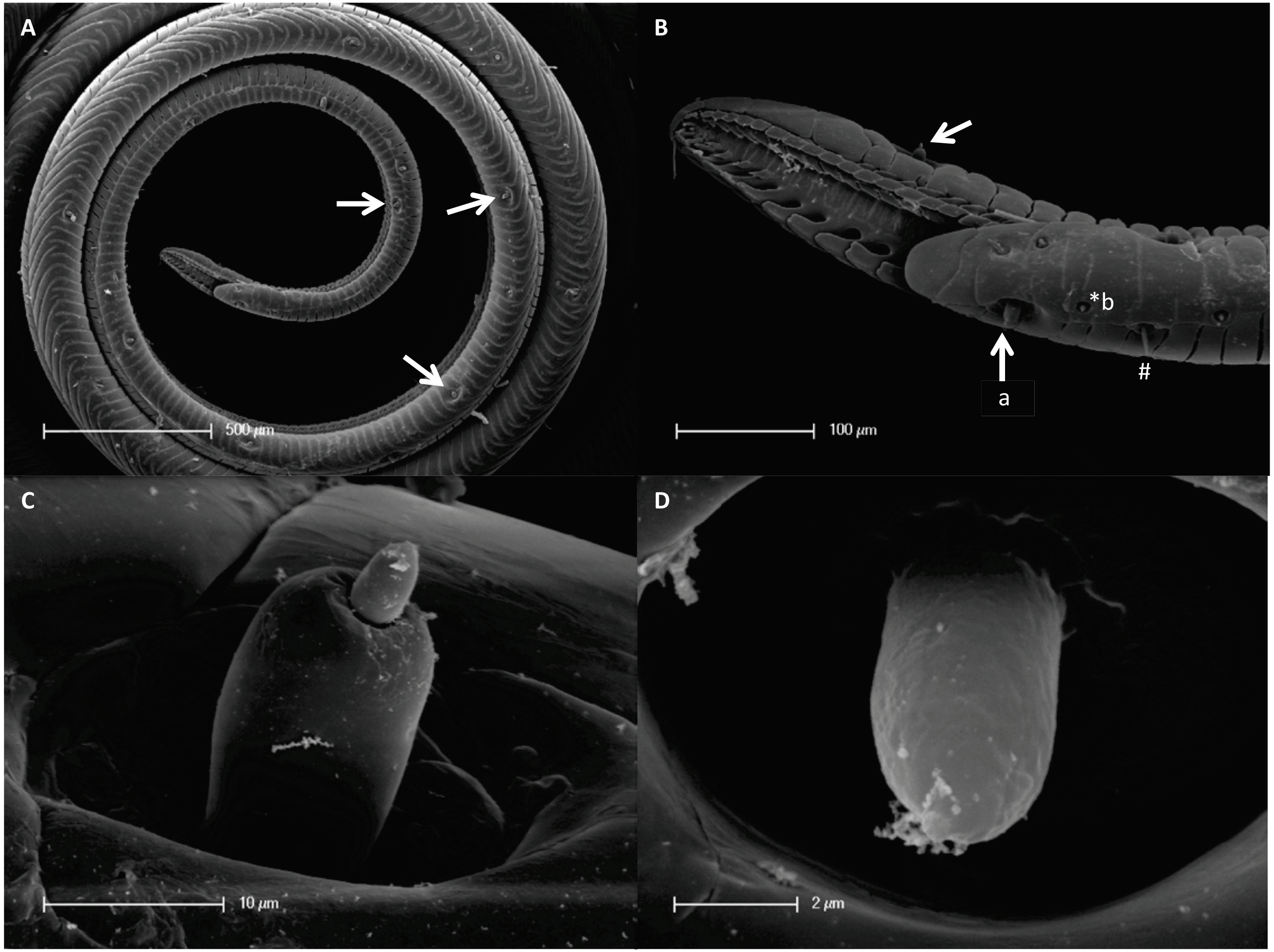


Figure III.1 Scanning electron micrographs of a proboscis of female M. sexta. (A) Overview of the distal part of the proboscis showing numerous sensillae along its length (white arrows). (B) Tip of the proboscis with the two maxilla uncoiled showing the alimentary canal between them. At least 3 types of sensillae can be distinguished: styloconic (white arrows), basiconic (*) and chetiform (#). Single sensillum recordings were conducted on sensilla indicated as "a" and "b". (C) Close-up of a styloconic sensillum. (D) Close-up of a basiconic sensillum. Scale bars are in the bottom left corner of each micrograph.

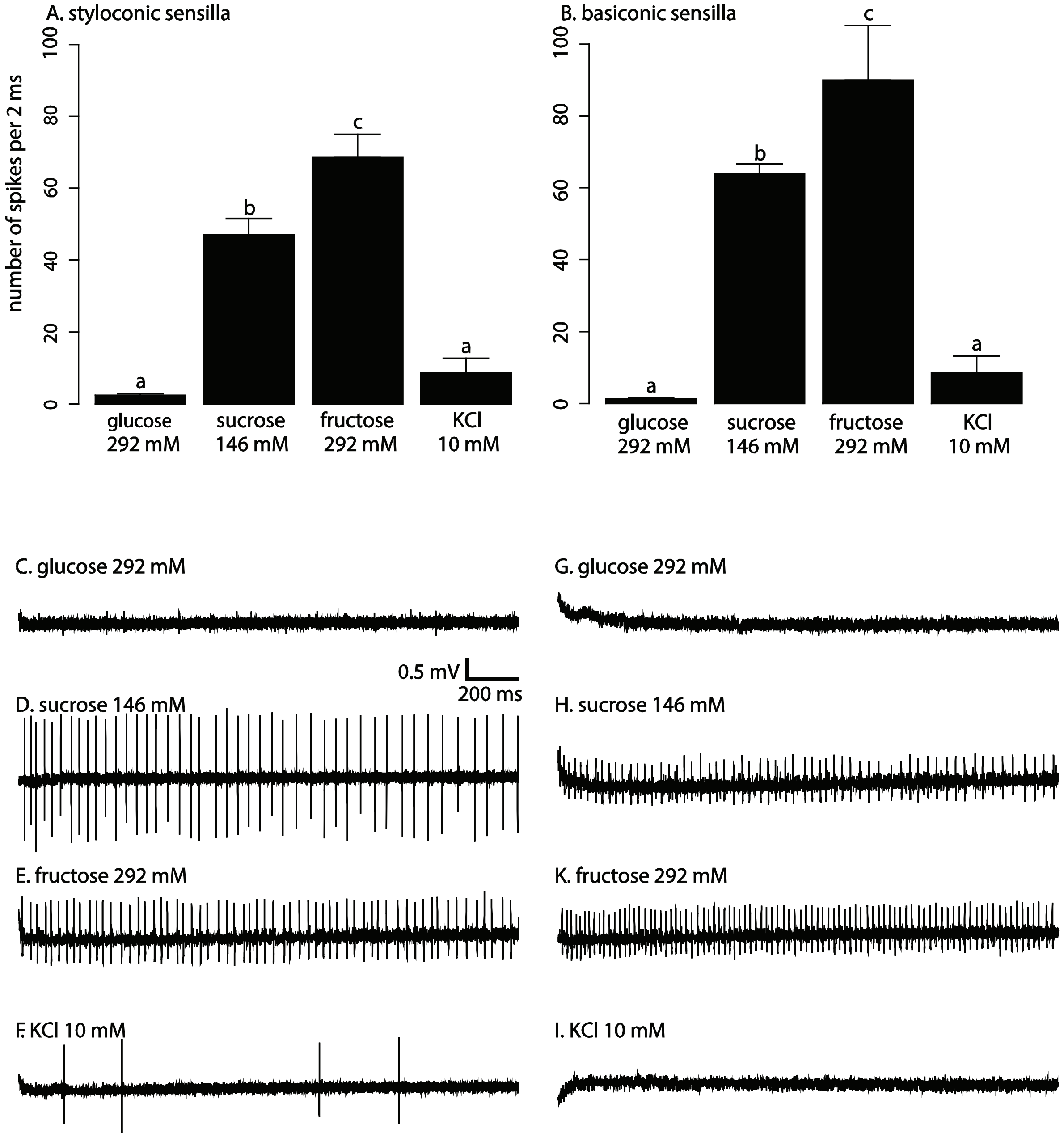


Figure III.2 Single sensillum recordings from styloconic (A, n = 6 moths) and basiconic (B, n = 3-4 moths) sensilla on the proboscis tip of *M. sexta* stimulated with 292 mM glucose, 146 mM sucrose and 292 mM fructose in 10mM KCl, and to 10 mM KCl. Letters above the bars indicate statistically significant differences between treatments (GLM, $p<0.001$). Examples of the responses of sensillar neurones (left traces from a styloconic sensillum and right traces from a basiconic sensillum) to the different sugar treatments are represented in C.

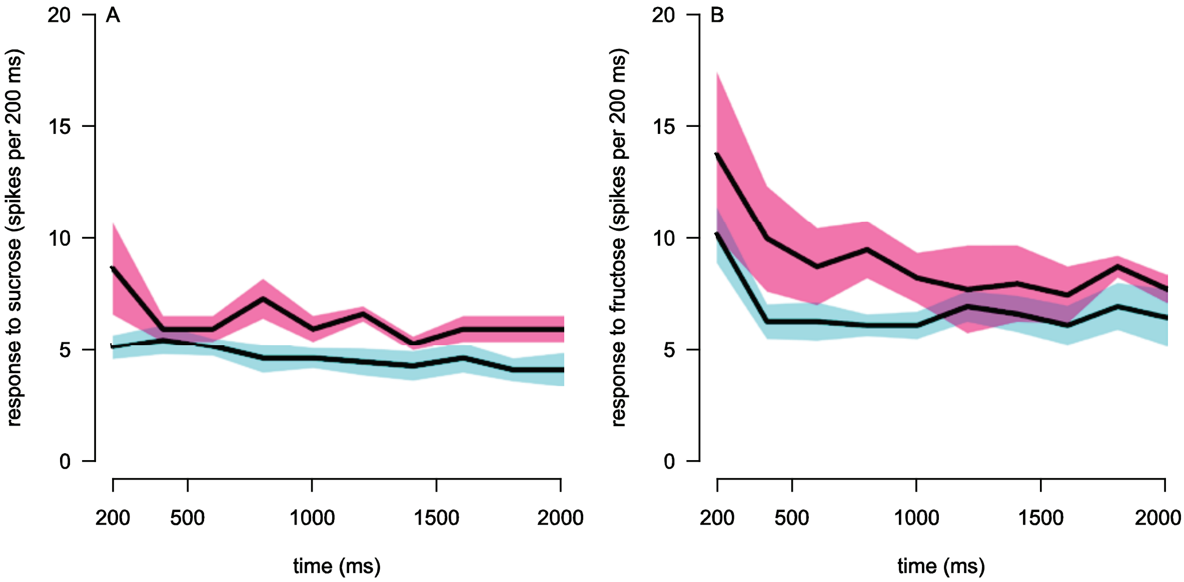


Figure III.3 Mean ± sem of the spike frequency over 2 s recordings to sucrose (A) and fructose (B) from neurones in 6 styloconic sensilla (blue) and from neurones in 3-4 basiconic sensilla (red) of *M. sexta.*

Feeding preference

Free flying moths were presented with the choice to feed on two artificial flowers filled with different sugar treatments (Figure III.4). Each moth tested succeeded in tasting each treatment. They probed both flowers several times by flying between them, but the main intake was made during their first visit to a suitable treatment. When moths had the choice between 10 % sucrose and water, all of them fully emptied the sweet flower (506 µL ± 4.2) and none imbibed water (6.9 µL ± 4.0). As 10 % sucrose is strongly phagostimulant, lower concentrations of sugars were tested to reduce intake by moths so as to avoid them being replete after visiting the first flower. When the sucrose concentration in a flower was reduced to 2 % and compared with water, moths drank only 188 µL ± 29 of sugar solution and only 2.3 µL ± 0.88 water. At a sucrose concentration of 1 %, moth intake was decreased further to 145.6 µL ± 26 compared to 23.1 µL ± 6.7 water (Wilcoxon's test, V = 13, P < 0.01). Moths are able to discriminate between two concentrations of sucrose (1 % versus 2 %) and fed preferentially on the treatment of highest nutritive value (sucrose 1 % 33.3 µL ± 8.6; sucrose 2 % 269.1 µL ± 63.7; Wilcoxon's test, V = 2, *P* < 0.01). When glucose and sucrose was compared at the same density, *M. sexta* clearly preferred to feed on sucrose (glucose 2 %:

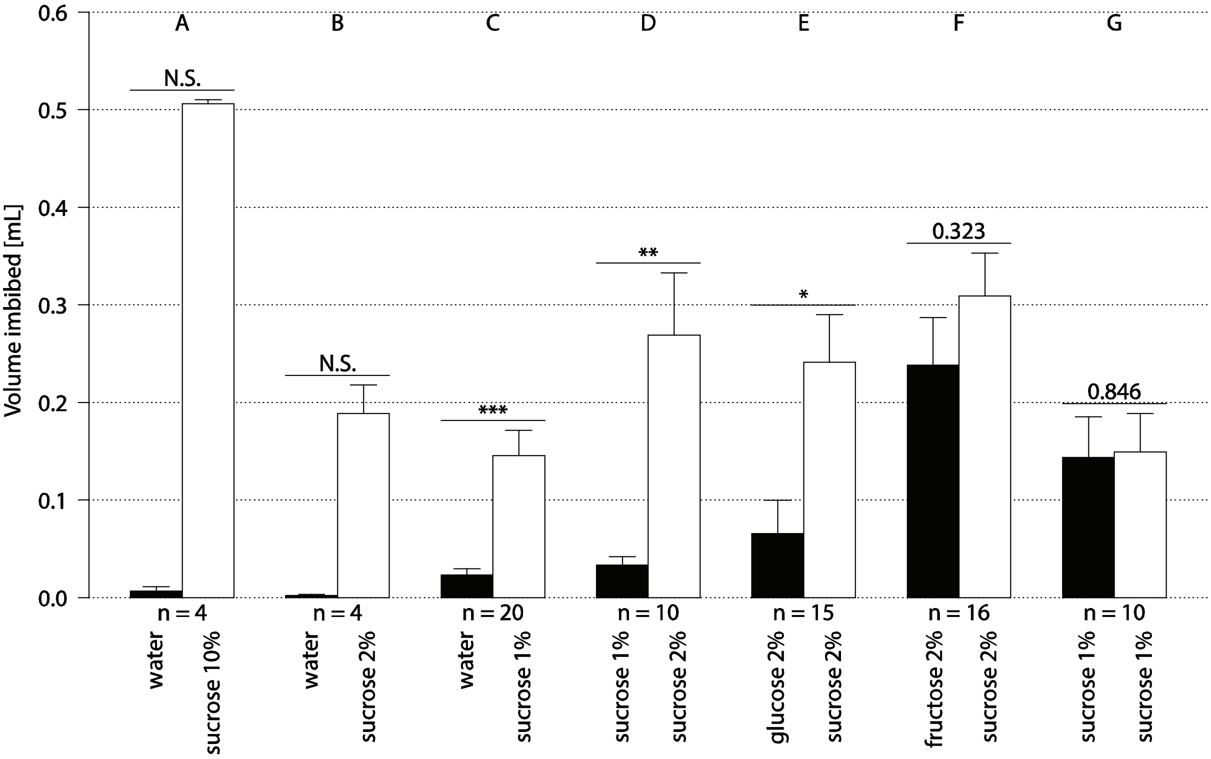


Figure III.4 Feeding preferences of *M. sexta* in two-choice experiments where two artificial flowers filled with 500 µL of a treatment were presented to a free flying moth. Bars represent the mean amount of liquid drunk ± s.e.m. and asterisks above treatments indicate significant differences (paired Wilcoxon-test; ***: $P<0.001$, **$P<0.01$, *: $P<0.05$). *P*-values are shown when treatments are not different.

65.8 µL ± 34; sucrose 2 %: 241.3 µL ± 48.7; Wilcoxon's test, V = 23, P < 0.05). However, moths could not differentiate between sucrose and fructose tested at the same density (fructose 2 % 238.4 µL ± 48.4; sucrose 2 % 309.3 µL ± 63.7; Wilcoxon's test, V = 48, *P* = 0.32). When the same treatment was exposed to the moths in both flowers, i.e. sucrose 1 %, moths fed equally on each artificial flower (143.8 µL ± 41.5 versus 149.1 µL ± 39.6, Wilcoxon's test, V = 25, *P* = 0.85). Remarkably, *M. sexta* tends to imbibe a defined volume of sucrose at a given concentration in solution when it is the preferred treatment (Figure III.4). Furthermore, the moths imbibed approximately half the amount of sucrose 1 % (146.0 µL ± 18.8) than compared with sucrose 2 % (285.2 µL ± 37.0; Wilcoxon's test, W = 556.5, *P* < 0.01).

Discussion

In this study, the sensilla of the tip on the proboscis of *M. sexta* are described and characterised in terms of their response to sugars. The feeding preference of the moth to these primary compounds was recorded and we show a correlation between the neuronal input from the proboscis and the behavioural response to feeding on sugars.

M. sexta bears several types of sensilla on the proboscis including styloconic sensillae set in a lateral row along the proboscis and basiconic sensilla. These sensilla are more numerous at the tip of the proboscis. We tested the electrophysiological responses of both styloconic and basiconic sensilla to sugar solutions. Surprisingly, both types of sensilla responded similarly with a firing rate for fructose > sucrose > glucose. Stimulation of a single styloconic or basiconic sensilla is sufficient to promote a behavioural response of the insect in that each time a styloconic or basiconic sensillum was contacted with a solution of sucrose or fructose the moth moved the maxillae or salivated. Städler and Seabrook (1975) showed that styloconic sensilla at the tip of the proboscis of the spruce budworm responded to sucrose, but not to host specific chemicals that serve to initiate oviposition. Styloconic sensillae on the proboscis of three Lepidopteran species, *S. littoralis, H. virescens* and *H. armigera*, show a higher response to sugars than to amino acids and sugar alcohols (Blaney and Simmonds, 1988). These authors observed a differential response pattern for sucrose, fructose and glucose with a higher firing rate of the proboscis sensillar neurones to fructose than to glucose, but lower than for sucrose in all the three tested species. However, the preferences of these Lepidopteran species were less clear in behavioural experiment, especially between sucrose and fructose.

We show here that adult *M. sexta* are able to taste low concentrations of sucrose and to discriminate between them. Whereas *M. sexta* is capable to imbibe large volumes of sugar solutions (maximum measured was 940 µL), intake is reduced at low sugar concentrations. At lower concentrations the ratio between the nutritive value of the meal and its volume would be too poor and the energetic costs to fly with a charged crop too high. As moths still forage even after ingestion of a meal this indicates they are not replete. Several factors affect foraging behaviour of insects, including food quality and the amount collected. Haemolymph composition is one of the most crucial factors in the modulation of foraging behaviour as it provides a continuously updated state of the insect's nutritional needs (Simpson and Raubenheimer, 1993). It has been shown that the responsiveness of the gustatory sensory system of the migratory locust, *Locusta migratoria*, to sugars and amino acids is modulated by the level of these nutrients in haemolymph (Abisgold and Simpson, 1988). Furthermore, the feeding behaviour of grasshoppers and caterpillars is influenced by the levels of amino acids and sugars in haemolymph (Abisgold and Simpson, 1987; Simpson and Simpson, 1992). Meal termination is induced by the trade-off between the excitatory effect of a phagostimulant and the inhibition induced by other compounds such as alkaloids and the graded adaptation of the gustatory sensory system. Simpson and Simpson (1992) propose that the gustatory sensory system measures the difference in concentration of key nutrients between the food and haemolymph. In our case intake should be regulated by such a mechanism in *M. sexta* since the volume ingested is correlated with sucrose concentration. The balance between haemolymph levels of sugars and food intake is reached earlier for the lower concentration of sugar such that the negative feedback from the haemolymph on the higher neuronal centres and/or taste receptors terminates feeding. However, as moths continued to forage after a feeding bout in our tests and even tried to feed on the same flower during subsequent visits, feeding was inhibited as the balance point between the concentration of sugar solution and the haemolymph level was reached. Edgecomb et al. (1994) and Josens et al. (1998) also found that the imbibed volume is correlated to sugar concentration in *Drosophila melanogaster* and in the ant *Camponotus mus.*

Sugar quality also plays a role in the feeding preference of *M. sexta*. Wild *M. sexta* usually feed on several plant nectars rich in sucrose (Baker and Baker, 1983) such as *D. wrightii* (Raguso et al., 2003a), *Nicotiana sp*. (Kaczorowski et al., 2005) and *P. axillaris* (Hoballah et al., 2007). At low concentration, the moths were able to discriminate between the most common sugars found in nectar. Pure solutions of sucrose or fructose were preferred over glucose. Other pollinators such as the hummingbird hawkmoth *Macroglossum stellatarum* (Kelber, 2003), the peacock butterfly, *Inachis io* (Rusterholz and Erhardt, 1997) and the cabbage butterfly, *Pieris brassicae* (Romeis and Wackers, 2000) prefer sucrose over fructose and glucose. Moreover, Blaney and Simmonds (1988) found a similar behavioural preferences as the above authors and established a correlation with the electrophysiological response of receptors cells to sucrose and fructose over glucose in styloconic sensilla on the mouth parts of *S. littoralis* larvae and adults.

Taste in *M. sexta* is crucial to permit feeding on profitable nutrients. At low doses of sugar solutions, where the balance between energy intake and volume to bear in flight might be problematic, moths were more sensitive to sucrose and fructose over glucose and prefer to feed on the former. However, floral nectars are much more concentrated in carbohydrates and as such the quality of a reward may less affect the behaviour of the moths.

Chapter IV.

Volatile Hydrocarbons Increase the Response of Male Grapevine Moths, *Lobesia botrana*, and Codling Moths, *Cydia pomonella,* to their sex pheromone

Abstract

The grapevine moth *Lobesia botrana* and the codling moth *Cydia pomonella* are two major pests in vineyards and orchards. We have tested the behavioural response of males to short-chained alkanes in combination to underdosed sex pheromone in a wind tunnel. The response of *L. botrana* with heptane or octane is increased in a dose dependant manner with the maximum proportion of males contacting the source of pheromone at 10 ng/min of the two alkanes, where attraction was as high as with calling females. Moreover, heptane as solvent for the pheromone induced source contact and increased the response of males in the early phases of attraction, but *L. botrana* pheromone diluted in octane induced flight arrestment of the moths while progressing upwind. As in *L. botrana*, heptane released at 10 ng/min in combination with underdosed codlemone increased the response of *C. pomonella*. The alkanes were not attractive on their own, but elicited more take off and flight casting than the ethanol control. The effects of the plant volatile 2-phenylethanol was also tested on both species. Even though 2-phenylethanol does not attract moths on its own it increased attraction of both species in presence of pheromone. Addition of both heptane and 2-

phenylethanol to the sex pheromones of *L. botrana* and *C. pomonella* did not improve the response, showing an underlying redundancy of these products when added together. Finally, alkanes and 2-phenylethanol shortened the time taken by males to reach the source of pheromone. The large range of alkane concentrations affecting attraction of these two moth species offers a promising perspective for control of field populations using mating disruption.

Introduction

Herbivorous insects are responsible for considerable annual economic loss in fruit production. The grapevine moth *Lobesia botrana* Den. et Schiff. (Lepidoptera; Tortricidae) is responsible for damage to grapes in vineyards across the Asia, Africa, Europe and recently into North and South America. The codling moth *Cydia pomonella* Linnaeus (Lepidoptera; Tortricidae) is another major worldwide pest of pome fruits such as apple, pear and walnut. In both species, fruit damage caused by larvae favour subsequent infestation by bacteria and fungi resulting in unmarketable products.

Identification of sex pheromone male attractants is well documented in both species (Arn et al., 1988; El-Sayed et al., 1999c; Mcdonough et al., 1993; Roelofs et al., 1971). As insecticides cause increasing resistance in pest moths (Mota-Sanchez et al., 2008; Reyes et al., 2007) pheromone release for mating disruption provides a complementary manner for controlling moth pest in crops. Mating disruption consists in reducing the probability of males to find females by attracting them to sex pheromone lures (see Witzgall et al., 2008). Though this method of control has demonstrated its efficiency for several moth species (Cardé and Minks, 1995), pheromone synthesis at an industrial scale remains costly.

Plant volatiles are important olfactory cues for moth species (Ansebo et al., 2004; Schoonhoven, 2005; Tasin et al., 2007). Females may use host plant compounds for oviposition and to find an appropriate mating site. Grape odour mixtures elicit upwind flights in gravid *L. botrana* (Tasin et al., 2010; Tasin et al., 2006) as well as in males (von Arx et al., 2011). Apple volatiles induce calling behaviour in virgin codling moth females and induced oviposition in gravid individuals (Yan et al., 1999). Moreover, single apple volatiles such as pear ester (E,Z)-2,4-decadienoate (Light et al., 2001), (E,E)-farnesol or (E)-β-farnesene attracts both male and female *C. pomonella* (Coracini et al., 2004; Yang et al., 2005).

In an effort to reduce pheromone quantities released in the field, plant volatiles can be added to underdosed sex pheromone mixtures to assess if they can serve to increase attraction of phytophagous moth pests to their pheromone. In wind tunnel, attraction of *Eupoecilia ambiguella* to underdosed sex pheromone increases by adding either (E)-β-caryophyllene, methyl salicylate, (Z)-3-hexen-1-ol or (+)-terpinen-4-ol, all host plant volatiles (Schmidt-Buesser et al., 2009). Similarly, more *C. pomonella* males contact the source of sex pheromone with the host plant volatiles R(+)-limonene, linalool, (E)-β-farnesene or pear ester added (Schmera and Guerin, 2012) and *L. botrana* males is better attracted to its sex pheromone in the presence of (E)-β-caryophyllene, (Z)-3-hexenyl acetate, 1-hexanol, or 1-octen-3-ol (von Arx et al., 2012b). However, although attraction of males to sex pheromones with plant compounds added in wind tunnel tests show promising results, only few encouraging reports have been obtained in the field. Traps baited with pear ester and codlemone have shown success in attracting *C. pomonella* males in the field (Knight et al., 2005; Knight and Light, 2005b; Light and Knight, 2006) but the formulation of attracting blends remains a challenge (see Witzgall et al., 2008).

Short-chain alkanes are interesting plant volatiles in that they can serve to dilute pheromone products of low polarity and their volatility varies as a function of the number of carbons. Moreover, moths can perceive C_{15}-C_{21} alkanes in host plant headspace (Bengtsson et al., 2001; Tasin et al., 2005). Here, we show how the response of *L. botrana* and *C. pomonella* to their sex pheromones can be synergised by short chain alkanes used as solute or solvent.

Materials and Methods

Insects

A colony of *L. botrana* (originally obtained from a laboratory culture at Agroscope, Changins, Switzerland) was maintained in a climate chamber at 65% RH and 25°C during the photophase (16 h) and at 85% RH and 18°C during the scotophase (8 h). The larval stages were reared on a semi-artificial medium (Rauscher et al., 1984). Pupae of *C. pomonella* were obtained from Andermatt Biocontrol AG (Grossdietwil, Switzerland). Pupae of both species were sexed and placed on a cloth mesh over a dish filled with water for emergence. Males emerged daily into cages (BugDorm, 30x30x30cm, MegaView Science Education Services Co., Taiwan). A sugar solution (10% sucrose) in a glass vial stoppered with a cotton wool wick was provided to males suspended from the top of the cage.

Synthetic chemicals

The sex pheromone components of *L. botrana* used were (E,Z)-7,9-dodecadienyl acetate (E7Z9-12:Ac,>97%), (E,Z)-7,9-dodecadien-1-ol (E7Z9-12:OH, >94%) and (Z)-9-dodecenyl acetate (Z9-12:Ac, 99.9%) from Plant Research International (Netherlands). The sex pheromone of *C. pomonella* was (E,E)-8, 10-dodecadien-1-ol (E8E10-12:OH or codlemone, >99.8%) from Siegfried Ltd (Zofingen, Switzerland). The plant secondary compounds tested were 2-phenylethanol (>99%), heptane (>99%), octane (>99.8%) and nonane (>99%) all from Fluka (Buchs, Switzerland). Ethanol was used as solvent (analysis grade, >99.8%; Merck AG, Dietikon, Switzerland).

Electroantennogram recording

An excised grapevine moth male antenna, whose distal tip was cut, was held between two glass electrodes filled with 0.1M KCl in a humidified charcoal-filtered air stream (90–100% RH, 23 ± 2°C) flowing at 1 m/s via a glass water-jacketed tube (7 mm ID). The tube outlet was about 0.8 cm from the antennal preparation. The method of electroantennogram (EAG) recording was as described in Schmidt-Busser et al. (2011). The responsiveness of the antenna was tested with a 1 s air puff from 10µg of (Z)-3-hexenol on a filter paper strip in a 5ml stimulus syringe (Taneja and Guerin, 1997). The EAG responses of the antenna to hexane, heptane, octane, nonane, decane, dodecane and tetradecane were tested by injecting 0.1, 0.5 and 1 µL of pure alkanes on a filter paper strip already enclosed in the 5 mL stimulus syringe to prevent further evaporative loss of the volatile alkanes. Five to seven antennae from different individuals were tested.

Wind tunnel bioassay

The wind tunnel (flight section 60 x 60 x 195 cm) was constructed of non-reflecting glass. Air was blown by a centrifugal fan (Fischbach GmbH, Neunkirchen, Germany) through a section of charcoal cartridges at a flow rate of 30 cm/s. The resulting air was sucked by another fan and cleaned by an additional set of charcoal filters placed at the downwind end of the wind tunnel. Overhead illumination was provided by high frequency fluorescent lights (36W, >1kHz, Philips) running the length of the tunnel. Light was dispersed using a Perspex Prisma® crystal-clear plastic sheet under the fluorescent tubes and intensity was regulated with a potentiometer and brown paper to produce light to ca. 6 lux along the wind tunnel floor. Below the tunnel floor, black shapes of irregular form were placed on a white sheet as optomotor cues. The wind tunnel was housed in a walk-in climate chamber (Schaller Uto AG, Bern, Switzerland) that allowed the air stream to be maintained at 18 ± 0.2 °C and 85 ± 2% RH during experiments. Three to four day-old male moths were placed individually in glass tubes (125 mm long, 24.2 mm o.d., 21 mm i.d.) at the beginning of the scotophase, 15 min prior to experiments. Males moths were presented on a stand (25 cm high) placed in the centre of the wind tunnel and 25 cm from its downwind end. Moth behaviour was scored for (1) no activation, (2) activation, (3) take off, (4) upwind flight, (5) passing the midline of the wind tunnel, (6) close in within 10 cm of the source and (7) contacting the source (see Annexe 3

F) as well as the time to each behavioural step by means of the OBSERVER software package (version 5.0, Noldus Information Technology, Wageningen, The Netherlands). The behavioural response of males was recorded for two minutes. Males that were not activated within one minute or landed on a wall of the wind tunnel for a minimum of 5 s were removed from the wind tunnel. Moths that were not able to fly properly were discarded (less than 3% of the tested insects).

Odour release and air flow laminarisation

All treatments were released using a piezo sprayer modified from El-Sayed et al. (1999b). The ultrasound evaporator consisted of a syringe pump (Model KDS-200-CE, kdScientific Holliston, USA) that pumped the test solution at 10 µl/min from a 5 ml gas-tight syringe (Hamilton type 81527, Milian SA, Meyrin, Switzerland) containing a solution of known amounts of the test products into PTFE micro tubing (1.5 m long, 1.02 mm o.d., 0.56 mm i.d., Hamilton type 90674) connected by a PTFE micro tubing connector (2 cm long, 1.57 mm o.d., 0.97 mm i.d., Hamilton type 20919) to a borosilicate glass capillary (100 mm long, 1 mm o.d., type GC100-10, Clark Electromedical Instruments, Pangbourne, England) with a drawn out tip (10-20 mm tip length, 30-40 µm i.d. tip opening) and sealed with two layers of heat-shrink tubing. A frequency generator (Wavetek FG-5000A, Willtek Communications GmbH, Ismaning, Germany) producing a square-wave signal ca. 92 kHz of 40V in amplitude was connected to a piezo-ceramic disc (25 mm diameter, Philips PXE5 25/2.0) that held the glass capillary mounted on one face of the disc. This caused the capillary to oscillate and produce an aerosol of the test solution. The oscillating glass capillary was installed in an aperture in the upwind honeycomb structure (see below). The capillary tip was protected from the approaching moths by a small metal grid (see below) that was cleaned after exposure to each treatment (Figure IV.1).

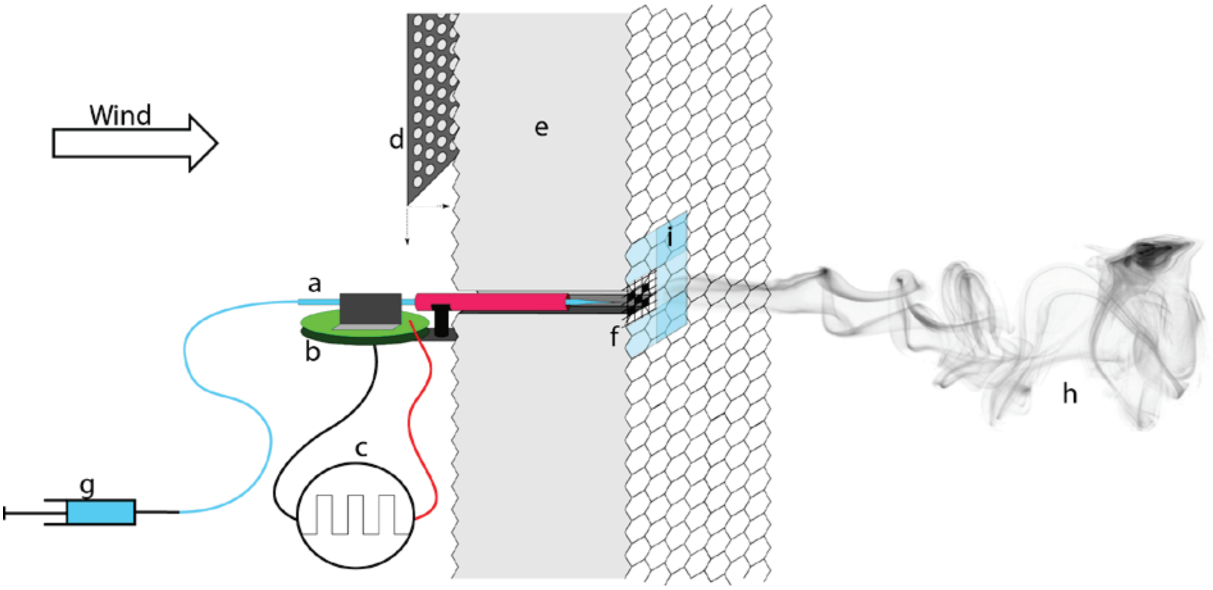


Figure IV.1 Setup of the piezoelectric sprayer and air flow laminarisation system. The piezo sprayer includes a glass capillary (a) mounted on a ceramic chip (b) that vibrates when voltage is applied to both faces using a frequency generator (c). A metallic cylinder (red) protects the capillary. The sprayer mounted on a perforated grid (d) is screwed to a honeycomb structure (e) which renders the air flow laminar. The downwind surface of the honeycomb structure is covered with mosquito netting (not shown), except for the aperture containing the capillary where a metal grid sits (f). A syringe pump (g) pushes the test solution into the capillary via a Teflon tube. Oscillations of the capillary tip serve to nebulize the test solution in the wind tunnel (h). A frame of sticky tape (19 mm wide, i) is placed around the metal grid to generate localized turbulence locally to increase the plume diameter to 10 cm at ca. 8 cm from the source.

The attraction of males to calling *L. botrana* females was tested by placing 4 virgin females in a 110 mm long 26 mm i.d. glass tube closed with a honeycomb (12 mm thick, 5 mm cell size) on its downwind end and with cotton wool (30 mm thick) at its upwind end. This tube was connected to an external charcoal-filtered air supply flowing at 6 L/min through a 6 mm i.d. Teflon tube providing an output flow of 30 cm/s through the 26 mm diameter outlet.

The wind tunnel laminar air flow was generated using an aluminium honeycomb structure (9.525 mm cell diameter, 5.7 x 60 x 60 cm) covered with a perforated steel screen (1mm thick, 3mm round holes, 51% of air passage, Schäfer, Neunkirchen, Germany) on its upwind surface and with a white cotton netting (1 mm mesh) on its downwind side to prevent moths from flying through the honeycomb. A 1 cm diameter aperture was perforated at the centre of the structure to accommodate the glass capillary tip. A metal grid (15 x 15 mm, mesh 2 mm) was placed on the plume exit from the aperture. Plume structure is critical on the performance of moths to undertake upwind flight. Mafra-Neto and Cardé (1994) demonstrated that a large turbulent plume induced more source contact by moths than a continuous ribbon plume. To increase the plume size, a frame of transparent sticky tape (19 mm wide) bordered the metallic grid. This generated turbulence locally around the plume to increase its diameter to ca. 8 cm at 10 cm from the release point (Figure IV.1). The resulting plume reached the downwind end of the wind tunnel at its centre with a diameter of ca. 15cm.

Treatments tested

L. botrana

In a first step, the ternary sex pheromone blend (TB) of E7Z9-12:Ac, E7Z9-12:OH and Z9-12:Ac at 100:20:5 was presented to male *L. botrana* at 0.1 pg/min, 1 pg/min and 10 pg/min of the most abundant component to determine its optimal concentration. Secondly, an underdosed level of TB of 0.1 pg/min was added to dilutions of heptane and octane released at 100 pg/min, 10 ng/min, 1 pg/min in ethanol and as pure products. Nonane was only tested at a release rate of 100 pg/min because of its low solubility in ethanol. 2-Phenylethanol was tested at 100 pg/min in combination with the underdosed pheromone in ethanol. Finally, the most attractive dose of heptane (10 ng/min) was admixed to 2-phenylethanol (100 pg/min) and the underdosed pheromone in ethanol. Pure heptane, octane, nonane, ethanol, a dilution of 2-phenylethanol released at 100 pg/min in ethanol and 10ng/min heptane in ethanol were tested as controls. Between 29-157 males were tested per treatment.

C. pomonella

Codlemone was tested at 1 pg/min and 100 pg/min to estimate an underdosed concentration to attract male *C. pomonella* to which to add test products. Heptane was released at 10 ng/min or in pure from and 2-phenylethanol was released at 100 pg/min admixed to the underdosed sex pheromone (1 pg/min). A ternary blend of heptane released at 10ng/min, 2-phenylethanol at 100 pg/min and underdosed codlemone in ethanol was also tested. Pure heptane and ethanol were tested as controls. Between 58-78 males were tested per treatment.

Statistical analysis

Comparisons between treatments within behavioural elements of the response of *L. botrana* males were performed by fitting general linear models (GLM) with a logit link function. Analysis of deviance based on the asymptotic distribution was used to test whether behavioural responses were dependent on treatments. Multiple comparisons were made by applying Tukey-contrasts when GLMs were significant ($P < 0.05$). When treatments attracted all moths or no moths within a behavioural element, an individual was removed or one was added, respectively, to make comparisons possible. Time-event analyses were performed by applying Cox proportional hazard regressions within the behavioural elements upwind flight and contact. A generalized estimation equation model with an exchangeable correlation matrix structure (Zeger and Liang, 1986) was applied to analyse the evolution of attraction within a treatment. All the data were analysed with the statistical package R 2.15.1 (R Core Team, 2012).

Results

Electroantennogram responses of *L. botrana* males to alkanes

The sensitivity of the antennal olfactory cells to hexane, heptane, octane, nonane, decane, dodecane and tetradecane were compared in EAG recordings from *L. botrana* males (Figure IV.2). Antennae responded to all tested alkanes in a dose dependent manner, except for tetradecane as this hydrocarbon is only slightly volatile at ambient temperatures. The sensitivity of antennae was lower for the smaller and bigger alkanes with a maximal sensitivity for heptane, octane, nonane and decane which evoked about the same EAG responses as the positive control at the highest dose tested.

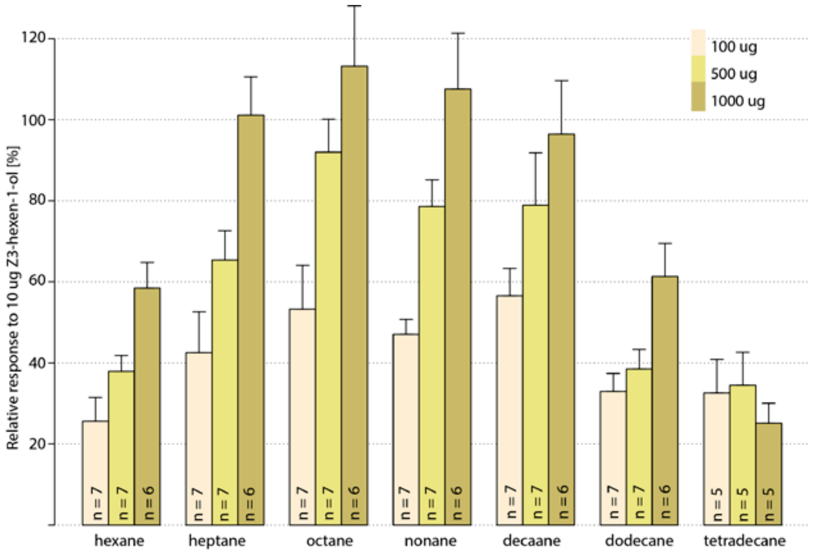


Figure IV.2 Mean ± SEM electroantennogram responses of *L. botrana* male antennae to alkanes at 100, 500 and 1000 µg source doses. Responses are normalized using 10 µg of Z3-hexen-1-ol as reference (100 %). N is the number of individual antennae tested.

Behavioural responses of *L. botrana* to its pheromone in combination with alkanes and 2-phenylethanol

Firstly, the adequate dose of the pheromone TB to be tested with other products in mixtures was established. Males responded to the TB in a dose dependant manner in the range tested (0.1, 1, 10 pg/min). At the optimal dose of 1 pg/min, 63% of males reached the source of pheromone, significantly higher than at the lower dose of 0.1 pg/min (46%) or at the higher dose of 10 pg/min (47%; Figure IV.3). At the lower dose, moths were less attracted than at the optimal dose for all behavioural steps. At 10 pg/min, the TB appears to be overdosed since the level of males responding falls gradually from take-off in comparison to the 1 pg/min dose and reaches the same level as with underdosed TB for moths arriving at the source. However, the TB was significantly less efficient than calling females from the casting phase of flight to source contact (78%). The underdosed concentration of 0.1pg/min was selected to test the effect of adding of alkanes and 2-phenylethanol in an attempt to increase pheromone attraction to males.

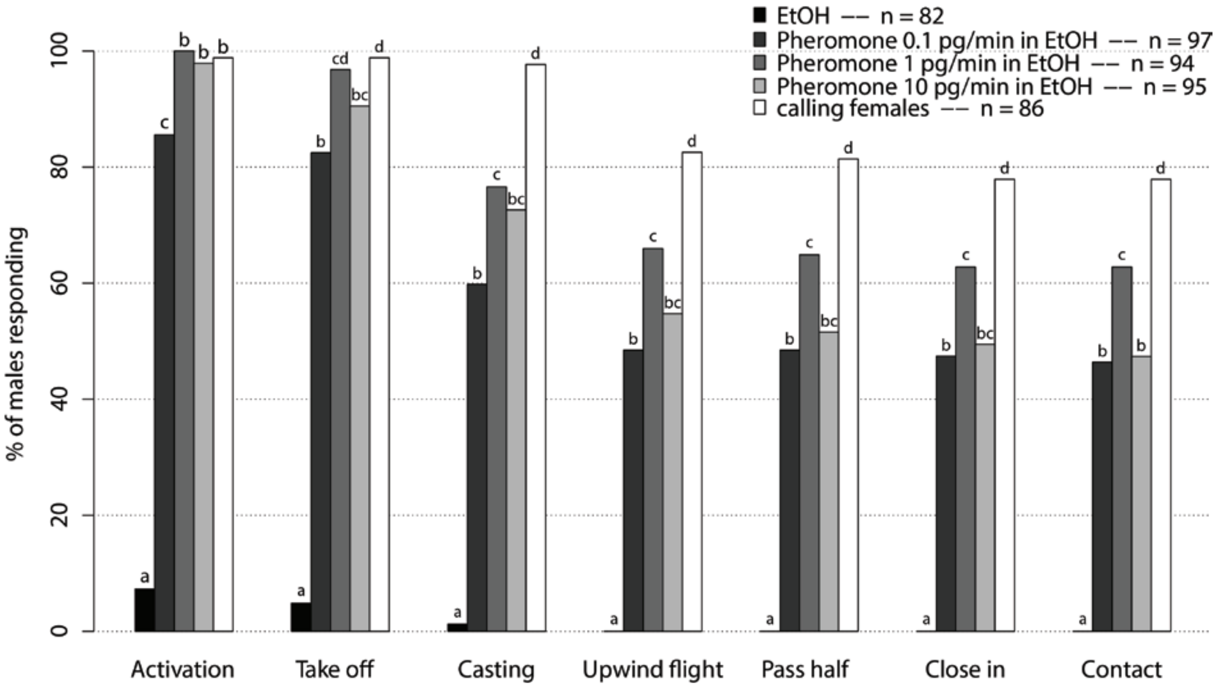


Figure IV.3 Behavioural responses of grapevine moth males to 3 concentrations of the TB (0.1 pg/min, 1 pg/min, 10 pg/min) diluted in ethanol (grey), to ethanol alone (black) and to 1-4 calling females (white). Letters assigned within a behavioural element indicate statistically significant differences (GLM, $P < 0.05$); n is the number of moths tested for each treatment.

When heptane was added at a release rate of 100pg/min to the underdosed TB, the number of males achieving a behaviour increased at all steps, and especially contacting the source, was significantly higher than the underdosed pheromone (46% to 60%,Figure IV.4). At higher doses of 10 ng/min and 1 µg/min of heptane combined with the underdosed TB, significantly more males reached the source (76% and 74%, respectively). The latter treatments were as attractive as calling females (78%). When the TB was diluted directly in heptane, 45% of males reached the source, no different to the same concentration of pheromone in ethanol. However, this mixture was significantly more efficient in recruiting moths at the take-off and casting behavioural steps compared to the same level of pheromone in ethanol. While heptane did not elicit attraction by itself, moths were more likely to be activated (ethanol 7%, heptane 17%) and to take off (ethanol 5%, heptane 9%) than with ethanol alone (Figure IV.3 and Figure IV.4).

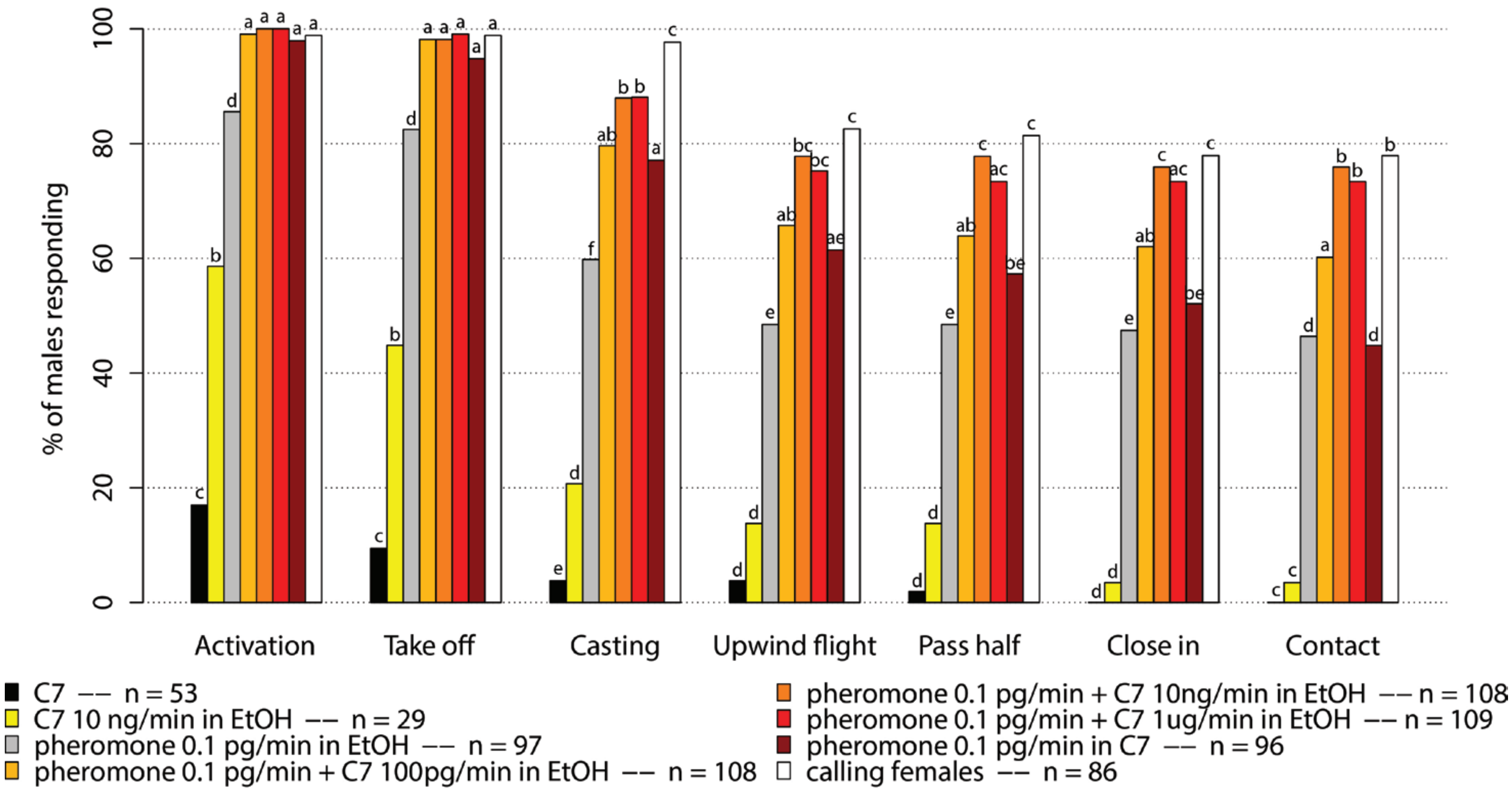


Figure IV.4 Behavioural responses of grapevine moth males to underdosed TB (0.1 pg/min) in ethanol (grey), to 3 concentrations of heptane released at 100 pg/min, 10 ng/min and 1 μg/min and pure with underdosed TB diluted in ethanol (orange to red), to the underdosed TB diluted in heptane (brown), to heptane alone (black), to 10 ng/min heptane in ethanol (yellow) and to 1-4 calling females (white). Letters assigned within a behavioural element indicate statistically significant differences (GLM, $p<0.05$); n is the number of moths tested for each treatment.

We choose to test the effect of 2-phenylethanol on the attraction of *L. botrana* males as it occurs in several host plants of this species. 2-Phenylethanol released at 100 pg/min in ethanol did not attract males, but 27% of them took flight (Figure IV.5). When the same release rate was added to the underdosed TB all the behavioural steps were increased and reach up to 66% of contact at source, significantly higher than to the pheromone alone (46%). When 2-phenylethanol was added to the TB combined with 10 ng/min of heptane, slightly more moths reached the source than without 2-phenylethanol (83% vs 76%) but not in significantly higher numbers. However, this blend recruited significantly more males during the casting phase (96%) than without 2-phenylethanol (88%) and was as performant as calling females (98%) for this behavioural element.

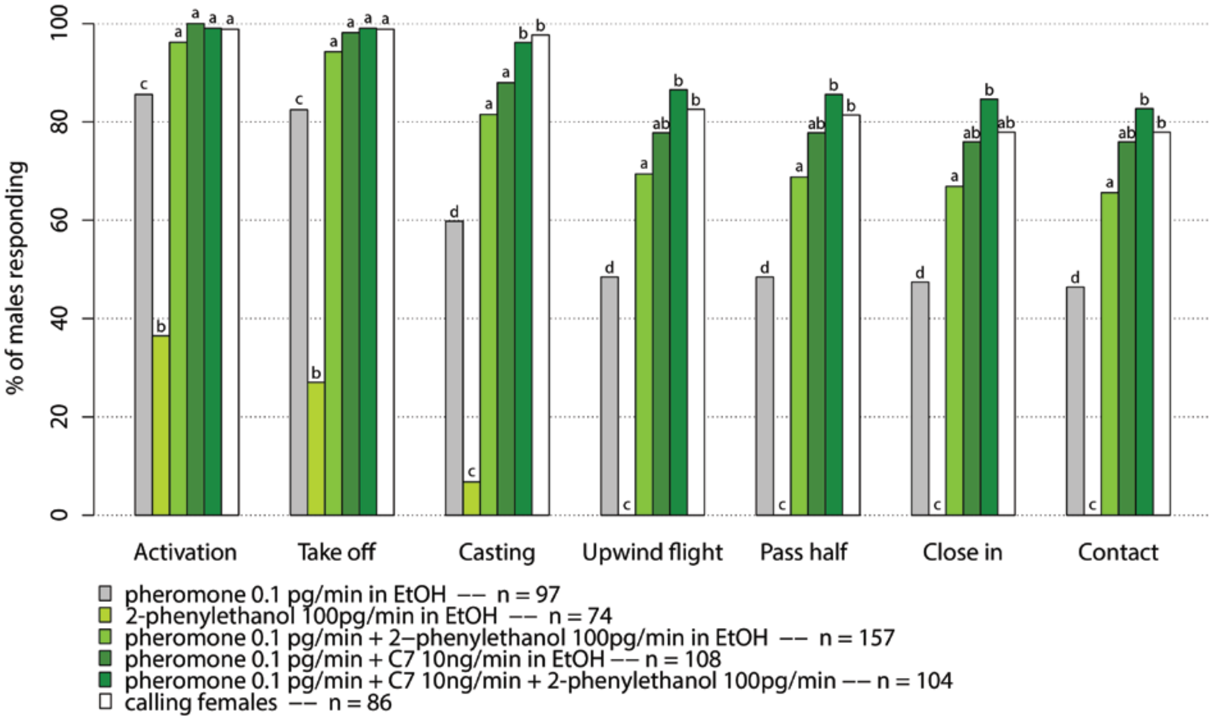


Figure IV.5 Behavioural responses of grapevine moth males to 2-phenylethanol released at 100 pg/min in ethanol (light green), with the underdosed TB at 0.1 pg/min in ethanol (green), or admixed to heptane at 10 ng/min and to the underdosed TB in ethanol (dark green), to underdosed TB (grey), to underdosed TB combined with heptane at 10 ng/min (forest green) and to 1-4 calling females (white). Letters assigned within a behavioural element indicate statistically significant differences (GLM, *P* <0.05); n is the number of moths tested for each treatment.

When octane was added to the underdosed TB a similar pattern of responses as to mixtures with heptane was observed (Figure IV.6 and Figure IV.7). As for heptane, octane was not attractive on its own but was more likely to activate males and induce them to fly. Octane significantly increased the attraction rate of males as it affected more moths in all behavioural steps and increased the proportion of moth contacting the source from 46% to 63% when released at 100pg/min with the underdosed TB (Figure IV.7). The effect of octane was increased even more by elevating the dose of octane to reach a maximum of 91% moths casting and 79% of males contacting the source at a release rate of 10 ng/min octane, not significantly different to calling females. The response decreased at higher doses of octane and the final approach was almost destroyed when the TB was added to pure octane with 24% of males arriving within 10 cm of the source but only 10% contacted it. When nonane was added to the underdosed TB at a release rate of 100 pg/min it significantly increased the proportion of males contacting the source (58%) compared to the same dose of pheromones alone (46%). Nonane was not soluble in ethanol for testing at higher doses.

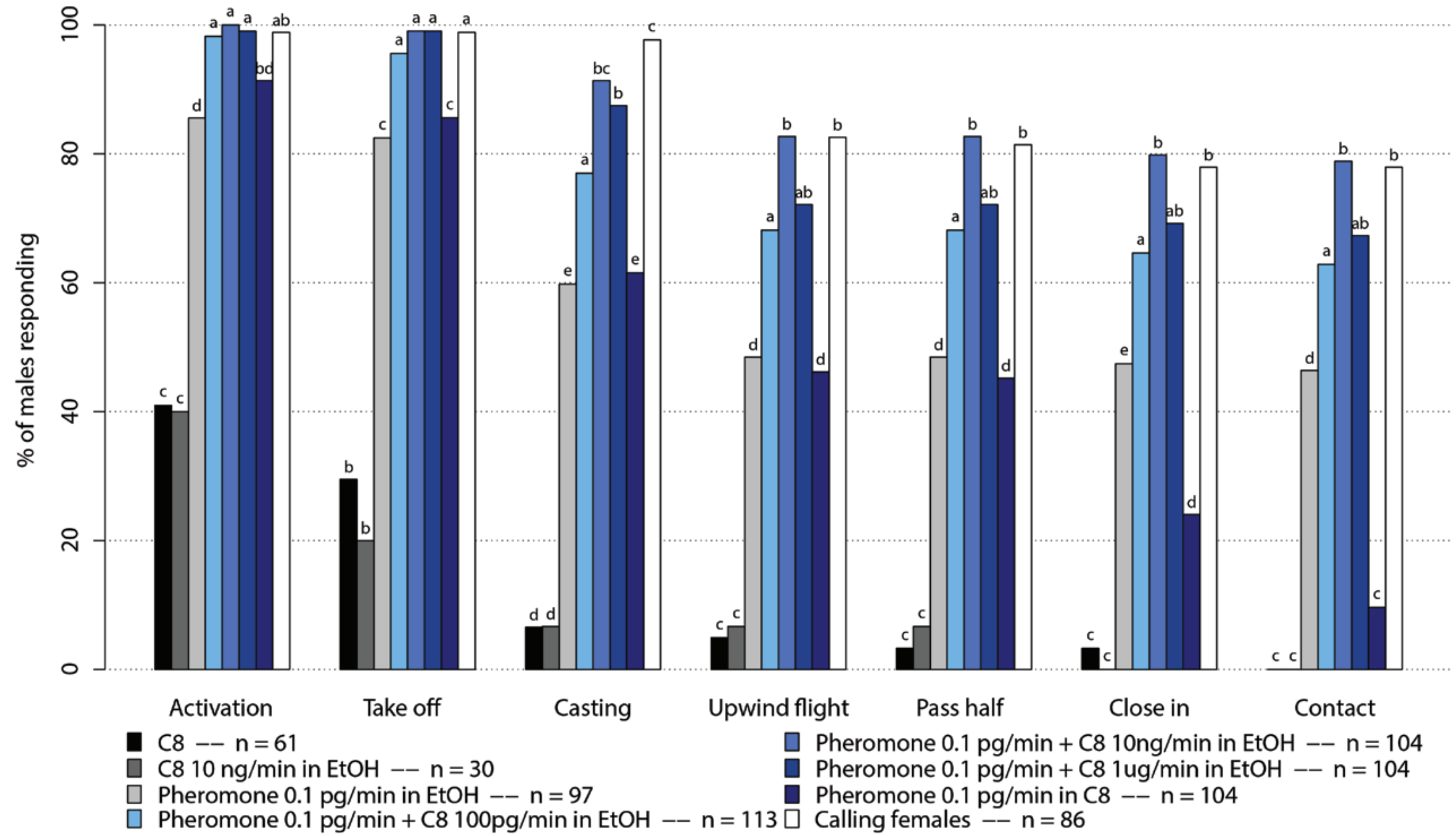


Figure IV.7 Behavioural responses of grapevine moth males to octane alone (black), to octane at 10 ng/min in ethanol (darkest blue), to the underdosed TB in ethanol (grey), to 3 release rate of octane (100 pg/min, 10 ng/min, 1 µg/min) with an underdosed TB (0.1 pg/min) diluted in ethanol (light blue, blue dark blue, respectively), to underdosed TB in octane (navy blue), and to 1-4 calling females (white). Letters assigned within a behavioural element indicate statistically significant differences (GLM, $P < 0.05$); n is the number of moths tested for each treatment.

Moths were highly sensitive to all the treatments, except for the controls without pheromone, as at least 86% were activated and almost all of them took flight (>82%). However, significantly fewer moths undertook casting (Generalized estimation equation model, $P < 0.05$) except to calling females and to 2-phenylethanol in combination with heptane. Moths that lost the plume flew either downwind or to the ceiling of the wind tunnel. The remaining moths that flew upwind in the plume of odour reached the source of pheromone, as there was no difference between the proportion of moths flying upwind and those which contacted the source (Generalized estimation equation model, $P < 0.05$). Attraction of *L. botrana* males to all treatments was highly repeatable across flight sessions as there was only little variation between days (Figure IV.8).

Table IV.1 Relative speeds of the responses to TB and codlemone *of L. botrana* and *C. pomonella* males beginning to fly upwind, i.e. lock on to the plume, and contact the source in response to their pheromones and plant volatiles. Values in yellow indicate positive relative speeds and blue highlights negative relative speeds compared with TB released at 0.1 pg/min (Cox proportional hazard regressions).

L. botrana	**relative speed up to begin upwind flight**	**P value**	**relative speed up to source contact**	**P value**
TB 0.1 pg/min	1	NA	1	NA
TB 1 pg/min	1.59	<0.05	1.56	<0.05
TB 10 pg/min	1.18	0.44	1.03	0.9
TB 0.1 pg/min + C7 100 pg/min	1.64	<0.01	1.39	0.09
TB 0.1 pg/min + C7 10 ng/min	2.17	<0.001	1.92	<0.001
TB 0.1 pg/min + C7 1 µg/min	1.92	<0.001	1.89	<0.001
TB 0.1 pg/min in C7	1.37	0.12	0.93	0.73
TB 0.1 pg/min + C8 100 pg/min	1.61	<0.01	1.41	0.08
TB 0.1 pg/min + C8 10 ng/min	2.33	<0.001	2.22	<0.001
TB 0.1 pg/min + C8 1 µg/min	1.82	<0.01	1.59	<0.05
TB 0.1 pg/min in C8	0.86	0.47	0.15	<0.001
TB 0.1 pg/min + 2-phenylethanol 100pg/min	1.67	<0.01	1.54	<0.05
TB 0.1 pg/min + 2-phenylethanol 100pg/min + C7 10 ng/min	2.27	<0.001	1.96	<0.001

C. pomonella				
Codlemone 1 pg/min	1	NA	1	NA
Codlemone 100 pg/min	3.15	<0.001	3.18	<0.001
Codlemone 1 pg/min + C7 10 ng/min	1.93	<0.01	1.84	<0.05
Codlemone 1 pg/min + 2-phenylethanol 100 pg/min	1.92	<0.01	1.55	0.09
Codlemone 1 pg/min + C7 10 ng/min + 2-phenylethanol 100 pg/min	2.62	<0.001	2.18	<0.01
Codlemone 1 pg/min in C7	1.6	0.06	0.86	0.61

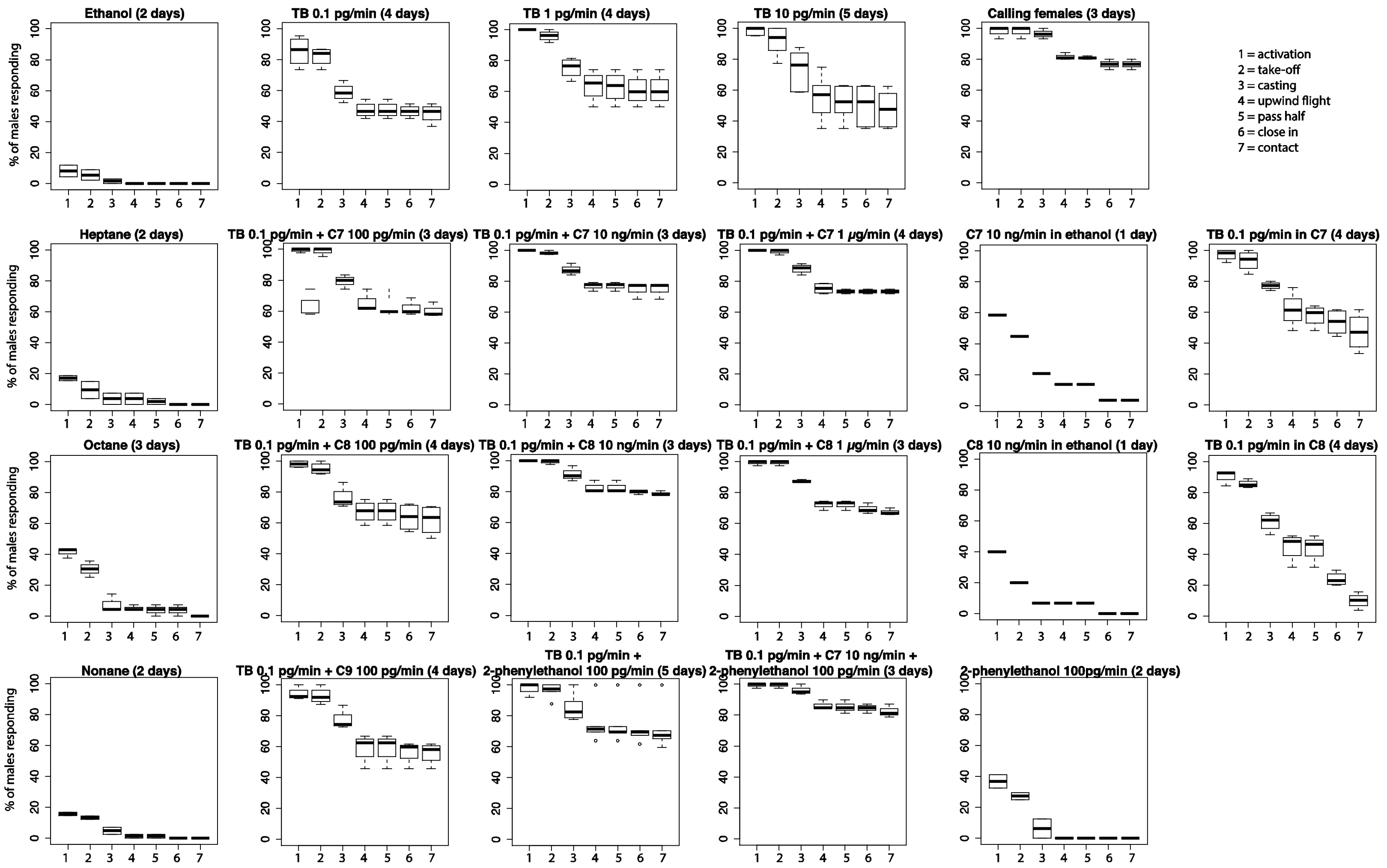


Figure IV.8 Consistency of the response across days of experiments with *L. botrana* males responding to their sex pheromone alone (TB) and in combination with plant volatiles. For explanation of behavioural criteria 1-7, see Materials and Methods.

Males *L. botrana* lock on the plume and begin to fly towards the source of pheromone released at the optimal dose of 1 pg/min 1.59 times faster than toward the underdosed TB (0.1 pg/min) and overdosed (10 pg/min) pheromone (Figure IV.9A,Table IV.1). Consequently, moths contacted the source 1.56 times faster than the underdosed TB (Figure IV.9B). Dilutions of heptane with the underdosed TB increased the speed of the response of the moths to upwind flight by a factor from 1.64 to 2.17 times compared to the underdosed TB (Figure IV.9C). Moreover, males contacted the source up to 1.92 times faster in response to the two highest release rates of heptane (10 ng/min and 1 µg/min heptane; Figure IV.9D). In presence of dilute octane with the underdosed TB males flew towards the plume and reached the source faster than in presence of the underdosed TB (Figure IV.9E & F). Octane released at 10 ng/min with underdosed TB was not only the most effective octane dilution but elicited the fastest response with moths arriving at the source 2.22 times faster than with underdosed TB. Both heptane and octane used as solvent with the underdosed TB, i.e. TB in heptane or octane as solvent, elicited as many upwind flights as the underdosed TB in ethanol and the timing of behavioural steps were the same. When 2-phenylethanol at a release rate of 100 pg/min was added to the underdosed pheromone it increased the speed at which moths found the plume (1.67 times, Figure IV.9G) compared to the underdosed pheromone, as well as when this blend was admixed with heptane released at 10 ng/min (2.27 times) and they also arrived at the source faster (Figure IV.9H). The females were not compared to the underdosed TB as the plume structures were different.

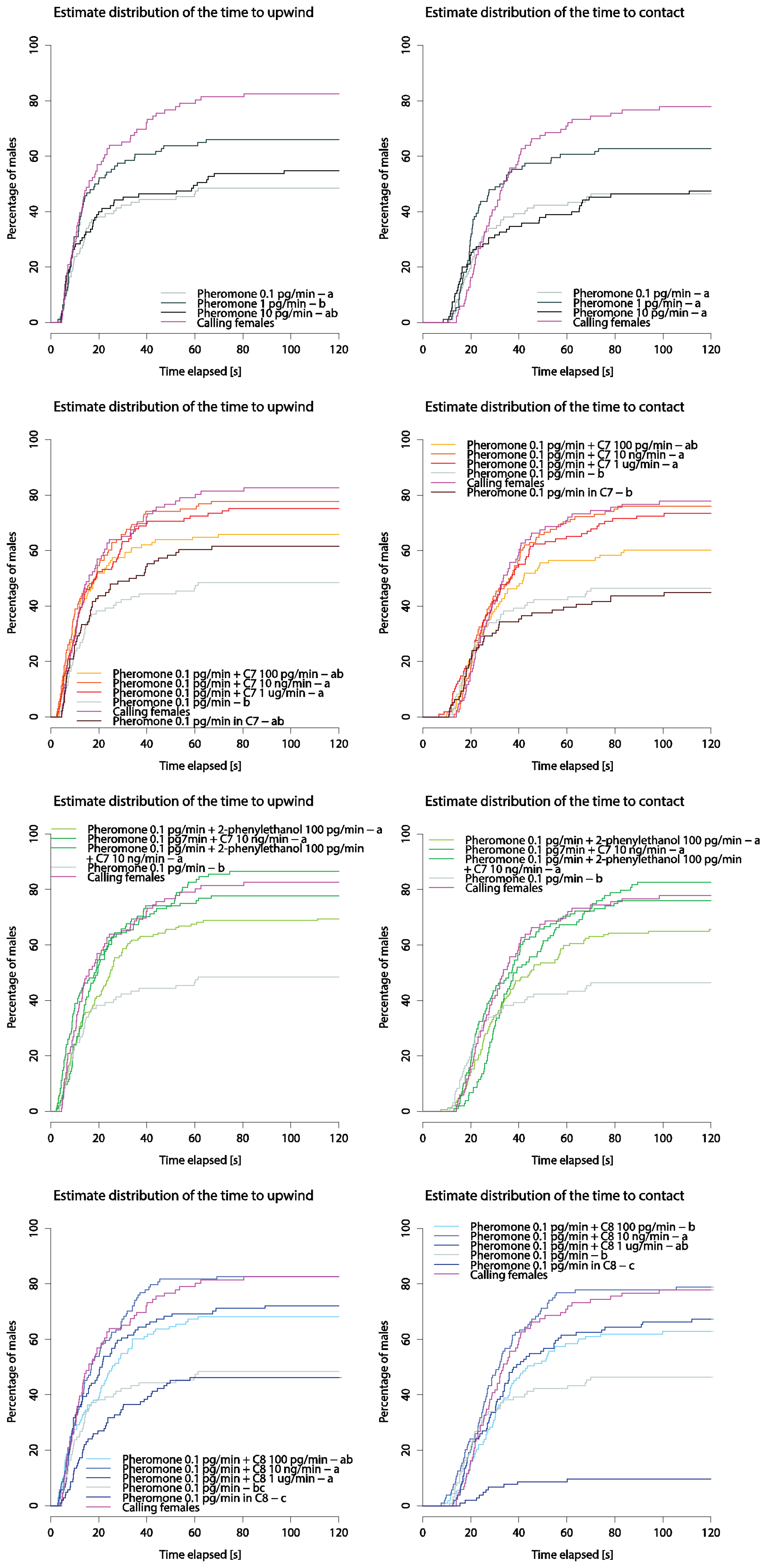


Figure IV.9 Cumulative percentage of male *L. botrana* locking on the plume of pheromone (A,C,E and G) and contacting the source (B, D, F and H) over a time period of 120 s in response to TB at 0.1, 1 and 10 pg/min (A and B), to TB:heptane mixtures (C and D), to TB:octane mixtures (E and F) and to TB:2-phenylethanol mixtures (G and H). Letters assigned to a treatment indicate significant differences (Cox proportion hazard models followed by a Tukey contrast, P<0.05).

Behavioural responses of *C. pomonella* to its pheromone in combination with heptane and 2-phenylethanol

We first set out to establish the adequate dose of codlemone to combine with the test products. Males *C. pomonella* responded to codlemone presented in ethanol in a dose dependant manner. Almost all males were activated and took off at the low dose of 1 pg/min, but the proportion of moths casting sharply decreased to 63%. The optimal dose of 100 pg/min codlemone elicited more casting and 85% of the males were attracted to the source, ca. 2 times more than at the underdosed codlemone release rate of 1 pg/min (45%; Figure IV.10). When heptane was added at a release rate of 10 ng/min to underdosed codlemone in ethanol, moths were more likely to be attracted by the treatment as it induced significantly more castings than the pheromone alone (85% vs. 64%, $P < 0.01$) and subsequently more moths reached the source of pheromone (69%, $P < 0.01$). When underdosed codlemone was diluted directly in heptane, moths were also more likely to fly upwind than with the pheromone diluted in ethanol as more males passed halfway in the wind tunnel (63% vs. 45%, $P < 0.05$). However, contact at the source was inhibited and only as many arriving at the source as with pheromone presented in ethanol was recorded (Figure IV.10).

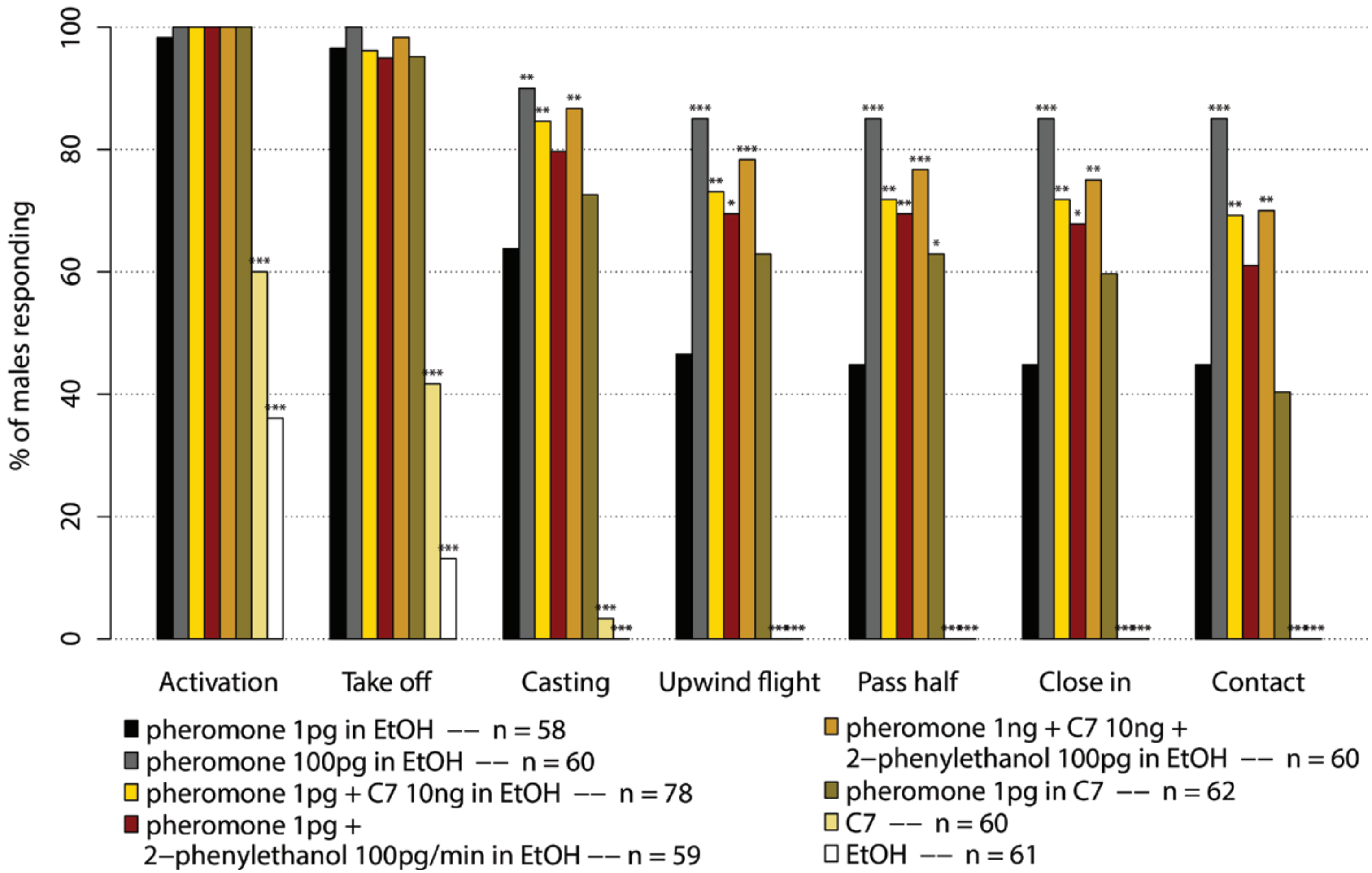


Figure IV.10 Behavioural responses of *C. pomonella* males to underdosed codlemone (released at 1 pg, black), to an optimal rate of codlemone release (100 pg/min, dark grey) diluted in ethanol, to codlemone released at 1 pg/min admixed to heptane at 10 ng/min (gold), to codlemone at 1 pg/min admixed to 2-phenylethanol at 100 pg/min (dark red), to codlemone at 1 pg/min admixed to heptane at 10 ng/min and 2-phenylethanol at 100 pg/min (orange), to codlemone at 1 pg/min diluted in heptane (bronze), to heptane alone (light yellow) and to ethanol alone (white). Asterisks assigned within a behavioural element indicate statistically significant differences to codlemone released at 1 pg/min in ethanol (GLM); n is the number of moths tested for each treatment.

The blend of 2-phenylethanol released at 100 pg/min with underdosed codlemone induced an increase in attraction of *C. pomonella* through more upwind flights by males than to the codlemone alone (69% vs. 47%, $P < 0.05$, Figure IV.10). However, contact with the source was not significantly higher than with codlemone alone (61% vs. 45%, $P = 0.081$). When heptane, released at 10 ng/min and 2-phenylethanol released at 100 pg/min were added to underdosed codlemone, attraction of the moths to the source was improved compared to the pheromone alone as more moths performed castings (80% vs. 64%, $P < 0.01$) and reached the source of odour (70% vs. 45%, $P < 0.01$, Figure IV.10). However, this ternary blend was not significantly more attractive than heptane released at 10 ng/min with codlemone.

Moths were slightly activated by ethanol (36%) and heptane (60%) alone. However, neither of these treatments induced the males to fan their wings prior to take-off and the time to activation was higher than to all the treatments with codlemone (ethanol = 33.0 ± 4.0 s; heptane = 22.6 ± 2.5 s; codlemone 1 pg/min = 2.1 ± 0.7 s). While time to activation of individual moths in presence of ethanol alone is uniformly distributed over time (Kolmogorov-Smirnov, $D = 0.147$, $P = 0.82$), the distribution of the time to activation of individual moths exposed to heptane alone is significantly different to a theoretical uniform distribution (Kolmogorov-Smirnov, $D = 0.306$, $P < 0.05$) and shorter than for ethanol. Among the moths that took off, none of them flew upwind to these two treatments.

As for *L. botrana*, codlemone highly activated *C. pomonella* (>98%) and most of them took off (>95%). However, the proportion of casting moths was significantly reduced as well as the proportion of males which locked on to the plume at the underdosed codlemone level, except for codlemone diluted in heptane (Generalized estimation equation model, $P < 0.05$). Almost all moths flying upwind reached the source of underdosed codlemone, optimal codlemone dose and to the mixture of underdosed codlemone plus heptane released at 10 ng/min. The proportion of moths making contact with the source dropped for mixtures including 2-phenylethanol and when heptane was used as a solvent (Figure IV.10).

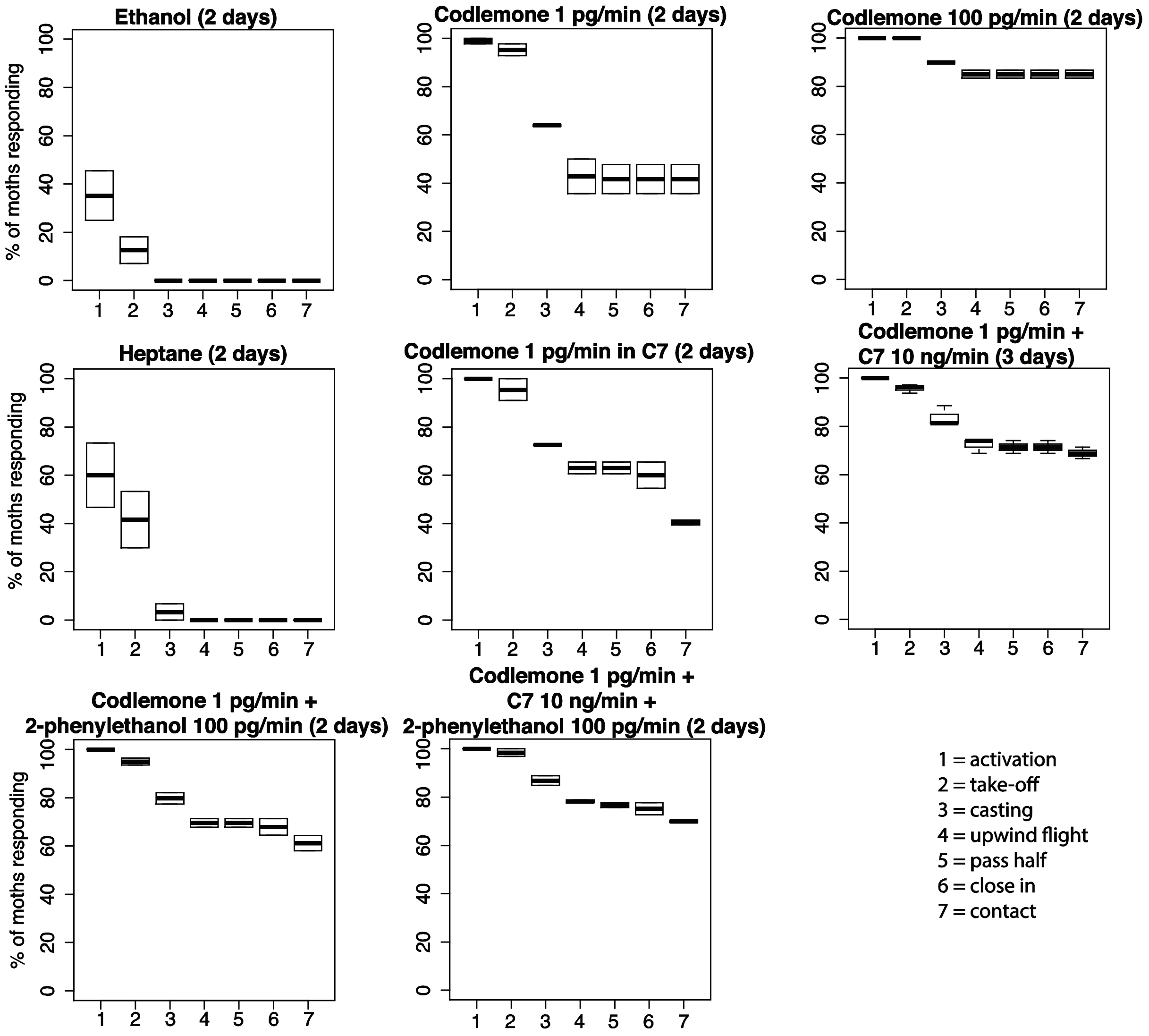


Figure IV.11 Consistency of the response across days in experiments with *C. pomonella* males responding to codlemone alone and in combination with plant volatiles. For explanation of behavioural criteria 1-7, see Materials and Methods.

Attraction of *C. pomonella* males to all treatments was highly repeatable across flight sessions as there was only little variation between days (Figure IV.11).

C. pomonella responding to codlemone released at 100 pg/min, to underdosed codlemone in combination with dilute heptane and/or with 2-phenylethanol locked on the plume from 1.92 to 3.15 times significantly faster than to the underdosed codlemone alone (Table IV.1 and Figure IV.12). Moreover, these moths also contacted the source significantly faster, except for the underdosed codlemone plus 2-phenylethanol released at 100 pg/min. When codlemone was directly diluted in heptane males did not respond faster than to underdosed codlemone in ethanol.

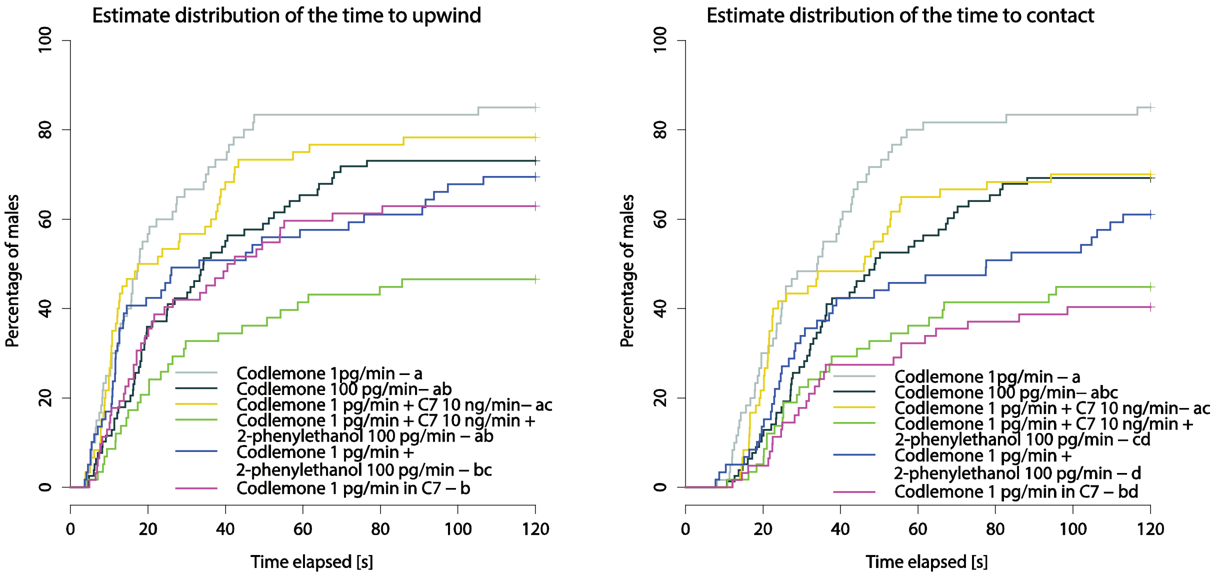


Figure IV.12 Cumulative percentage of male *C. pomonella* locking on the plume (A) and contacting the plume (B) over a time period of 120 s in response to codlemone and codlemone:plant volatile mixtures. Letters assigned to treatments indicate significant differences (Cox proportion hazard models followed by a Tukey contrast, P<0.05).

Discussion

This study focuses on the effect of volatile alkanes and 2-phenylethanol on the attraction of *L. botrana* and *C. pomonella* males to their sex pheromones in a wind tunnel. We show that *L. botrana* can perceive short-chain alkanes through EAG recordings. Dilute blends of pheromone with heptane increases the attraction of *L. botrana* and *C. pomonella* to their sex pheromones in a dose dependant manner. While heptane used as a solvent with pheromone is only as effective as ethanol at inducing source contacts, it shows greater efficiency in recruiting moths in the initial flight phases. When used as a solute with pheromone in ethanol, octane increased the overall attraction level in *L. botrana*. We have also demonstrated that the host plant volatile 2-phenylethanol increases the attraction of both species to their sex pheromone. Moreover, all these plant volatiles shortened the time to react to the pheromone for both species.

Alkane perception by phytophagous insects and occurrence in plants

Volatile alkanes are produced by numerous plants, including host plants of *L. botrana* and *C. pomonella* such as the grape *Vitis vinifera*, apple *Malus domestica*, pear *Pyrus communis* and cherries *Prunus mahaleb* (Baraldi et al., 1999; Bengtsson et al., 2001; Mastelic et al., 2006; Tasin et al., 2005). Alkanes from undecane to heneicosane are often detected over plants using headspace collection followed by solvent desorbtion. However, smaller alkanes may evaporate with the solvent and are probably not detected in such samplings. Other techniques such as thermal desorbtion, which requires no solvent, reveals smaller alkanes including heptane and octane in several pome fruits (Baraldi et al., 1999), and we show here that these molecules evoke EAG responses in *L. botrana* males in a dose dependent manner. Tasin et al. (2005) also recorded substantial EAG responses to C_{15}-C_{21} in host plant headspace extracts from female *L. botrana* antennae with gas chromatography-linked EAG, but these compounds evoked only marginal EAG responses from female *C. pomonella* (Bengtsson et al., 2001). On the one hand, short chain alkanes are usually released in quite low quantities by plants and as such are not really plant species specific. Furthermore, the detection threshold of moth antennae is rather high and not tuned to a particular hydrocarbon in *L. botrana*. Therefore, alkanes could be mimicking the effects of other plant volatiles, as shown in the yellow fever mosquito, *Aedes aegypti*, where heptane vapour generates strong responses from carbon dioxide receptors on the maxillary palps (Kellogg, 1970), or specific receptors for alkanes may be located on other organs.

Alkanes influence male *L. botrana* and *C. pomonella* flight responses to their sex pheromones and shorten their reaction time

Attraction of the grapevine moth was tested to a ternary pheromonal blend of *L. botrana* at 3 concentrations and to the pheromone released from calling females. Moth were more attracted to the medium concentration of TB released at 1 pg/min as lower and higher release rates appeared to be under- and over-dosed, respectively. El-Sayed et al. (1999c) obtained a similar dose response curve with an optimum at the same release rate. However, none of the release rates tested here were as efficient as calling females. Even though the ternary blend is sufficient to induce robust responses of males, the olfactory message seems to be incomplete or the ratio between compounds may be suboptimal resulting in males having trouble to orientate in the plume (Witzgall and Arn, 1990). On the other hand, the difference between the ternary blend and females could be partly ascribed to dissimilarity in the pheromone plume generated by the two release methods.

The attraction of *C. pomonella* males to codlemone was tested at two doses. The higher dose of 100 pg/min attracted 2 times more males than a 100 fold less dose. Schmera and Guerin (2012) recorded an attraction optimum at the same concentration in the wind tunnel, but the response decreased more sharply at lower doses with less than 10% of moths contacting the source at 1 pg/min. The optimal release rate of codlemone is almost achieved at 100 pg/min as it attracted 85% of males. El-Sayed et al. (1999a) showed that 1 ng/min of codlemone released from a piezo sprayer was as attractive as a single calling female in a two-choice experiment in a wind tunnel. Calling females release codlemone at a rate of 7 ng/hr (Backman et al., 1997).

We chose to study the interaction between pheromones and hydrocarbons by using underdosed pheromone concentrations as it would have been difficult to observe any additional effect of the hydrocarbons where optimal doses of pheromone already attract a high proportion of males. Dilute heptane, octane and nonane enhanced the attraction of male *L. botrana* to underdosed pheromone at all the behavioural steps and up to 1.7 fold more moths contacted the source when the alkanes were released at 10 ng/min, levels of attraction equal to that of calling females. Consequently, the effect of dilute heptane on the attraction of *L. botrana* to its pheromone may be underestimated, considering that the response already reaches over 75%, a level that is difficult to exceed in the wind tunnel, even with calling females. In the same way, heptane increased the response of the codling moth males to codlemone by 1.6 fold also at a release rate of 10 ng/min. Comparably, host plant volatiles such as pear ester or β-farnesene released at 100pg/min increase the response of codling moth by ca. 1.7 fold in a wind tunnel (Schmera and Guerin, 2012). Moreover, (E)-β-caryophyllene released at 100 pg/min increases the attraction of the pheromone by 1.7 fold for *L. botrana* (von Arx et al., 2012b).

Heptane was not different to ethanol when used as solvent to dilute the pheromone for *L. botrana* as well as for *C. pomonella*. This was not the case for octane where *L. botrana* stopped its upwind flight behaviour before reaching the source. Such flight arrestment behaviour was already observed in *E. ambiguella* (Schmidt-Buesser et al., 2009) and *C. pomonella* (Witzgall et al., 2001) with overdosed pheromone. Such a turbulent plume generated here is sensed by the flying insect as intermittent bursts of odour whose density is higher near the source (Kaissling, 1997; Murlis and Jones, 1981), thus the concentration of octane in the vicinity of the source may be too high.

Heptane and octane were not attractive on their own and thus may act as pheromone synergists. Host plant volatiles have already been established to enhance the behavioural response of males to sex pheromones in moths species. Compounds such as (E)-β-caryophyllene, (Z)-3-hexenyl acetate and 1-hexanol induce only low levels of attraction of *L. botrana* males when released at 100 pg/min (von Arx et al., 2011) but increase the attraction to pheromone by up to 1.7 fold (von Arx et al., 2012b). Similarly, linalool, (Z)-3-hexen-1-ol and (E)-β-farnesene are not attractive for *C. pomonella* males but enhance its response to codlemone by about 2 fold when released at 100 pg/min (Yang et al., 2004). The synergistic effect may occur at level of the pheromone-specific olfactory neurones. Ochieng et al. (2002) showed that the neurones specific for detection of the sex pheromone in the corn earworm *Helicoverpa zea* males is not sensitive to host plant compounds, but its response to codlemone was increased in presence of plant volatiles. Moreover, plant volatiles increase the response to pheromone of the macroglomerular complex which is dedicated to pheromone perception in the antennal lobe of *C. pomonella* (Trona et al., 2013).

The plant volatile 2-phenylethanol was also tested with the moth pheromones. This compound occurs in many plants species, including host plants of *L. botrana* and *C. pomonella* (Baraldi et al., 1999; Buchbauer et al., 1993; Buchbauer et al., 1994). Similar to the alkanes, 2-phenylethanol did not attract males on its own even though it induced activation in *L. botrana*, but increased attraction to the pheromone by 1.4 times when released at 100 pg/min. However, 2-phenylethanol did not significantly increase source contact by *C. pomonella* to codlemone, but improved upwind flight. Moreover, addition of 2-phenylethanol to heptane and pheromone did not increase the response by the moths. These two compounds may have redundant effects when combined. Alternatively, the already high attraction level achieved by heptane added to the pheromone may not permit a correct evaluation of the additive effect of 2-phenylethanol to the mixture. Field trials using traps baited with 2-phenylethanol attracted apple fruit moth, *Argyresthia conjugella,* males and females (Bengtsson et al., 2006). However, this compound was not attractive for this species in a wind tunnel (Knudsen et al., 2008), presumably because the dose released was inappropriate. Indeed, this molecule may also act as a deterrent for insects since its emission by the alpine skypilot, *Polemonium viscosum,* prevents floral damage caused by ants and decreases pollinator visitation (Galen et al., 2011).

Host plant volatiles not only increased the response of the moths to pheromones, but also increased the speed at which they react. All the dilute tested volatiles induced the moths to lock on to the plume and fly upwind 1.5 to 3 times faster than the underdosed pheromone alone and this shortening of response time is maintained up to contact with the source. In other words, plant volatiles increased the efficiency of the moths in finding the plume as they lock on to it faster. Subsequently, most of the moths which locked on the plume arrived at the source. The most critical behavioural elements in plume following to the source are casting and onset of upwind flight as most of the moths failing to arrive at the source already failed to locate the plume. However, alkanes used as solvent did not serve to accelerate the behaviour compared with pheromone in ethanol. At these high alkane concentrations, moths do not interpret the olfactory message carried by these compounds as being that of plants because of the high quantity. Both species use plant compounds to better orientate toward a calling female and to optimise the flight response to arrive earlier at the source thus increasing the probability to be the first to mate. Schmidt-Buesser et al. (2009) showed that (+)-trpinen-4-ol and (E)-β-caryophyllene released at 500 ng/min shortened the activation time *E. ambiguella* in response to underdosed pheromone. Similarly, limonene, methyl salicylate and hexanol decreased the reaction time of *C. pomonella* males to underdosed codlemone and the time until upwind flight was shortened by admixing pear ester, β-farnesene or limonene to underdosed codlemone (Schmera and Guerin, 2012).

Alkanes such as heptane, octane and nonane have a positive behavioural effect on the attraction of *L. botrana* at very low doses of sex pheromone (100 pg/min) and increase its response to pheromone by 14% at a ratio pheromone:heptane of 1:1000 and by 30% with a ratio of 1:100’000. By comparison, attraction of *C. pomonella* to codlemone is increased by 24% in response to a ratio of 1:10’000 of codlemone:heptane.

Host plant volatiles have already demonstrated their potential to improve the control of pests in orchards using mass trapping and in mating disruption methods for *C. pomonella* (Knight et al., 2005; Knight and Light, 2005b; Light and Knight, 2006). However, the step between wind tunnel tests and field application remains challenging as one has to ascertain the quantity of products to release in the field. The ratio between plant compounds is crucial (Tasin et al., 2006) in order to assure a relevant olfactory message and the range of effective quantities that affect behaviours is narrow (Schmidt-Buesser et al., 2009; von Arx et al., 2012b; Yang et al., 2004). Here, we show how heptane provides a good candidate as a pheromone synergist for improvement of diffusers for mating disruption as it 1) recruits as many moths as calling females when combined with underdosed pheromone, especially in the early stages of attraction, 2) is efficient over a wide spectrum of concentrations and is at least as effective as ethanol when used as a solvent, thus facilitating the formulation of the blend, 3) accelerates the attraction behaviour of males, and 4) permits to reduce the quantity of pheromone released to achieve the same effect and so reduce costs linked to use of pheromone for moth pest control.

Chapter V.

General Discussion

The outcomes of the present thesis focuses on two major aspects of insect life: their quest for food and the search for a mate. We show here how these behaviours can be influenced by chemical messages from resources and the surrounding environment.

The fragile equilibrium of the reproductive isolation in *Petunia* relies largely in its capacity to develop a well-defined mutualistic interaction with pollinators by using a specific panel of floral traits. We showed that scent significantly governs the relationship between *Petunia* and hawkmoths such as *M. sexta*. Scent production is promoted by a rather simple genetic architecture in Petunia. Two loci are responsible of the major part of the regulation of the benzenoids and phenylpropanoids that compose the fragrance bouquet of *Petunia*. By replacing the two silenced loci in *P. exserta* by those from *P. axillaris*, fragrance can be restored to the former. On the other hand, *P. axillaris* becomes scentless when the loci of *P. exserta* are inserted. By associating both fragrance and colour, four phenotypes were formed for this work. We showed that *M. sexta* foraged preferentially on scented flowers, be they white or red. *M. sexta* is a night-active moth and, consequently, long range resource detection relies on its ability to reliable sense olfactory massages. We showed that its long antennae are highly tuned to methyl benzoate, benzyl alcohol and methyl salicylate. These compounds are also released by other plants on which *M. sexta* forages such as *Nicotiana* sp. and *Datura wrightii* and contribute largely to the sweet smell of these flowers with methyl benzoate and benzyl alcohol being produced in copious amounts. However, fewer moths were interested to visit red flowers than in a white flower background. The photoreceptors of the compound eyes of *M. sexta* are highly sensitive at low electromagnetic wavelengths (ultraviolet to green), but detect red colours poorly (Cutler et al., 1995). Foraging on red scented flowers might rely more on olfactory cues than on vision. Opposing both preferred visual and olfactory information on two different plants, i.e. a red scented flower and a white unscented flower, brought about confusion, as no flower was preferred. Apparently, the behaviour of *M. sexta* is highly tuned to both olfactory and visual stimuli expressed by *P. axillaris*, as both scent and colour seem to have the same power of attraction. In native *P. axillaris* populations, where mutations occur, when scent production and/or colour are altered in some individuals *M. sexta* should be more likely to focus on flowers possessing both olfactory and visual attractive cues and ignore flowers expressing only one of these cues. In this case, cross-pollination is avoided and genotypes of the different varieties are not mixed. The pollination of the mutants is therefore favoured either by other guilds of pollinators or by auto-pollination leading to a new plant variety. Such a sensitive selection, in addition to the rather few genes involved in floral trait expression such as scent, may be sufficient to explain (partly, at least) the particular pollination biology of Petunia and the diversity of flower phenotypes between genetically closely related species.

Nectar is another floral trait that maintains pollinator consistency. Pollinator insects are able to associate floral cues with nutritive resources (Perry and Barron, 2013). Learning to recognise an adequate feeding site is profitable for both partners, as the pollinator can identify food from a distance and thus spare energy while foraging on unsuitable flowers and, on the other hand, rewarding flowers encourage pollinators to visit conspecifics to insure pollination (Brandenburg et al., 2009). *M. sexta* has mouth-part gustatory receptors tuned for its physiological needs for sugar. Moreover, we have shown that *M. sexta* is able to detect and differentiate between different sugar concentration and qualities and that meal preference correlates well with the sensitivity of receptor cells in the sensillae situated on their proboscis. Indeed, fructose is the sugar eliciting the highest response from receptor cells in basiconic and styloconic sensilla on the tip of the proboscis of *M. sexta* and is as phagostimulatory as sucrose which elicits a slightly lower sensillar response. Glucose did not activate sensillar neurones of the proboscis and was less preferred. The correlation between both peripheral sensory input of nutrient quality and the following feeding behaviour underlies the nutritional needs of the moth. *M. sexta* is therefore foraging for fructose and/or sucrose (as sucrose is an association of glucose and fructose molecules). Interestingly, plants pollinated by *M. sexta* produce copious amounts of nectar rich in sucrose and poor in glucose.

Appetence in insects can be modulated by the concentration of sugars in haemolymph. The digestion of nectar feeders remains quite simple as only disaccharides such as sucrose are digested through hydrolysis by a sucrase to produce fructose and glucose. Absorption of these nutrients by the midgut mainly involves a passive transfer into the haemolymph through a negative concentration gradient between the two compartments. Some evidence indicates that sucrose can be directly absorbed through the hindgut by a sucrose transporter in *D. melanogaster* (Meyer et al., 2011; Turunen, 1985) and be subsequently rapidly hydrolysed in the haemolymph in *Anthonomus grandis* (Nettles et al., 1971). This sucrose transporter actively transfers sucrose into haemolymph and thus might be more efficient than passive glucose absorption. Fructose is then converted into glucose in the haemolymph and glucose is then rapidly metabolised into trehalose for energy storage in the fat body cells through glycolysis (Reyes-DelaTorre et al., 2012). This serves to maintain a low concentration of glucose in the haemolymph to avoid creating osmotic pressure and to facilitate further absorption from the midgut (Treherne, 1958). Feeding terminates when the concentration of sugars in nectar and in the haemolymph is balanced. The insect needs to wait until the concentration of glucose into the haemolymph drops through the synthesis of trehalose before the next feeding bout. As such, sucrose provides a potentially higher energy index than glucose as it can be digested into monosaccharaides which are rapidly absorbed by diffusion into the haemolymph and, even when glucose is balanced, more sucrose can be absorbed before digestion. Moreover, fructose specific receptors have been found in the brain of *D. melanogaster* and these sensors serve to regulate the satiation level of the fly as a function of the concentration of fructose in the haemolymph. We have shown that the volume of sucrose imbibed is correlated with concentration when fed with sucrose solutions. These outcomes support the hypothesis that feeding is terminated when sugar balance between haemolymph and the midgut is achieved rather than being controlled by abdomen extension. Consequently, one might hypothesise that differences in preference between sugars might also be governed by the amount of a given sugar in haemolymph prior to ingestion. Even when the level of glucose in haemolymph is higher than for sucrose, moths would prefer to feed on sucrose as it is able to absorb more energy per molecule consumed. However, sugar preference in favour of sucrose seems to be rather generalized in insects and the reason of this preference remains unclear to date. Finally, each sugar intake renders the moth heavier and the energy expenditure needed to fly is consequently higher, especially when hovering. In follows that, the ratio between the energetic value of a meal and the dead weight provided by the amount of water (i.e. the dilution factor) is a determining factor.

Plant volatiles are also used by insects to locate host plants to rendezvous with a mate or to identify an adequate site for oviposition. It is demonstrated here how two major fruit pests, *L. botrana* and *C. pomonella* use plant volatiles to facilitate the location of a mate. Indeed, by adding a single product such as heptane, octane, nonane or 2-phenylethanol to a pheromone, the attraction of the males is increased as a function of the dose of the plant compound delivered. We show that the pheromone quantity can be reduced by a factor of ten fold and still maintain an attraction capacity similar to an optimal dose of pheromone by adding heptane, octane, nonane or 2-phenylethanol at low doses for *L. botrana*. Moreover, the attraction levels reached are similar to those of 1-4 calling females when the concentration of heptane or octane was increased. Furthermore, the time needed to reach the source of odour was reduced by 1.5 to 3 times. These results show how *L. botrana* and *C. pomonella* take advantage of plant volatiles to better orientate into a plume of pheromone. A higher chance to reach a calling female within a reduced flight time is of considerable advantage as it increases the chance of the male to arrive at a female first for mating with a minimum energy investment.

These findings are of potential interest for the control of populations of moth pests in the field by using mating disruption. The mode of action of this control method has been under debate for several years. Researchers have proposed different mechanisms for mating disruption ranging from camouflage, false-trail following, desensitization or adaptation (Miller et al., 2006a; Miller et al., 2006b; Witzgall et al., 2008). To date, most evidences favour false-trail following as the main factor involved in mating disruption. Here, females are directly in competition with artificial pheromone sources for recruiting males. Males are, however, misled by the pheromonal dispensers and are less likely to locate females. Additionally, they fly from dispenser to dispenser and the energy cost for such effort is considerable. The efficacy of mating disruption relies on four factors. First, the population density should be rather low. Second, the density of the artificial sources of pheromone and the plumes generated should cover the area being controlled. Third, the surface of the treated area must be of a minimal size, and fourth, attractiveness of dispensers must be high relative to females. Indeed, if the dispensers do not compete efficiently with females males will be more likely to find a mate, especially when the population density is high. The use of plant compounds to increase the attractiveness of dispensers is therefore of interest. However, whereas it is rather simple to test the adequate ratio between the different constituents of a plant volatile/pheromone mixture under controlled conditions in the wind tunnel, the translation into a relevant formulation for field dispensers can be quite challenging. Most dispensers consist of a plastic matrix that releases the pheromone blend over time and the release rate depends on the structure of the matrix, the nature of the compounds and temperature. Consequently, such dispensers are complicated to use when several compounds from different chemical classes have to be released together. Puffers that create an aerosol of semiochemicals are an interesting solution for the release of pheromones with other compounds diluted in solvent as the mixture is directly sprayed at the correct dose at regular intervals during the flight period of males and is not weather dependent. Puffers have notably proven their efficiency for mating disruption of the orangeworm *Amyelois transitella* in almond, walnut, and pistachio orchards (Shorey and Gerber, 1996). Using puffers, the main question is the formulation. We have shown that heptane increases the attraction of the pheromones for *L. botrana* and *C. pomonella* over a wide range of concentrations under the controlled conditions of the wind tunnel. Moreover, the efficacy of the pheromone is enhanced for recruiting males when the pheromone is diluted in heptane. Therefore, heptane offers a particularly good candidate to increase the efficiency of artificial dispensers such as a puffer where the formulation does not need strictly defined ratios.

Adding host plant compounds to pheromones however raises questions regarding the relevancy of such an approach since these compounds are already released in large amounts by plants in monocultures. Therefore, plant compounds released by dispensers could be masked by the volatiles from the surroundings to such as an extent that males would be no longer be affected by the plant volatiles from the dispensers. However, each plant part has its own olfactory signature (Masante-Roca et al., 2007; Tasin et al., 2006) and the amount of volatiles released vary according to environmental conditions (Vallat et al., 2005). Moreover, female *L. botrana* are known to use these defined olfactory messages to orientate towards grapes and buds for oviposition. Males presumably also use such olfactory cues to find a rendezvous site and are therefore able to track odours within monocultures. Furthermore, the success of pheromone based on mating disruption enhanced by plant volatiles is well documented for *C. pomonella* using codlemone in combination with pear ester (Knight et al., 2011; Knight et al., 2005; Knight and Light, 2005a). An interesting alternative to using plant compounds that are already present in a monoculture is the release of volatiles forming the olfactory signature of other host plants. Such volatiles should contrast well with the locally dominant olfactory message and be easily detected against the background.

Throughout this thesis we have explored many facets of insect-plant interactions. We have shown the strong relationship between *P. axillaris* and *M. sexta* through the contribution of flower fragrance in the preservation of this interaction. However, this mutualism is also considered as versatile, since the relationship is easily altered through slight plant genetic mutations. Pollination mechanisms have been examined from the plant side and from the point of view of the pollinator’s needs. The differential sensitivity of the taste cells of *M. sexta* well reflects its nutritional preferences. Finally, the potential of alkanes and other plant compounds for improving the efficacy of mating disruption for the control of moth pest populations in orchards and vineyards has been described.

CITED LITERATURE

Abisgold, J. D. and Simpson, S. J. (1987). The Physiology of Compensation by Locusts for Changes in Dietary Protein. *Journal Of Experimental Biology* **129**, 329-346.

Abisgold, J. D. and Simpson, S. J. (1988). The Effect of Dietary-Protein Levels and Hemolymph Composition on the Sensitivity of the Maxillary Palp Chemoreceptors of Locusts. *Journal Of Experimental Biology* **135**, 215-229.

Alarcon, R., Davidowitz, G. and Bronstein, J. L. (2008). Nectar usage in a southern Arizona hawkmoth community. *Ecological Entomology* **33**, 503-509.

Ando, T., Kokubun, H., Watanabe, H., Tanaka, N., Yukawa, T., Hashimoto, G., Marchesi, E., Suarez, E. and Basualdo, I. L. (2005). Phylogenetic analysis of *Petunia* sensu Jussieu (Solanaceae) using chloroplast DNA RFLP. *Annals of Botany* **96**, 289-297.

Ando, T., Nomura, M., Tsukahara, J., Watanabe, H., Kokubun, H., Tsukamoto, T., Hashimoto, G., Marchesi, E. and Kitching, I. J. (2001). Reproductive isolation in a native population of *Petunia* sensu Jussieu (Solanaceae). *Annals of Botany* **88**, 403-413.

Ansebo, L., Coracini, M. D. A., Bengtsson, M., Liblikas, I., Ramirez, M., Borg-Karlson, A. K., Tasin, M. and Witzgall, P. (2004). Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *Journal of Applied Entomology* **128**, 488-493.

Arn, H., Rauscher, S., Guerin, P. and Buser, H.-R. (1988). Sex pheromone blends of three tortricid pests in European vineyards. *Agriculture, Ecosystems & Environment* **21**, 111-117.

Ashman, T. L., Bradburn, M., Cole, D. H., Blaney, B. H. and Raguso, R. A. (2005). The scent of a male: The role of floral volatiles in pollination of a gender dimorphic plant. *Ecology* **86**, 2099-2105.

Backman, A. C., Bengtsson, M. and Witzgall, P. (1997). Pheromone release by individual females of codling moth, *Cydia pomonella*. *Journal of Chemical Ecology* **23**, 807-815.

Baker, H. G. and Baker, I. (1983). Floral Nectar Sugar constituents in Relation to Pollination Type. In *Handbook of Experimental Pollination Biology*, eds. E. C. Jones and J. R. Little): Scientific and Academic Editions.

Baraldi, R., Rapparini, F., Rossi, F., Latella, A. and Ciccioli, P. (1999). Volatile organic compound emissions from flowers of the most occurring and economically important species of fruit trees. *Physics and Chemistry of the Earth Part B-Hydrology Oceans and Atmosphere* **24**, 729-732.

Becher, P. G. and Guerin, P. M. (2009). Oriented responses of grapevine moth larvae *Lobesia botrana* to volatiles from host plants and an artificial diet on a locomotion compensator. *Journal of Insect Physiology* **55**, 384-393.

Bengtsson, M., Backman, A. C., Liblikas, I., Ramirez, M. I., Borg-Karlson, A. K., Ansebo, L., Anderson, P., Lofqvist, J. and Witzgall, P. (2001). Plant odor analysis of apple: Antennal response of codling moth females to apple volatiles during phenological development. *Journal of Agricultural and Food Chemistry* **49**, 3736-3741.

Bengtsson, M., Jaastad, G., Knudsen, G., Kobro, S., Bäckman, A.-C., Pettersson, E. and Witzgall, P. (2006). Plant volatiles mediate attraction to host and non-host plant in apple fruit moth, *Argyresthia conjugella*. *Entomologia Experimentalis Et Applicata* **118**, 77-85.

Blaney, W. M. and Simmonds, M. S. J. (1988). Food Selection in Adults and Larvae of 3 Species of Lepidoptera: a Behavioral and Electrophysiological Study. *Entomologia Experimentalis Et Applicata* **49**, 111-121.

Boatright, J., Negre, F., Chen, X. L., Kish, C. M., Wood, B., Peel, G., Orlova, I., Gang, D., Rhodes, D. and Dudareva, N. (2004). Understanding in vivo benzenoid metabolism in petunia petal tissue. *Plant Physiology* **135**, 1993-2011.

Bovey, R., Baggiolini, M., Bolay, A., Corbaz, R., Mathys, G., Meylan, A., Murbach, R., Pelet, F., Savary, A. and Trivelli, G. (1972). La défense des plantes cultivées. Lausanne: Association suisse des ingenieurs agronomes.

Bradshaw, H. D. and Schemske, D. W. (2003). Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* **426**, 176-178.

Brandenburg, A. and Bshary, R. (2011). Variable responses of hawkmoths to nectar-depleted plants in two native *Petunia axillaris* (Solanaceae) populations. *Arthropod-Plant Interactions* **5**, 141-148.

Brandenburg, A., Dell'Olivo, A., Bshary, R. and Kuhlemeier, C. (2009). The sweetest thing Advances in nectar research. *Current Opinion in Plant Biology* **12**, 486-490.

Brandenburg, A., Kuhlemeier, C. and Bshary, R. (2012a). Hawkmoth Pollinators Decrease Seed Set of a Low-Nectar *Petunia axillaris* Line through Reduced Probing Time. *Current Biology* **22**, 1635-1639.

Brandenburg, A., Kuhlemeier, C. and Bshary, R. (2012b). Innate Adjustment of Visitation Behavior to Rewarding and Reward-Minimized *Petunia axillaris* (Solanacea) Plants by Hawkmoth *Manduca sexta* (Sphingidae). *Ethology* **118**, 654-661.

Brantjes, N. B. M. (1978). Sensory responses to flowers in night-flying moths. In *The pollination of flowers by insects*, (ed. A. J. Richards), pp. 14-19: Academic Press for the Linnean Society of London.

Buchbauer, G., Jirovetz, L., Wasicky, M. and Nikiforov, A. (1993). Headspace and essential oil analysis of apple flowers. *Journal of Agricultural and Food Chemistry* **41**, 116-118.

Buchbauer, G., Jirovetz, L., Wasicky, M. and Nikiforov, A. (1994). Headspace Analysis of *Vitis vinifera* (Vitaceae) Flowers. *Journal of Essential Oil Research* **6**, 311-314.

Cardé, R. T. and Minks, A. K. (1995). Control of Moth Pests by Mating Disruption - Successes and Constraints. *Annual Review of Entomology* **40**, 559-585.

Castellucci, F. (2013). Statistical report on world vitiviniculture 2013: International Organisation of Vine and Wine.

Chess, S. K. R., Raguso, R. A. and LeBuhn, G. (2008). Geographic divergence in floral morphology and scent in *Linanthus dichotomus* (Polemoniaceae). *American Journal of Botany* **95**, 1652-1659.

Christensen, T. and Hildebrand, J. (1987). Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth *Manduca sexta*. *Journal of Comparative Physiology A* **160**, 553-569.

Coracini, M., Bengtsson, M., Liblikas, I. and Witzgall, P. (2004). Attraction of codling moth males to apple volatiles. *Entomologia Experimentalis Et Applicata* **110**, 1-10.

Cutler, D. E., Bennett, R. R., Stevenson, R. D. and White, R. H. (1995). Feeding Behaviour In The Nocturnal Moth *Manduca sexta* Is Mediated Mainly By Blue Receptors, But Where Are They Located In The Retina. *Journal Of Experimental Biology* **198**, 1909-1917.

Daly, K. C. and Smith, B. H. (2000). Associative olfactory learning in the moth *Manduca sexta*. *Journal Of Experimental Biology* **203**, 2025-2038.

Degen, T., Chevallier, A. and Fischer, S. (2005). Evolution de la lutte phéromonale contre les vers de la grappe. *Revue suisse de viticulture arboriculture horticulture* **37**, 273-280.

Dell'Olivo, A., Hoballah, M. E., Gubitz, T. and Kuhlemeier, C. (2011). Isolation Barriers between Petunia Axillaris and *Petunia integrifolia* (Solanaceae). *Evolution* **65**, 1979-1991.

Dodson, C. H., Dressler, R. L., Hills, H. G., Adams, R. M. and Williams, N. H. (1969). Biologically active compounds in orchid fragrances. *Science* **164**, 1243-9.

Dudareva, N. and Pitchersky, E. (2006). Biology of Floral Scent: Taylor & Francis.

Edgecomb, R. S., Harth, C. E. and Schneiderman, A. M. (1994). Regulation of Feeding Behavior in Adult *Drosophila melanogaste*r Varies with Feeding Regime and Nutritional State. *Journal Of Experimental Biology* **197**, 215-235.

El-Sayed, A., Bengtsson, M., Rauscher, S., Lofqvist, J. and Witzgall, P. (1999a). Multicomponent sex pheromone in codling moth (Lepidoptera : Tortricidae). *Environmental Entomology* **28**, 775-779.

El-Sayed, A., Godde, J. and Arn, H. (1999b). Sprayer for quantitative application of odor stimuli. *Environmental Entomology* **28**, 947-953.

El-Sayed, A., Gödde, J., Witzgall, P. and Arn, H. (1999c). Characterization of Pheromone Blend for Grapevine Moth, *Lobesia botrana* by Using Flight Track Recording. *Journal of Chemical Ecology* **25**, 389-400.

Faegri, K. and Van der Pijl, L. (1979). The Principles of Pollination Ecology. New-York: Oxford: Pergamon.

Fraser, A. M., Mechaber, W. L. and Hildebrand, J. G. (2003). Electroantennographic and behavioral responses of the sphinx moth *Manduca sexta* to host plant headspace volatiles. *Journal of Chemical Ecology* **29**, 1813-1833.

Galen, C., Kaczorowski, R., Todd, S. L., Geib, J. and Raguso, R. A. (2011). Dosage-Dependent Impacts of a Floral Volatile Compound on Pollinators, Larcenists, and the Potential for Floral Evolution in the Alpine Skypilot *Polemonium viscosum*. *American Naturalist* **177**, 258-272.

Galliot, C., Hoballah, M. E., Kuhlemeier, C. and Stuurman, J. (2006a). Genetics of flower size and nectar volume in Petunia pollination syndromes. *Planta* **225**, 203-212.

Galliot, C., Stuurman, J. and Kuhlemeier, C. (2006b). The genetic dissection of floral pollination syndromes. *Current Opinion in Plant Biology* **9**, 78-82.

Glendinning, J. I., Jerud, A. and Reinherz, A. T. (2007). The hungry caterpillar: an analysis of how carbohydrates stimulate feeding in *Manduca sexta*. *Journal Of Experimental Biology* **210**, 3054-3067.

Goyret, J. (2010). Look and touch: multimodal sensory control of flower inspection movements in the nocturnal hawkmoth *Manduca sexta*. *The Journal of Experimental Biology* **213**, 3676-3682.

Goyret, J., Markwell, P. M. and Raguso, R. A. (2007). The effect of decoupling olfactory and visual stimuli on the foraging behavior of *Manduca sexta*. *Journal Of Experimental Biology* **210**, 1398-1405.

Goyret, J., Markwell, P. M. and Raguso, R. A. (2008a). Context- and scale-dependent effects of floral CO_2 on nectar foraging by *Manduca sexta*. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **105**, 4565-4570.

Goyret, J., Pfaff, M., Raguso, R. A. and Kelber, A. (2008b). Why do *Manduca sexta* feed from white flowers? Innate and learnt colour preferences in a hawkmoth. *Naturwissenschaften* **95**, 569-576.

Goyret, J. and Raguso, R. A. (2006). The role of mechanosensory input in flower handling efficiency and learning by *Manduca sexta*. *Journal Of Experimental Biology* **209**, 1585-1593.

Gübitz, T., Hoballah, M. E., Dell'Olivo, A. and Kuhlemeier, C. (2009). Petunia as a Model System for the Genetics and Evolution of Pollination Syndromes. In *Petunia*, (ed. T. S. Gerats, J.).

Guerenstein, P. G. and Guerin, P. M. (2001). Olfactory and behavioural responses of the blood-sucking bug *Triatoma infestans* to odours of vertebrate hosts. *Journal Of Experimental Biology* **204**, 585-597.

Guerenstein, P. G., Yepez, E. A., van Haren, J., Williams, D. G. and Hildebrand, J. G. (2004). Floral CO_2 emission may indicate food abundance to nectar-feeding moths. *Naturwissenschaften* **91**, 329-333.

Gurba, A., Harraca, V., Perret, J. L., Casera, S., Donnet, S. and Guerin, P. M. (2012). Three-dimensional flight tracking shows how a visual target alters tsetse fly responses to human breath in a wind tunnel. *Physiological Entomology* **37**, 250-257.

Heitler, W. J. (2012). DataView. St-Andrews, Scotland: University of St Andrews.

Herrera, C. M., de Vega, C., Canto, A. and Pozo, M. I. (2009). Yeasts in floral nectar: a quantitative survey. *Annals of Botany* **103**, 1415-1423.

Herrera, C. M., Garcia, I. M. and Perez, R. (2008). Invisible floral larcenies: Microbial communities degrade floral nectar of bumble bee-pollinated plants. *Ecology* **89**, 2369-2376.

Hoballah, M. E., Gubitz, T., Stuurman, J., Broger, L., Barone, M., Mandel, T., Dell'Olivo, A., Arnold, M. and Kuhlemeier, C. (2007). Single gene-mediated shift in pollinator attraction in Petunia. *Plant Cell* **19**, 779-790.

Hoballah, M. E., Stuurman, J., Turlings, T. C. J., Guerin, P. M., Connetable, S. and Kuhlemeier, C. (2005). The composition and timing of flower odour emission by wild Petunia axillaris coincide with the antennal perception and nocturnal activity of the pollinator Manduca sexta. *Planta* **222**, 141-150.

Honda, K. (1995). Chemical Basis of Differential Oviposition by Lepidopterous Insects. *Archives of Insect Biochemistry and Physiology* **30**, 1-23.

Irwin, R. E. and Adler, L. S. (2008). Nectar Secondary Compounds Affect Self-Pollen Transfer: Implications for Female and Male Reproduction. *Ecology* **89**, 2207-2217.

Jones, K. N. and Reithel, J. S. (2001). Pollinator-mediated selection on a flower color polymorphism in experimental populations of Antirrhinum (Scrophulariaceae). *American Journal of Botany* **88**, 447-454.

Josens, R. B., Farina, W. M. and Roces, F. (1998). Nectar feeding by the ant *Camponotus mus*: intake rate and crop filling as a function of sucrose concentration. *Journal of Insect Physiology* **44**, 579-585.

Kaczorowski, R. L., Gardener, M. C. and Holtsford, T. P. (2005). Nectar traits in *Nicotiana* section *Alatae* (Solanaceae) in relation to floral traits, pollinators, and mating system. *American Journal of Botany* **92**, 1270-1283.

Kaczorowski, R. L., Seliger, A. R., Gaskett, A. C., Wigsten, S. K. and Raguso, R. A. (2012). Corolla shape vs. size in flower choice by a nocturnal hawkmoth pollinator. *Functional Ecology* **26**, 577-587.

Kaissling, K. E. (1997). Pheromone-controlled anemotaxis in moths. In *Orientation and Communication in Arthropods*, (ed. M. Lehrer), pp. 343-374. Basel: Birkhäuser Verlag.

Kelber, A. (2003). Sugar preferences and feeding strategies in the hawkmoth Macroglossum stellatarum. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **189**, 661-666.

Kelber, A., Balkenius, A. and Warrant, E. J. (2003). Colour vision in diurnal and nocturnal hawkmoths. *Integrative and Comparative Biology* **43**, 571-579.

Kellogg, F. E. (1970). Water Vapour and Carbon Dioxide Receptors in *Aedes aegypti*. *Journal of Insect Physiology* **16**, 99-&.

Kent, K. S., Harrow, I. D., Quartararo, P. and Hildebrand, J. G. (1986). An Accessory Olfactory Pathway in Lepidoptera - the Labial Pit Organ and Its Central Projections in Manduca-Sexta and Certain Other Sphinx Moths and Silk Moths. *Cell and Tissue Research* **245**, 237-245.

Kessler, D. and Baldwin, I. T. (2006). Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of Nicotiana attenuata. *Plant Journal* **49**, 840-854.

Kessler, D., Diezel, C., Clark, D. G., Colquhoun, T. A. and Baldwin, I. T. (2013a). Petunia flowers solve the defence/apparency dilemma of pollinator attraction by deploying complex floral blends. *Ecology Letters* **16**, 299-306.

Kessler, S., Vlimant, M. and Guerin, P. M. (2013b). The sugar meal of the African malaria mosquito Anopheles gambiae and how deterrent compounds interfere with it: a behavioural and neurophysiological study. *The Journal of Experimental Biology* **216**, 1292-1306.

Kite, G. C. and Leon, C. (1995). Volatile compounds emitted from flowers and leaves of Brugmansia × candida (Solanaceae). *Phytochemistry* **40**, 1093-1095.

Klahre, U., Gurba, A., Hermann, K., Saxenhofer, M., Bossolini, E., Guerin, P. M. and Kuhlemeier, C. (2011). Pollinator Choice in Petunia Depends on Two Major Genetic Loci for Floral Scent Production. *Current Biology* **21**, 730-739.

Knight, A., Haworth, J., Lingren, B. and Hebert, V. (2011). Combining pear ester with codlemone improves management of codling moth. *IOBC-WPRS Bulletin* **72**, 145-149.

Knight, A. L., Hilton, R. and Light, D. M. (2005). Monitoring codling moth (Lepidoptera : Tortricidae) in apple with blends of ethyl (E, Z)-2,4-decadienoate and codlemone. *Environmental Entomology* **34**, 598-603.

Knight, A. L. and Light, D. M. (2005a). Developing action thresholds for codling moth (Lepidoptera : Tortricidae) with pear ester- and codlemone-baited traps in apple orchards treated with sex pheromone mating disruption. *Canadian Entomologist* **137**, 739-747.

Knight, A. L. and Light, D. M. (2005b). Dose-response of codling moth (Lepidoptera : Tortricidae) to ethyl (E, Z)-2,4-decadienoate in apple orchards treated with sex pheromone dispensers. *Environmental Entomology* **34**, 604-609.

Knudsen, G. K., Bengtsson, M., Kobro, S., Jaastad, G., Hofsvang, T. and Witzgall, P. (2008). Discrepancy in laboratory and field attraction of apple fruit moth Argyresthia conjugella to host plant volatiles. *Physiological Entomology* **33**, 1-6.

Knudsen, J. T. and Tollsten, L. (1993). Trends in Floral Scent Chemistry in Pollination Syndromes - Floral Scent Composition in Moth-Pollinated Taxa. *Botanical Journal of the Linnean Society* **113**, 263-284.

Koeduka, T., Orlova, I., Baiga, T. J., Noel, J. P., Dudareva, N. and Pichersky, E. (2009). The lack of floral synthesis and emission of isoeugenol in Petunia axillaris subsp parodii is due to a mutation in the isoeugenol synthase gene. *Plant Journal* **58**, 961-969.

Kolosova, N., Gorenstein, N., Kish, C. M. and Dudareva, N. (2001). Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants. *Plant Cell* **13**, 2333-2347.

Kondo, M., Oyama-Okubo, N., Ando, T., Marchesi, E. and Nakayama, M. (2006). Floral Scent Diversity is Differently Expressed in Emitted and Endogenous Components in Petunia axillaris Lines. *Ann Bot* **98**, 1253-1259.

Krömer, T., Kessler, M., Lohaus, G. and Schmidt-Lebuhn, A. N. (2008). Nectar sugar composition and concentration in relation to pollination syndromes in Bromeliaceae. *Plant Biology* **10**, 502-511.

Kulcheski, F. R., Muschner, V. C., Lorenz-Lemke, A. P., Stehmann, J. R., Bonatto, S. L., Salzano, F. M. and Freitas, L. B. (2006). Molecular phylogenetic analysis of Petunia Juss. (Solanaceae). *Genetica* **126**, 3-14.

Landolt, P. J. (1989). Attraction of the Cabbage Looper to Host Plants and Host Plant Odor in the Laboratory. *Entomologia Experimentalis Et Applicata* **53**, 117-124.

Landolt, P. J. and Phillips, T. W. (1997). Host plant influences on sex pheromone behavior of phytophagous insects. *Annual Review of Entomology* **42**, 371-391.

Light, D. M. and Knight, A. (2006). Kairomone-augmented mating disruption control for codling moth in Californian walnuts and apples. *IOBC/WPRS Bulletin*.

Light, D. M., Knight, A. L., Henrick, C. A., Rajapaska, D., Lingren, B., Dickens, J. C., Reynolds, K. M., Buttery, R. G., Merrill, G., Roitman, J. et al. (2001). A pear-derived kairomone with pheromonal potency that attracts male and female codling moth, *Cydia pomonella* (L.). *Naturwissenschaften* **88**, 333-338.

Lorenz-Lemke, A. P., Mader, G., Muschner, V. C., Stehmann, J. R., Bonatto, S. L., Salzano, F. M. and Freitas, L. B. (2006). Diversity and natural hybridization in a highly endemic species of Petunia (Solanaceae): a molecular and ecological analysis. *Molecular Ecology* **15**, 4487-4497.

Maeda, H., Shasany, A. K., Schnepp, J., Orlova, I., Taguchi, G., Cooper, B. R., Rhodes, D., Pichersky, E. and Dudareva, N. (2010). RNAi Suppression of Arogenate Dehydratase1 Reveals That Phenylalanine Is Synthesized Predominantly via the Arogenate Pathway in Petunia Petals. *Plant Cell* **22**, 832-849.

Mafra-Neto, A. and Cardé, R. T. (1994). Fine-Scale Structure of Pheromone Plumes Modulates Upwind Orientation of Flying Moths. *Nature* **369**, 142-144.

Majetic, C. J., Raguso, R. A. and Ashman, T. L. (2009). The sweet smell of success: floral scent affects pollinator attraction and seed fitness in Hesperis matronalis. *Functional Ecology* **23**, 480-487.

Masante-Roca, I., Anton, S., Delbac, L., Dufour, M. C. and Gadenne, C. (2007). Attraction of the grapevine moth to host and non-host plant parts in the wind tunnel: effects of plant phenology, sex, and mating status. *Entomologia Experimentalis Et Applicata* **122**, 239-245.

Mastelic, J., Jerkovic, I. and Mesic, M. (2006). Volatile constituents from flowers, leaves, bark and wood of *Prunus mahaleb* L. *Flavour and Fragrance Journal* **21**, 306-313.

Mcdonough, L. M., Davis, H. G., Chapman, P. S. and Smithhisler, C. L. (1993). Response of Male Codling Moths (*Cydia pomonella*) to Components of Conspecific Female Sex-Pheromone Glands in Flight Tunnel Tests. *Journal of Chemical Ecology* **19**, 1737-1748.

Mevi-Schutz, J. and Erhardt, A. (2005). Amino acids in nectar enhance butterfly fecundity: A long-awaited link. *American Naturalist* **165**, 411-419.

Meyer, H., Vitavska, O. and Wieczorek, H. (2011). Identification of an animal sucrose transporter. *Journal of Cell Science* **124**, 1984-1991.

Miller, J. R., Gut, L. J., de Lame, F. M. and Stelinski, L. L. (2006a). Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): Case studies. *Journal of Chemical Ecology* **32**, 2115-2143.

Miller, J. R., Gut, L. J., de Lame, F. M. and Stelinski, L. L. (2006b). Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part I): Theory. *Journal of Chemical Ecology* **32**, 2089-2114.

Mota-Sanchez, D., Wise, J. C., Poppen, R. V., Gut, L. J. and Hollingworth, R. M. (2008). Resistance of codling moth, Cydia pomonella (L.) (Lepidoptera : Tortricidae), larvae in Michigan to insecticides with different modes of action and the impact on field residual activity. *Pest Management Science* **64**, 881-890.

Murlis, J. and Jones, C. D. (1981). Fine-Scale Structure of Odor Plumes in Relation to Insect Orientation to Distant Pheromone and Other Attractant Sources. *Physiological Entomology* **6**, 71-86.

Nettles, W. C., Parro, B., Sharbaug.C and Mangum, C. L. (1971). Trehalose and Other Carbohydrates in *Anthonomus grandis, Heliothis zea*, and *Heliothis virescens* during Growth and Development. *Journal of Insect Physiology* **17**, 657-675.

O'Brien, D. M., Fogel, M. L. and Boggs, C. L. (2002). Renewable and nonrenewable resources: Amino acid turnover and allocation to reproduction in lepidoptera. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **99**, 4413-4418.

Ochieng, S. A., Park, K. C. and Baker, T. C. (2002). Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea*. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **188**, 325-333.

Paré, P. W. and Tumlinson, J. H. (1997). De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiology* **114**, 1161-1167.

Pellmyr, O. and Thien, L. B. (1986). Insect Reproduction and Floral Fragrances - Keys to the Evolution of the Angiosperms. *Taxon* **35**, 76-85.

Perry, C. J. and Barron, A. B. (2013). Neural Mechanisms of Reward in Insects. *Annual Review of Entomology* **58**, 543-562.

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J. and Koes, R. (1999). Molecular Analysis of the anthocyanin2 Gene of Petunia and Its Role in the Evolution of Flower Color. *Plant Cell* **11**, 1433-1444.

R Core Team. (2012). R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Raguso, R. A. (2003). Floral scent, olfaction, and scent-driven foraging behavior. In *Cognitive Ecology of Pollination: Animal Behavior and Floral Evolution*, eds. L. Chittka and J. D. Thomson), pp. 83-105: Press Syndicate of the University of Cambridge.

Raguso, R. A. (2004). Flowers as sensory billboards: progress towards an integrated understanding of floral advertisement. *Current Opinion in Plant Biology* **7**, 434-440.

Raguso, R. A., Henzel, C., Buchmann, S. L. and Nabhan, G. P. (2003a). Trumpet flowers of the Sonoran Desert: Floral biology of *Peniocereus cacti* and sacred *Datura*. *International Journal of Plant Sciences* **164**, 877-892.

Raguso, R. A., LeClere, A. R. and Schlumpberger, B. O. (2005). Sensory flexibility in hawkmoth foraging behavior: Lessons from *Manduca sext*a and other species. *Chemical Senses* **30**, I295-I296.

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W. and McDade, L. A. (2003b). Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry* **63**, 265-284.

Raguso, R. A., Light, D. M. and Pickersky, E. (1996). Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. *Journal of Chemical Ecology* **22**, 1735-1766.

Raguso, R. A. and Willis, M. A. (2002). Synergy between visual and olfactory cues in nectar feeding by naive hawkmoths, *Manduca sexta*. *Animal Behaviour* **64**, 685-695.

Raguso, R. A. and Willis, M. A. (2003). The importance of olfactory and visual cues in nectar foraging by nocturnal hawkmoths. In *Evolution and Ecology Taking Flight: Butterflies as Model Systems*, eds. W. B. Watt C. L. Boggs and P. R. Ehrlich), pp. 43-65. Chicago.

Raguso, R. A. and Willis, M. A. (2005). Synergy between visual and olfactory cues in nectar feeding by wild hawkmoths, *Manduca sexta*. *Animal Behaviour* **69**, 407-418.

Rauscher, S., Arn, H. and Guerin, P. (1984). Effects of Dodecyl Acetate and Z-10-Tridecenyl Acetate on Attraction of *Eupoecilia ambiguella* Males to the Main Sex-Pheromone Component, Z-9-Dodecenyl Acetate. *Journal of Chemical Ecology* **10**, 253-264.

Reyes-DelaTorre, A., Peña-Rangel, M. T. and Riesgo-Escovar, J. R. (2012). Carbohydrate Metabolism in Drosophila: Reliance on the Disaccharide Trehalose. In *Carbohydrates – Comprehensive Studies on Glycobiology and Glycotechnology*, (ed. C.-F. Chang): InTech.

Reyes, M., Franck, P., Charmillot, P. J., Ioriatti, C., Olivares, J., Pasqualin, E. and Sauphanor, B. (2007). Diversity of insecticide resistance mechanisms and spectrum in European populations of the Codling moth, *Cydia pomonella*. *Pest Management Science* **63**, 890-902.

Riffell, J. A., Alarcon, R., Abrell, L., Davidowitz, G., Bronstein, J. L. and Hildebrand, J. G. (2008). Behavioral consequences of innate preferences and olfactory learning in hawkmoth-flower interactions. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **105**, 3404-3409.

Roelofs, W., Comeau, A., Hill, A. and Milicevic, G. (1971). Sex attractant of the codling moth: characterization with electroantennogram technique. *Science* **174**, 297-9.

Romeis, J. and Wackers, F. L. (2000). Feeding responses by female *Pieris brassicae* butterflies to carbohydrates and amino acids. *Physiological Entomology* **25**, 247-253.

Rusterholz, H. P. and Erhardt, A. (1997). Preferences for nectar sugars in the peacock butterfly, *Inachis io*. *Ecological Entomology* **22**, 220-224.

Sanders, C. J. (1997). Mechanisms of Mating Disruption in Moths. In *Insect Pheromone Research*, eds. R. Cardé and A. Minks), pp. 333-346: Springer US.

Sanes, J. R. and Hildebrand, J. G. (1976). Structure and development of antennae in a moth, *Manduca sexta*. *Developmental Biology* **51**, 282-299.

Schmera, D. and Guerin, P. M. (2012). Plant volatile compounds shorten reaction time and enhance attraction of the codling moth (*Cydia pomonella*) to codlemone. *Pest Management Science* **68**, 454-461.

Schmidt-Buesser, D., von Arx, M. and Guerin, P. M. (2009). Host plant volatiles serve to increase the response of male European grape berry moths, *Eupoecilia ambiguella*, to their sex pheromone. *Journal of Comparative Physiology a-Neuroethology Sensory Neural and Behavioral Physiology* **195**, 853-864.

Schmidt-Busser, D., von Arx, M., Connetable, S. and Guerin, P. M. (2011). Identification of host-plant chemical stimuli for the European grape berry moth *Eupoecilia ambiguella*. *Physiological Entomology* **36**, 101-110.

Schoonhoven, L. M. (1969). Gustation and Foodplant Selection in Some Lepidopterous Larvae. *Entomologia Experimentalis Et Applicata* **12**, 555-564.

Schoonhoven, L. M. (2005). Insect-plant relationships: the whole is more than the sum of its parts. *Entomologia Experimentalis Et Applicata* **115**, 5-6.

Schuurink, R. C., Haring, M. A. and Clark, D. G. (2006). Regulation of volatile benzenoid biosynthesis in petunia flowers. *Trends in Plant Science* **11**, 20-25.

Shields, V. D. C. and Hildebrand, J. G. (1999a). Fine structure of antennal sensilla of the female sphinx moth, *Manduca sexta* (Lepidoptera : Sphingidae). I. Trichoid and basiconic sensilla. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* **77**, 290-301.

Shields, V. D. C. and Hildebrand, J. G. (1999b). Fine structure of antennal sensilla of the female sphinx moth, *Manduca sexta* (Lepidoptera : Sphingidae). II. Auriculate, coeloconic, and styliform complex sensilla. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* **77**, 302-313.

Shields, V. D. C. and Hildebrand, J. G. (2001). Recent advances in insect olfaction, specifically regarding the morphology and sensory physiology of antennal sensilla of the female sphinx moth *Manduca sexta*. *Microscopy Research and Technique* **55**, 307-329.

Shorey, H. H. and Gerber, R. G. (1996). Use of Puffers for Disruption of Sex Pheromone Connnunication Among Navel Orangeworm Moths (Lepidoptera: Pyralidae) in Almonds, Pistachios, and Walnuts. *Environmental Entomology* **25**, 1154-1157.

Simpson, S. J. and Raubenheimer, D. (1993). The Central Role of the Hemolymph in the Regulation of Nutrient Intake in Insects. *Physiological Entomology* **18**, 395-403.

Simpson, S. J. and Simpson, C. L. (1992). Mechanisms Controlling Modulation by Hemolymph Amino Acids of Gustatory Responsiveness in the Locust. *Journal Of Experimental Biology* **168**, 269-287.

Spitzer-Rimon, B., Marhevka, E., Barkai, O., Marton, I., Edelbaum, O., Masci, T., Prathapani, N. K., Shklarman, E., Ovadis, M. and Vainstein, A. (2010). EOBII, a Gene Encoding a Flower-Specific Regulator of Phenylpropanoid Volatiles' Biosynthesis in *Petunia*. *Plant Cell* **22**, 1961-1976.

Städler, E. and Seabrook, W. D. (1975). Chemoreceptors On The Proboscis Of The Female Eastern Spruce Budworm: Electrophysiological Study. *Entomologia Experimentalis Et Applicata* **18**, 153-160.

Stehmann, J. R. (1987). *Petunia exserta* (Solanaceae): Uma nova espécie do Rio Grande do Sul, Brasil. *Napaea* **2**, 19-21.

Stehmann, J. R. and Semir, J. (2005). New species of *Calibrachoa* and *Petunia* (Solanaceae) from subtropical south America. . In *Festschrift for William G.Darcy: the Legacy of a Taxonomist* eds. R. C. Keating V. C. Hollowell and T. B. Croat). Saint Louis, Missouri

Stuurman, J., Hoballah, M. E., Broger, L., Moore, J., Basten, C. and Kuhlemeier, C. (2004). Dissection of floral pollination syndromes in petunia. *Genetics* **168**, 1585-1599.

Tabata, J., Noguchi, H., Kainoh, Y., Mochizuki, F. and Sugie, H. (2007). Sex pheromone production and perception in the mating disruption-resistant strain of the smaller tea leafroller moth, *Adoxophyes honmai*. *Entomologia Experimentalis Et Applicata* **122**, 145-153.

Taneja, J. and Guerin, P. M. (1997). Ammonia attracts the haematophagous bug Triatoma infestans: Behavioural and neurophysiological data on nymphs. *Journal of Comparative Physiology a-Sensory Neural and Behavioral Physiology* **181**, 21-34.

Tasin, M., Anfora, G., Ioriatti, C., Carlin, S., De Cristofaro, A., Schmidt, S., Bengtsson, M., Versini, G. and Witzgall, P. (2005). Antennal and behavioral responses of grapevine moth *Lobesia botrana* females to volatiles from grapevine. *Journal of Chemical Ecology* **31**, 77-87.

Tasin, M., Backman, A. C., Anfora, G., Carlin, S., Ioriatti, C. and Witzgall, P. (2010). Attraction of Female Grapevine Moth to Common and Specific Olfactory Cues from 2 Host Plants. *Chemical Senses* **35**, 57-64.

Tasin, M., Backman, A. C., Bengtsson, M., Ioriatti, C. and Witzgall, P. (2006). Essential host plant cues in the grapevine moth. *Naturwissenschaften* **93**, 141-144.

Tasin, M., Backman, A. C., Coracini, M., Casado, D., Ioriatti, C. and Witzgall, P. (2007). Synergism and redundancy in a plant volatile blend attracting grapevine moth females. *Phytochemistry* **68**, 203-209.

Thiery, D. and Moreau, J. (2005). Relative performance of European grapevine moth (*Lobesia botrana*) on grapes and other hosts. *Oecologia* **143**, 548-557.

Thom, C., Guerenstein, P. G., Mechaber, W. L. and Hildebrand, J. G. (2004). Floral CO_2 reveals flower profitability to moths. *Journal of Chemical Ecology* **30**, 1285-1288.

Treherne, J. E. (1958). The Absorption and Metabolism of Some Sugars in the Locust, *Schistocerca gregaria* (Forsk). *Journal Of Experimental Biology* **35**, 611-625.

Trona, F., Anfora, G., Balkenius, A., Bengtsson, M., Tasin, M., Knight, A., Janz, N., Witzgall, P. and Ignell, R. (2013). Neural coding merges sex and habitat chemosensory signals in an insect herbivore. *Proc Biol Sci* **280**, 20130267.

Turunen, S. (1985). Absorption. In *Comprehensive insect physiology, biochemistry and pharmacology*, vol. 4 eds. G. S. Kerkut and L. I. Gilbert), pp. 241-277. London: Pergamon Press.

Vallat, A., Gu, H. N. and Dorn, S. (2005). How rainfall, relative humidity and temperature influence volatile emissions from apple trees in situ. *Phytochemistry* **66**, 1540-1550.

Varela, L. G., Welter, S. C., Jones, V. P., Brunner, J. F. and Riedl, H. (1993). Monitoring and Characterization of Insecticide Resistance in Codling Moth (Lepidoptera, Tortricidae) in 4 Western States. *Journal of Economic Entomology* **86**, 1-10.

Venail, J., Dell'Olivo, A. and Kuhlemeier, C. (2010). Speciation genes in the genus *Petunia*. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**, 461-468.

Verdonk, J. C., de Vos, C. H. R., Verhoeven, H. A., Haring, M. A., van Tunen, A. J. and Schuurink, R. C. (2003). Regulation of floral scent production in petunia revealed by targeted metabolomics. *Phytochemistry* **62**, 997-1008.

Verdonk, J. C., Haring, M. A., van Tunen, A. J. and Schuurink, R. C. (2005). ODORANT1 regulates fragrance biosynthesis in petunia flowers. *Plant Cell* **17**, 1612-1624.

Visser, J. H. (1986). Host Odor Perception in Phytophagous Insects. *Annual Review of Entomology* **31**, 121-144.

von Arx, M. (2013). Floral humidity and other indicators of energy rewards in pollination biology. *Communicative & Integrative Biology* **6**, e22750.

von Arx, M., Goyret, J., Davidowitz, G. and Raguso, R. A. (2012a). Floral humidity as a reliable sensory cue for profitability assessment by nectar-foraging hawkmoths. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **109**, 9471-9476.

von Arx, M., Schmidt-Busser, D. and Guerin, P. M. (2011). Host plant volatiles induce oriented flight behaviour in male European grapevine moths, *Lobesia botrana*. *Journal of Insect Physiology* **57**, 1323-1331.

von Arx, M., Schmidt-Busser, D. and Guerin, P. M. (2012b). Plant Volatiles Enhance Behavioral Responses of Grapevine Moth Males, *Lobesia botrana* to Sex Pheromone. *Journal of Chemical Ecology* **38**, 222-225.

Waser, N. M. and Price, M. V. (1981). Pollinator Choice and Stabilizing Selection for Flower Color in *Delphinium nelsonii*. *Evolution* **35**, 376-390.

Watanabe, H., Ando, T., Iida, S., Suzuki, A., Buto, K., Tsukamoto, T., Hashimoto, G. and Marchesi, E. (1996). Cross compatibility of *Petunia* cultivars and *P. axillaris* with native taxa of *Petunia* in relation to their chromosome number. *Journal of the Japanese Society for Horticultural Science* **65**, 625-634.

White, R. H., Stevenson, R. D., Bennett, R. R., Cutler, D. E. and Haber, W. A. (1994). Wavelength Discrimination And The Role Of Ultraviolet Vision In The Feeding-Behavior Of Hawkmoths. *Biotropica* **26**, 427-435.

White, R. H., Xu, H. H., Munch, T. A., Bennett, R. R. and Grable, E. A. (2003). The retina of *Manduca sexta*: rhodopsin expression, the mosaic of green-, blue- and UV-sensitive photoreceptors, and regional specialization. *Journal Of Experimental Biology* **206**, 3337-3348.

Wijsman, H. J. W. (1983). On the Interrelationships of Certain Species of *Petunia* 2. Experimental Data - Crosses between Different Taxa. *Acta Botanica Neerlandica* **32**, 97-107.

Witzgall, P. (1996). Modulation of pheromone-mediated flight in male moths. In *Pheromone research : New Directions*, eds. R. T. Cardé and A. K. Minks), pp. 265-274. New York: Chapman & Hall.

Witzgall, P. and Arn, H. (1990). Direct Measurement of the Flight Behavior of Male Moths to Calling Females and Synthetic Sex-Pheromones. *Zeitschrift Fur Naturforschung C-a Journal of Biosciences* **45**, 1067-1069.

Witzgall, P., Bengtsson, M., Rauscher, S., Liblikas, I., Backman, A. C., Coracini, M., Anderson, P. and Lofqvist, J. (2001). Identification of further sex pheromone synergists in the codling moth, Cydia pomonella. *Entomologia Experimentalis Et Applicata* **101**, 131-141.

Witzgall, P., Stelinski, L., Gut, L. and Thomson, D. (2008). Codling moth management and chemical ecology. *Annual Review of Entomology* **53**, 503-522.

Yan, F. M., Bengtsson, M. and Witzgall, P. (1999). Behavioral response of female codling moths, *Cydia pomonella*, to apple volatiles. *Journal of Chemical Ecology* **25**, 1343-1351.

Yang, Z. H., Bengtsson, M. and Witzgall, P. (2004). Host plant volatiles synergize response to sex pheromone in codling moth, *Cydia pomonella*. *Journal of Chemical Ecology* **30**, 619-629.

Yang, Z. H., Casado, D., Ioriatti, C., Bengtsson, M. and Witzgall, P. (2005). Pheromone pre-exposure and mating modulate codling moth (Lepidoptera : Tortricidae) response to host plant volatiles. *Agricultural and Forest Entomology* **7**, 231-236.

Zeger, S. L. and Liang, K. Y. (1986). Longitudinal Data-Analysis for Discrete and Continuous Outcomes. *Biometrics* **42**, 121-130.

Ziegler, R. and Schulz, M. (1986). Regulation of Carbohydrate Metabolism during Flight in *Manduca sexta*. *Journal of Insect Physiology* **32**, 997-1001.

APPENDIX

Annexe 1

Insight onto the olfactory sensory organ of *M. sexta*: the antennae

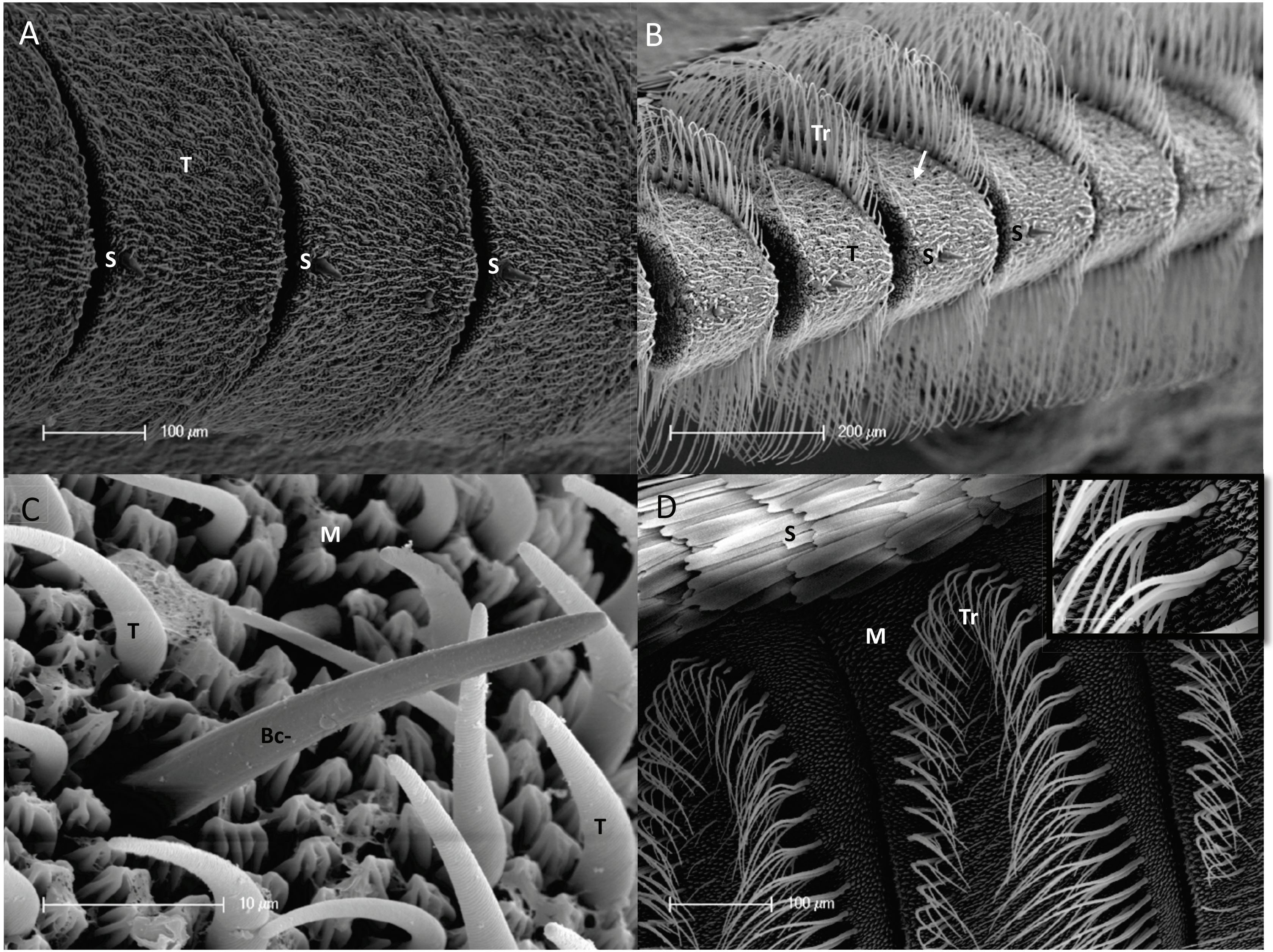
The antenna is the organ of olfaction in insects. Its morphology varies largely among Lepidopteran insects. From the extremely long and filamentous antennae of the fairy moth *Adela ridingsella* (Adelidae) to the highly branched antennae of the Hercules moth *Coscinocera hercules* (Saturniidae) the antennae offer a large panel of macroscopic structures and adaptations. Moreover, a sexual dimorphism is observed in most of nocturnal moth species as males possess larger antennae as females. Indeed, males express specific sensilla sensitive to sex pheromone used to locate mature females for mating over long distance in obscurity. The antennae are covered with numerous sensilla. These microscopic exvaginations of the cuticle permits the sensory neurons to expand from the core body of the insect. This cuticular adaptation increases the contact surface of the sensory system with the environment. A single annulus of the 80 that compose the *M. sexta* female antenna bears more than 2200 sensilla (Sanes and Hildebrand, 1976). A typical olfactory sensillum is formed by a cuticular sheath perforated with plenty of microscopic pores that ensure the passage of the volatile molecules from the surrounds into the sensillum. One to three neurones expand their dendrites along the sensillum lumen filled with lymph. The sensillum is nursed by three support cells (the thecogen, tormogen and trichogen cells) at its base that insure the formation and nutrition of the neurones and the preservation of the ionic balance of the cuticular lymph. Olfactory chemoreceptors detect volatile organic compounds with a variable sensitivity/selectivity, depending on the nature of the neurone. A typical example of highly selective with low threshold of sensitivity are the sensory neurones involved in the detection of the sex pheromone of conspecific females with a highly tuned response to these molecules (Christensen and Hildebrand, 1987). On the contrary, most of the other olfactory cells respond to a large variety of plant volatile compounds involved in the recognition of suitable food or oviposition. Olfactory neurones are occasionally accompanied with neurones sensitive to temperature gap (heat/cold receptors), non-volatile molecules (taste sensory cells), humidity (hygroreceptors) or cuticular deformations (mechanoreceptors). Lepidoptera express several types of olfactory sensilla over the antenna characterised by their external structure, internal organisation and function. At least 8 types of sensilla have been inventoried on female *M. sexta* antennae on their leading, ventral and dorsal surfaces (rear sides are covered by scales). Male antennae have the same types, but their structures slightly differ in size and relative abundance.

Trichoid sensilla are the most abundant. They are hair-like single-walled, multiporous sensilla divided in two subgroups: Type-A and Type-B (Type-I and Type-II for males, respectively; Figure S1A, B and D). Type-A trichoid sensilla are long hairs curved toward the distal extremity of the antenna. Circumferential cuticular ridges holding pores aligned draw circular pattern along the sensillum. Two unbranched dendrites are projected to the tip of the sensillum. Type-B trichoid sensilla have the same structure as Type-A trichoid sensilla, except a smaller size, cuticular ridges draw a helical pattern along the sensilla that contain 1 to 3 unbranched dendrites (Figure S1C) (Shields and Hildebrand, 1999a).

Basiconic sensilla are peg like structures with a single-walled, multiporous cuticle divided in two subgroups: Type-A and Type-B (Type-II and Type-I for males, respectively). Pores of Type-A basiconic sensilla are distributed along oblique rows along the sensillum. Seven to nine unbranched dendrites extend in the sensillar lumen. Type-B basiconic sensilla are shorter than Type-A basiconic sensilla and their pores are aligned in deep furrows along the sensillum (Figure S1C). The sensillum lumen holds 40-50 branched dendrites. In both sexes, basiconic sensilla are shorter than trichoid sensilla (Shields and Hildebrand, 1999a).

Coeloconic sensilla are very small structures divided in two types: Type-A and Type-B (Type-I and Type-II for males, respectively). Type-A coeloconic sensilla are double-walled, multiporous sensilla that resembles to a peg in a pit formed by 15-16 grooves and surrounded by 7-9 cuticular spines (Figure S1G and H). This sensillum type is innervated with 5 branched dendrites. The function of this type is associated with plant odours detection and probably also temperature decreases. Type-B coeloconic sensilla are single-walled, aporous sensilla resembling to a small bud with 3 unbranched dendrites (Shields and Hildebrand, 1999b).

Auriculate sensilla are single-walled, multiporous, ear (or spoon) like structure in shallow depressions (Figure S1F and G). Its two sensory cells divide into 50-60 branches and are tuned to plant odours. Another cuticular structure erects on the distal side of each annulus, the styliform complex (Figure S1E). This single-walled, aporous cuticular excrescence houses 4-7 sensilla with 3 unbranched dendrites each. The sensilla of the styliform complex are thermo- (cold) and hygro-receptors (rise and fall of relative humidity) (Shields and Hildebrand, 1999b).


A
T
S
S
S
100 µm
B
Tr
T
S
S
S
200 µm
C
M
T
Bc-
T
10 µm
D
S
M
Tr
100 µm

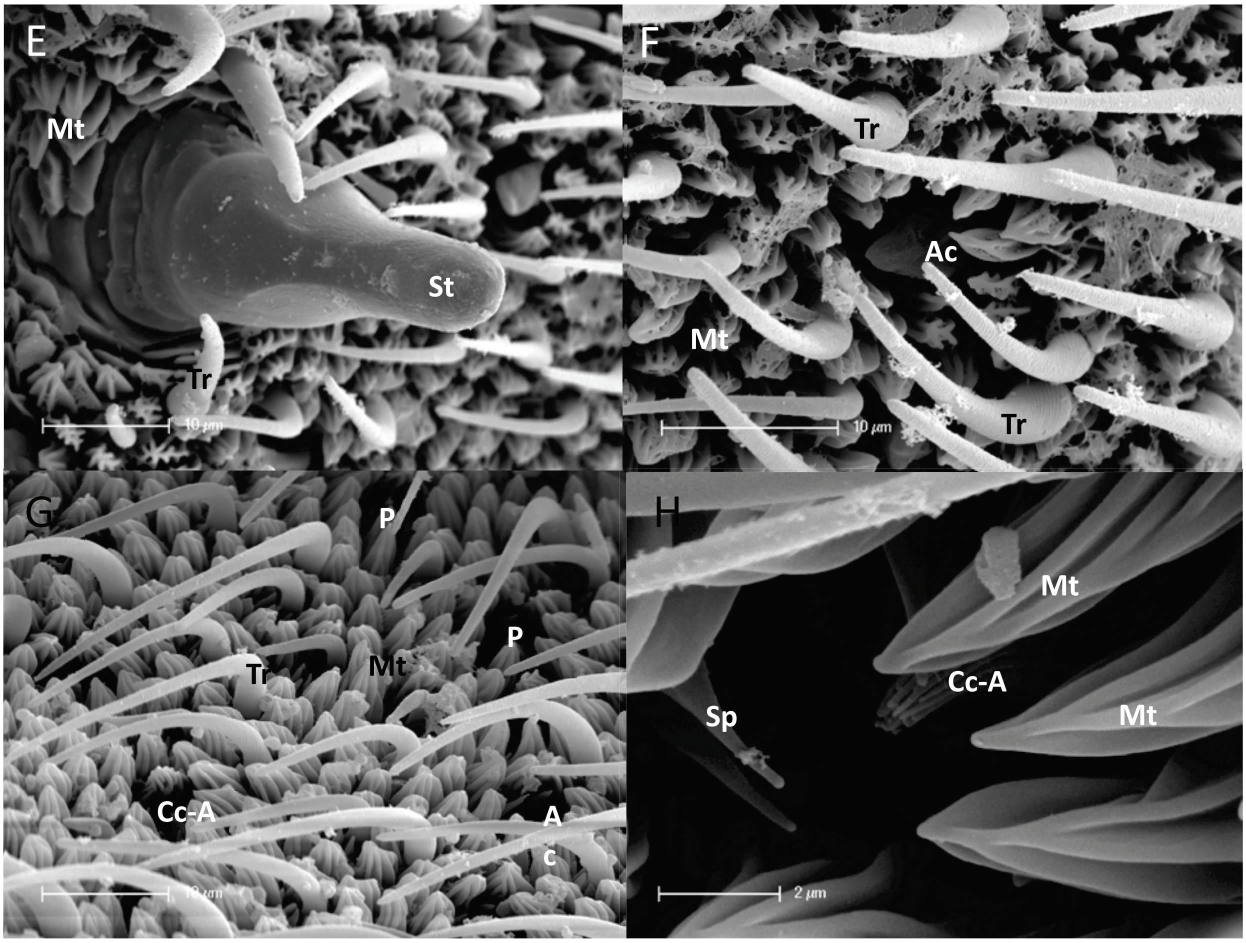


Figure S1 . Scaning electron micrographs (SEM) of the sensory apparatus of the antennae of *M. sexta.* A : overview of the leading surface of a female *M. sexta* antenna showing 3 annuli. Each annulus is covered by numerous trichoid sensilla (Tr) and a single styliform complex (St) on the distal extremity. B: overview of the leading surface of a male *M. sexta* antenna showing 6 annuli. The long pheromone specific Type-I trichoid sensilla (Tr-I) appear at the upper and lower surface of the antenna. Smaller trichoid sensilla (Tr) cover the entire leading surface of the antenna. As for females, a single styliform complex (St) erects on the distal part of each annulus. Small pits (arrow) appear on the upper part of each annulus. C: Close-up of a Type-B basiconic sensillum (Bc-B) with longitudinal ridges erecting from microtrichia (Mt) and among numerous trichoid sensilla (Tr) showing circular and helical ridges. D: Upper surface of a male annulus showing scales (Sc) on the rear surface and Type-I trichoid sensilla (Tr-I) placed in rows of 8 sensilla (see medallion). E: Close-up of an aporous styliform complex (St) placed among trichoid sensilla (Tr). F: Close-up of an auriculate sensillum (Ac) placed in a pit and covered by numerous trichoid sensilla (Tr). G: Overview of the upper surface of an annulus showing multiple pits (P) containing auriculate (Ac) and Type-A coeloconic (Cc-A) snesilla. H: Close-up of a Type-A coeloconic sensillum (Cc-A) in a pit and bordered by a crown of spines (Sp). For SEM preparations, see Materials and methods of Chapter III.

Annexe 2

Foraging preference of *M. sexta* for sibling violet flowers that are diverging in scent production

The genus *Petunia* displays three well defined pollination syndromes. Different pollinator's guilds are attracted by different flower patterns and so maintain species isolation by preventing any cross-pollination between plant species. The ancestral bee-pollinated *P. integrifolia* has a short and wide tube supporting a purple corolla and releasing only benzaldehyde (Hoballah et al., 2005). As many floral aspects diverge between *P. integrifolia* and *P. axillaris* (i.e. tube length and width, colour and scent) the foraging response of a hawkmoth pollinator may be triggered by a complex interaction between these floral traits. We showed that scent is a major floral trait in the definition of the segregation between white *P. axillaris* and red *P. exserta* as hawkmoths prefers to forage on scented flowers, independently of the flower colour (red or white; Figure II.7). However, as *P. axillaris* and *P. exserta* were discriminated only by two floral traits, i.e. colour and scent, the manipulation of one of these parameters produced strong effect on the foraging preference. To evaluate the role of scent on *M. sexta* using a more complex system, we conducted a choice experiment between two near isogenic lines where QTLs triggering scent production in *P. axillaris* (IS) and *P. exserta* (INS) were added in a *P. integrifolia* background. These plants strongly diverged from *P. axillaris* by at least 3 traits (see Figure II.1).

Introgression lines IS and INS showed typical bee pollination syndromes as they expressed similar floral traits as *P. integrifolia*, except for scent production. Floral light reflectance was similar for both lines (Figure S1A and C) with a reflectance peak at 430 nm that match with the "blue" photopigments of the compound eyes of *M. sexta* (Cutler et al., 1995). INS looks slightly darker than IS (Figure S1C). As QTLs responsible for fragrance in *P. axillaris* were replaced in IS, the latter line produced volatile compounds resembling those of *P. axillaris,* i.e. benzaldehyde, methyl benzoate, methyl salicylate, isoeugenol and benzyl alcohol (Figure S1B). Alternatively, INS received QTLs from *P. exserta* and showed only traces of isoeugenol (Figure S1B). When *M. sexta* was confronted with these plants that mismatched with the *P. axillaris* trait pattern, except for scent production in IS, 60 % of them chose to visit the scented plant at first (binomial exact test, P = 0.12) and 70 % of them extended their proboscis (binomial exact test, P<0.01). No statistical difference in terms of choice was observed between these plants due to the low number of replicates (n = 18), but a strong trend was observed as 11 moths visited the scented side at first and only 4 on the unscented side.

These results show that in a floral context where no trait matches the preferred combination of traits except for scent, *M. sexta* was attracted preferentially to the plants that produce fragrance where plants were placed closely to one another. In the case of *P. integrifolia*, hawkmoth pollinators are supposed to have driven speciation towards *P. axillaris*. Colour is governed by 6 loci in *Petunia* (Wijsman, 1983) and the inactivation of colour genes to produce white corolla occurred relatively recently and probably subsequently in the speciation process (Hoballah et al., 2007; Quattrocchio et al., 1999). As morphological floral traits such as limb surface (4 QTLs), tube length (6 QTLs) and nectar volume (4 QTLs) are highly linked (Galliot et al., 2006a; Venail et al., 2010) they may have mutated simultaneously and then diverged gradually toward a hawkmoth-adapted plant due to the high number of genes implicated. Since the genetics underlying production of scent involves only a few loci and remains rather simple (Klahre et al., 2011), the adaptation process towards a hawkmoth pollination syndrome may have been initiated by mutation of scent regulation. However, bees and other diurnal generalist pollinators are plentiful and are able to easily pollinate those mutants and so block the speciation process by diluting hybrids within populations of *P. integrifolia*.

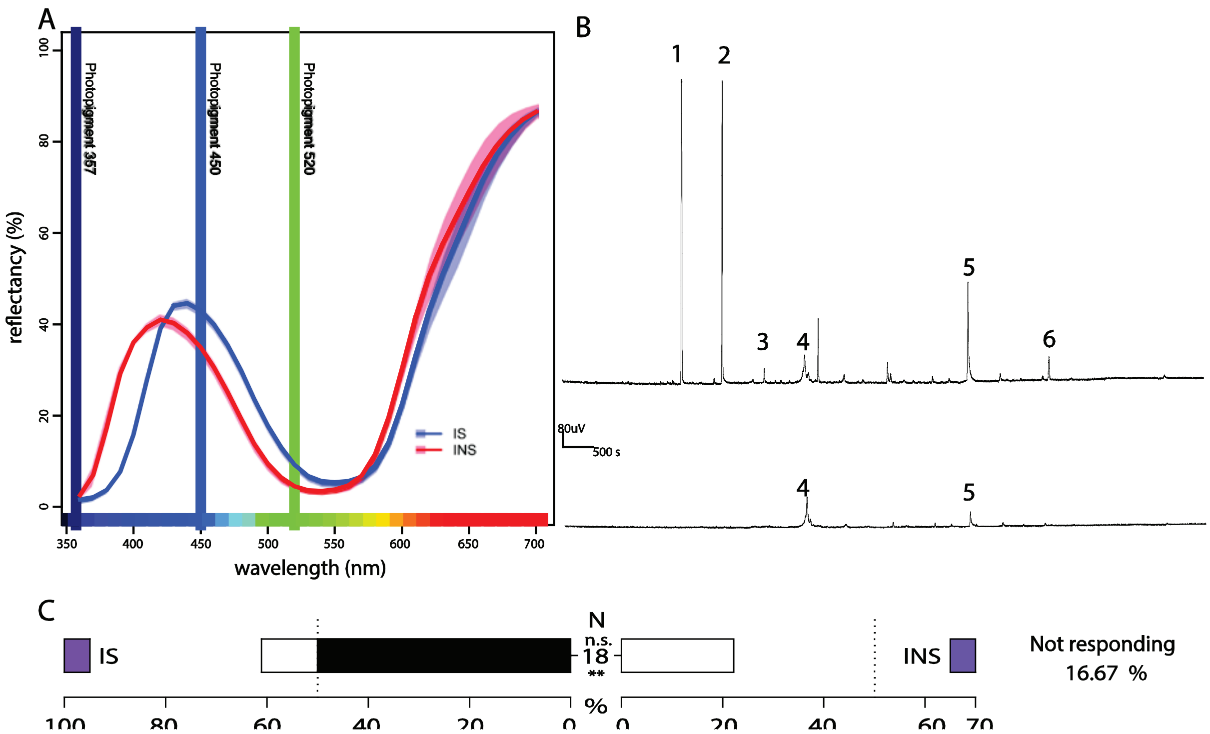


Figure S1 Floral traits and choice preferences of *M. sexta* to the scented (IS) and unscented (INS) violet introgression lines (measurement methods as in Chapter II). **A**: Spectral reflectance of the top side of the corolla of IS (n=4) and INS (n=3). The 3 vertical lines show the peak of light absorbance of the photoreceptors of *M. sexta* (from Cutler et al. (1995)) **B**: GC-FID trace of headspace collection of IS (upper trace) and INS (lower trace) on a 30 m FFAP column with (1) benzaldehyde, (2) methyl benzoate, (3) methyl salicylate, (4) column pollution, (5) isoeugenol and (6) benzylbenzoate. **C**: First choice preference of *M. sexta* between IS and INS (white boxes) and their related proboscis extension probability (black boxes) in a wind tunnel. N is the number of moth tested and the information above and below N represent, respectively, the significance for the first choice and first proboscis extension (exact binomial test). The coloured boxes depict the apparent colours of the corolla of each phenotype.

Annexe 3

Flight responses of European grapevine moth males, *L. botrana*, to calling females filmed in 3D

The flights of *L. botrana* males in response to 3 calling females were filmed in 3D in a 2.5 x 1 x 1 m (length x width x height) wind tunnel. Two cameras recording at 25 frames per second recorded the insect flight from above with a precision of 3 mm (for more information about the 3d tracking system see Gurba et al. (2012)). Light was provided from beneath by a 2.5 x 1 m panel of LEDs (Luniscontrol Gmbh, Lanzenhäusern, Bern, Switzerland) covered with sintered glass and with a light filter (Roscolab Ltd., Schloss Holte-Stukenbrock, North Rhine-Westphalia, Germany) with a cut off at 600 nm. Pheromone was released by 3 calling females enclosed in a glass tube (125 mm long, 24.2 mm o.d., 21 mm i.d.) whose openings were covered with cotton netting (1 mm mesh). The tube with females was held at 30 cm from the floor, 40 cm from the upwind end of the wind tunnel. Charcoal-filtered air was blown laminarly at 30 cm/s across the wind tunnel. The males were placed in glass tubes at 30 cm high, 40 cm from the downwind end and released as described in Chapter IV.

Attractive olfactory stimuli such as the pheromone are perceived by the antennal receptor cells and induce a behavioural response. Resting males are at first activated and begin to fan their wings. Take-off is usually immediately followed by the in-flight detection of the olfactory plume that elicits zigzags while maintaining position relative to the wind. This behaviour permits the male to define the structure and the edge of the plume of odour. Then, the male undertakes anemotactic flight upwind to the source of pheromone but may sometimes be arrested in its flight path to undertake another casting bout to reposition itself in the pheromone plume (Figure S1). Flight directionality depends on the nature of the stimulus. Witzgall (1996) showed that upwind flight by *L. botrana* males is straighter in response to calling females than to an optimized but incomplete synthetic pheromone blend. Male fights are confined to the plume of odour and casting occurs as soon as the moths lose the plume. Figure S2 illustrates well how the flight is restricted to a narrow cylinder of ca. 20 cm in diameter along the wind tunnel.

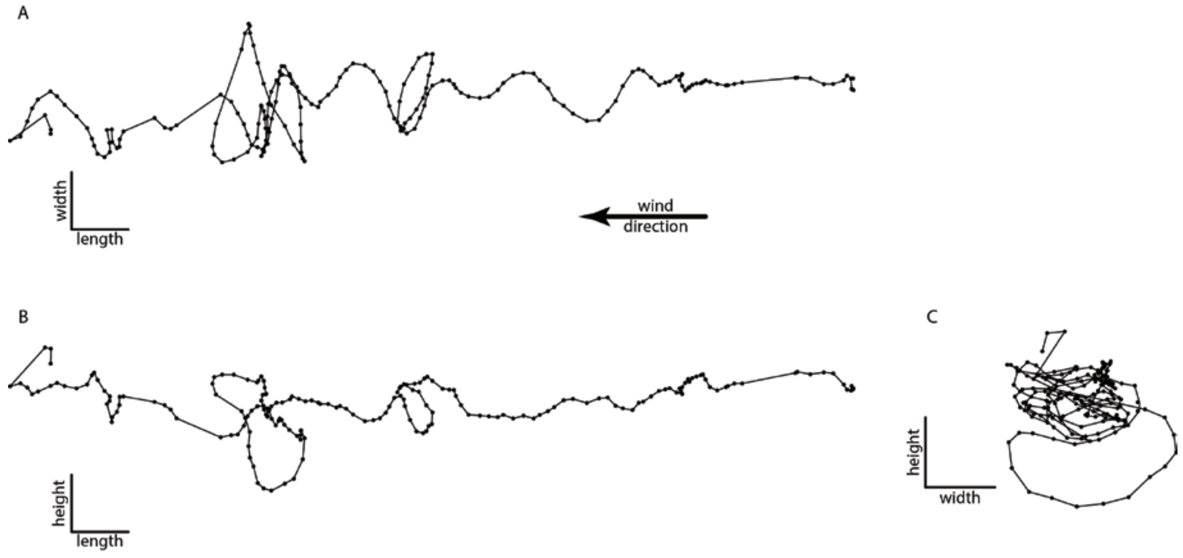


Figure S1 Example of a single upwind flight made by a *L. botrana* male in response to 3 calling females in the wind tunnel as viewed from the side (A), from the top (B) and from the upwind end of the wind tunnel (C). The recorded track begins after the casting flight sequence (left) and finishes when the male contacts the source (right). Points are the recorded positions of the moth along the wind tunnel and scale bars represent 10 cm. Note how most of the flight occurs within a well-defined cylinder (C). Scale bars represent 10 cm. Wind blew from right to left.

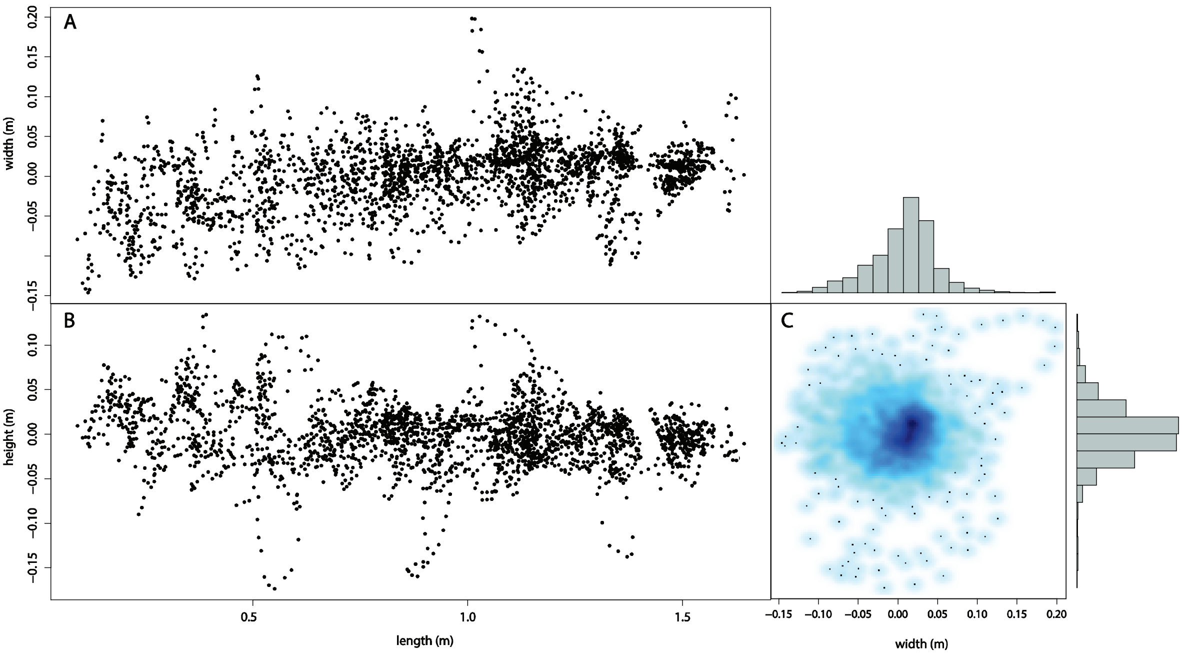


Figure S2 Superposition of 12 upwind flights made by *L. botrana* males in response to 3 calling females as viewed from the side (A), from the top (B) and the density of the flight positions of the 12 moths as viewed from the upwind end of the wind tunnel (C). The histograms in C show the distribution of the flight positions of the moths across and over the height of the wind tunnel. Wind blew from right to left.

Annexe 4

Foraging preference of *M. sexta* to 4 phenotypes of *Petunia*: a 3D overview

The flight of a single *M. sexta* male was filmed under similar light conditions as for *L. botrana* (see Annexe 3) and by using the 3D video recording setup. An array of 4 freshly cut flowers of *Petunia* differing both in scent production and colour (i.e. WS, WNS, RS and RNS; see Chapter II) and arranged as described in Figure S1 was placed at 1m from the upwind end of the wind tunnel at a height of 35 cm. The take-off platform of the moth was placed at the downwind end of the wind tunnel at a height of 10 cm.

Figure S1 Frontal view of the array of 4 freshly cut flowers held on a vertically held perforated plate in the wind tunnel. The white scented (WS, upper left) and the red scented (RS, upper right) phenotypes were 22 cm apart. The white non-scented (WNS, lower left) and the red non-scented (RNS, lower right) phenotypes were 8 cm apart. Five cm separated the lower row from the upper one.

Tracking constraints

The large size of the *M. sexta* generates a set of recording constraints as the 3D tracking system is designed to record only a single moving blob. However, the system detected the flying moth as a multitude of moving blobs. This artefact was resolved by adjusting the number of moving pixels that define a blob. However, the centre of gravity of the blob diverges from that of the insect resulting in the generation of noise (a moth flying in a straight line will be tracked as describing small zigzags). Moreover, the reflection of the moth on the side-walls can be seen by the camera that ends up tracking the reflection of the moth instead of the real one. To avoid this as much as possible, I needed to make the moth fly in the lower part of the wind tunnel. Despite all of these tracking improvements, many tracking position of the moths were missed, resulting in random gaps in the flight track.

Track analysis

To disentangle pertinent information from the tracks, kernel density estimates of the positions of the insect (i.e. an estimation of the density) were calculated to build a 3D histogram of the foraging paths of *M. sexta* (Figure S2). Four volumes were computed, each representing 5%, 10 %, 25% and 40% of the positions around the highest flight density regions. This three-dimensional representation of the track provides an honest estimate of the flight path positions and of the blob density that can be interpreted as the level of activity in space.

Moths were more active at the scented flowers

The position density of the foraging flight of *M. sexta* in response to the four phenotypes correlates well with the findings from the two choice assay described in Chapter II (see Figure II.7). Most of the positions of the moth were at the scented flowers (Figure S2). Moreover, *M. sexta* showed a more intense activity at the white scented flower than at the red scented one whereas the scentless flowers were barely visited. Nevertheless, there is a small spot of activity associated with the red unscented flower. This might be due to the difficulty of the moth to precisely locate the red scented flower. The poor visual information provided by the red corolla was probably lowered by the red lighting from the floor of the wind tunnel resulting in the moth lacking of visual information from the flower to guide the proboscis into the flower tube. As in Chapter II, we show that *M. sexta* is able to discriminate between two sibling plants diverging only in scent production, even when presented very close to each other. Most of the flight activity of the moth is centred in the vicinity of the flowers. At the downwind end of the wind tunnel, quite wide zone of activity is evident. This represents the casting behaviour during which the moth scan the wind in cross section to lock on to the plume of odour. Then the moth flew directly to the flower array.

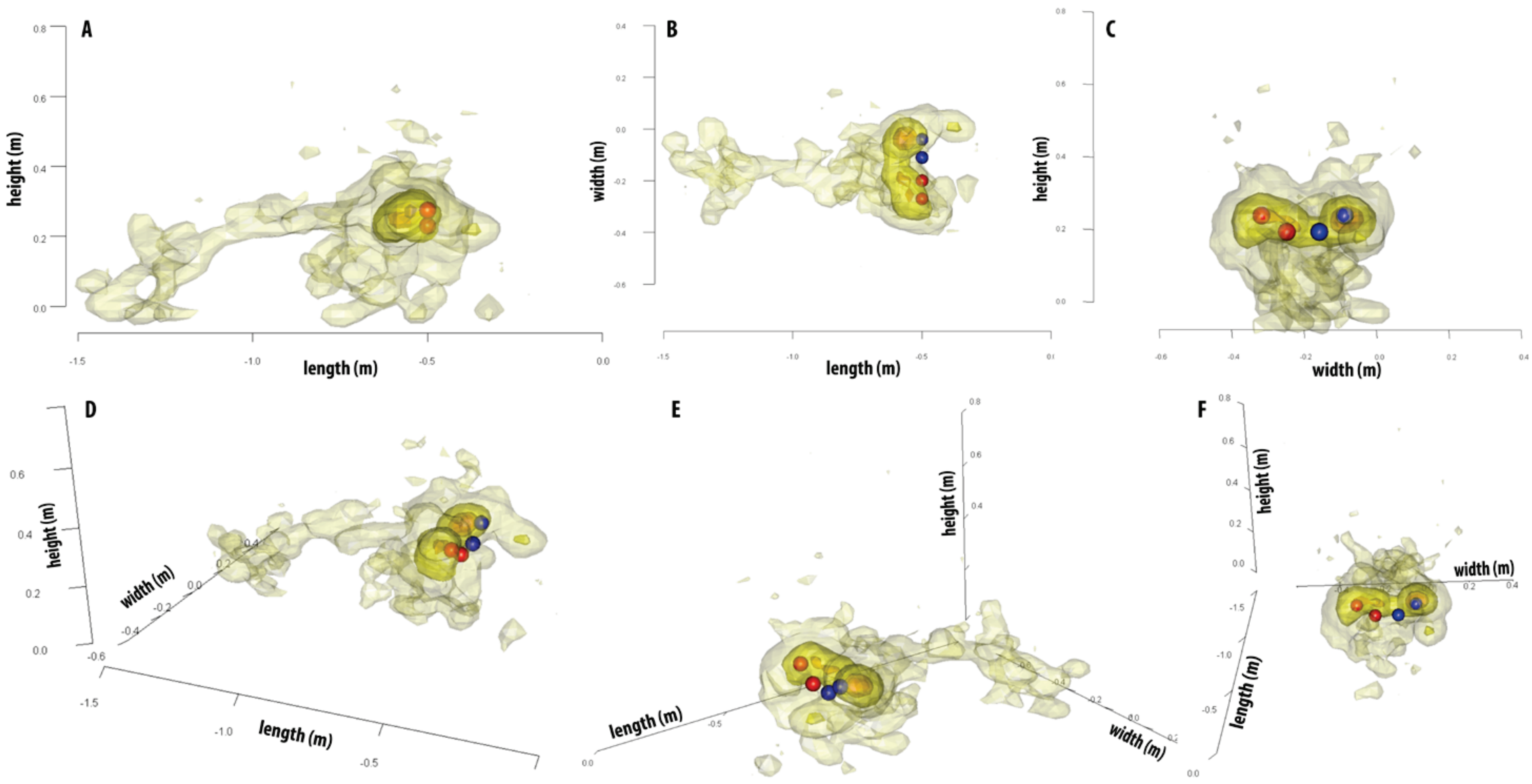


Figure S2 Overview of the foraging behaviour of a single *M. sexta* in response to 4 phenotypes of *Petunia* displayed as 3D density plots. Dark orange volumes represent 5%, light orange volumes 10%, dark yellow 25% and light yellow 40% of the positions around the highest density regions. The four spheres represent the positions of the flowers (see Figure S1): red spheres – red flowers; blue spheres – white flowers. Panels A, B, C, D, E and F depict the 3D representations of the positions of the flying moth at different angles. Wind blew from right to left in A and B.