BIONOMY AND HOST PLANT FINDING
IN
OIL COLLECTING BEES

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List of Publications

1) **Schäffler I., Dötterl S. 2011**. A day in the life of an oil bee: phenology, nesting, and foraging behavior. *Apidologie,* **42**: 409-424.

2) **Dötterl S., Milchreit K., Schäffler I. 2011**. Behavioural plasticity and sex differences in host finding of a specialized bee species. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **197**: 1119-1126.

3) **Schäffler I., Balao F., Dötterl S.** Floral and vegetative cues in oil-secreting and non-oil secreting *Lysimachia* species. *Annals of Botany*, doi: 10.1093/aob/mcs101.

In preparation for submission to Proceedings of the National Academy of Sciences:

4) **Schäffler I., Steiner K. E., Haid M., Gerlach G., Johnson S. D., Wessjohann L., Dötterl S.** Honest signaling by a private communication channel in a specialized pollination system.

Publication not included in this thesis:

5) **Dötterl S., Schäffler I. 2007.** Floral scent of oil-producing *Lysimachia punctata* as attractant for the oil-bee *Macropis fulvipes*. *Journal of Chemical Ecology*,**33**, 441-445.

Declaration of contribution to publications

The thesis contains four research articles. Together with my supervisor I developed the methods and discussed the results with all co-authors.

1st article

> Schäffler I., Dötterl S. 2011. **A day in the life of an oil bee: phenology, nesting, and foraging behavior.** Apidologie, 42: 409-424.

> Most of the field work and data analysis was done by myself. I prepared the first version of the manuscript. S. Dötterl contributed comments.

2nd article

> Dötterl S., Milchreit K., Schäffler I. 2011. **Behavioural plasticity and sex differences in host finding of a specialized bee species**. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology,* **197**: 1119-1126.

> Most of the data were collected by S. Dötterl and K. Milchreit. I prepared the figures and table for the manuscript, which was mainly written by S. Dötterl.

3rd article

> Schäffler I., Balao F., Dötterl S. 2012. **Floral and vegetative cues in oil and non-oil secreting *Lysimachia* species.** *Annals of Botany*, doi: 10.1093/aob/mcs101.

> All data were collected and most of them were analysed by myself. Phylogenetically controlled analyses were performed by F. Balao. I prepared the first version of the manuscript, the final version of which was prepared together with S. Dötterl and F. Balao.

4th article

Schäffler I., Steiner K. E., Haid M., Gerlach G., Johnson S. D., Wessjohann L., Dötterl S.

Honest signalling by a private communication channel in a specialized pollination system.

Most of the chemical and electrophysiological, and all behavioural experiments as well as the presentation of the results were performed by myself. L. Wessjohann and M. Haid conducted the purification of diacetin. K. Steiner and S. Dötterl provided flower samples of oil secreting species from South Africa, and G. Gerlach from the Botanical Garden München-Nymphenburg for my analyses. S. Johnson and S. Dötterl conducted electrophysiological experiments (EAG) with *Rediviva* in Pietermaritzburg, South Africa. I prepared the first version of the manuscript. The actual version of the manuscript was prepared together with S. Dötterl and M. Haid (method for purification of diacetin).

Content

Summary

The pollination system between oil offering flowers and oil collecting bees is one of the most specialised mutualistic interactions between insects and their host plants. It occurs in four floristic regions with about 1800 plant species and 400 bee species involved. The bees collect the fatty oils to provide their offspring and in some cases also to line the walls of their brood cells. The only oil flowers in the Holarctic are species in the genus *Lysimachia*. About 40 % of the species in this genus secrete floral oils and these species are almost exclusively pollinated by oil collecting bees of the genus *Macropis*, whereas non-oil species in this genus are regarded as being pollinated by generalised bees.

In the present work, I focused on (i) the bionomics of a flight cage population of *Macropis* oil bees, (ii) the visual (colour) and olfactory (scent) advertisement in oil and non-oil secreting *Lysimachia* species, (iii) the relative importance of visual and olfactory cues of *L. punctata* for their pollinating bees *M. fulvipes*, and (iv) the specific cues (scent compounds) mediating the interaction between *Lysimachia* and *Macropis*. I also tested whether cues mediating the *Lysimachia-Macropis* interaction may be important for other oil plants and oil bees.

The bees started to hatch in the flight cage when a certain temperature sum (temperature five weeks before hatching was most important) was reached, and although the date of emergence varied over the four years, it coincided with the start of the flowering period of *L. punctata*. The population was protandrous and the sex ratio balanced in three of the four observation years. Not only female but also male bees fed on pollen of their host plants after hatching, and for females seems pollen feeding to be the trigger to search appropriate nesting sites and construct a cell. Following excavating a cell, they collected floral oil to line their cell walls. After finishing the cell lining, they collected oil+pollen for the larval bred, and finally laid an egg. At good weather condition a female bee completed two cells per day. About 460 flowers (10 inflorescences) were needed to complete a cell for one larva. To sustain a viable population of 50-500 bees, 20 000-200 000 flowers (at least 400-4 000 inflorescences) are necessary.

Behavioural experiments with decoupled and combined visual and olfactory cues of *L. punctata* demonstrated that *Lysimachia*-inexperienced *M. fulvipes* females prefer olfactory over visual cues and primarily rely on olfactory cues to locate their host plants, whereas for experienced females the importance of visual cues was increased. In male bees visual cues play a more important role independent of experience. Overall, data demonstrate that the relative importance of visual and olfactory cues for locating host plants depends both on sex and experience of *M. fulvipes* bees.

In 17 different *Lysimachia* species we found 63 flower-specific compounds and 62 compounds were found in the vegetative scent samples. Vegetative and floral scent was species-specific and variability in floral but not vegetative scent was lower in oil compared to non-oil species. Although oil

species did not differ in either floral or vegetative scent from non-oil species we did find six floral scent compounds in oil species, which correlated with oil secretion. The petals of most yellow coloured oil secreting species appear green to bees, that of diverse coloured non-oil species appeared UV-blue, UV, UV-green, or blue-green, but never green to bees, whereas leaves in all species were similarly coloured. The bee green color of oil species correlated with oil secretion. Both floral scent compounds and the bee-green colour that correlated with oil secretion are likely selected by *Macropis* oil bees and may be involved in attracting these bees to the oil flowers.

Few compounds in the flower extract of *Lysimachia punctata* elicited antennal responses in *M. fulvipes* bees, among them diacetin, which was not known as a floral scent compound before this study. This compound was a key stimulus for attracting *M. fulvipes* bees in the bioassays, whereas others were needed to obtain attractiveness comparable to that of natural flower extract.

Diacetin not only occurs in scent samples of *L. punctata* flowers, instead I found it in many oil secreting species around the world. Similar to *M. fulvipes*, the South African oil bee *Rediviva neliana* responded to diacetin. In contrast, neither *Melitta haemorrhoidalis* non-oil bees, nor the honey bee responded in electroantennographic measurements to this compound. These findings point towards diacetin as a 'private communication channel' in the *Macropis-Lysimachia* and possibly also in other oil bee oil plant pollination systems. Diacetin is structurally similar to the floral oils and likely produced by similar metabolic pathways as the non-volatile fatty oils. Therefore it represents a reliable and honest cue for bees in search for oils.

Zusammenfassung

Das Bestäubungssystem zwischen Öl produzierenden Blüten und Blütenöl sammelnden Bienen schließt ungefähr 1800 Pflanzen- und 400 Bienenarten in vier Florenreichen ein. Es handelt sich um eine hochspezialisierte, mutualistische Interaktion. Die Bienen sammeln das fette Blütenöl, um ihre Nachkommen zu versorgen und in einigen Fällen auch, um ihre Brutzellen damit auszukleiden. In der Holarktis wird Blütenöl ausschließlich von einigen Arten aus der Gattung *Lysimachia* (Primulaceae) produziert, welche hauptsächlich von Öl sammelnden Bienen der Gattung *Macropis* bestäubt werden.

In meiner Arbeit konzentrierte ich mich (i) auf die Biologie der Ölbiene *Macropis fulvipes*, (ii) auf visuelle (Farbe) und olfaktorische (Duft) Signale in Öl sowie nicht-Öl produzierenden Arten der Gattung *Lysimachia*, (iii) die relative Bedeutung von Infloreszenzduft und -optik von *L. punctata* für *M. fulvipes* bei der Wirtspflanzenfindung und ich bestimmte (iv), welche Einzelkomponenten im Blütenduft für die Erkennung der Wirtspflanze entscheidend sind. Darüber hinaus testete ich, ob diese Signale auch in anderen Ölsystemen von Bedeutung sind. Die Beobachtungen der Bienen und die Verhaltensexperimente führten wir in einem 22 m^2 großen Flugkäfig durch.

Der Schlüpfzeitpunkt der Bienen variierte während der vier Jahre, war aber zur gleichen Zeit wie die Anthese der Wirtspflanzen. Erst ab einer gewissen Jahrestemperatursumme schlüpften die Bienen und besonders der Temperaturverlauf fünf Wochen vor dem Schlüpfen war ausschlaggebend. Das Geschlechterverhältnis der Population war ausgeglichen. Die männlichen Bienen schlüpften früher als die weiblichen in drei der vier Jahren. Nicht nur weibliche sondern auch männliche Bienen besuchten die Wirtspflanzen, um Pollen zu fressen. Dieses Verhalten löste bei den Weibchen das Anlegen einer Brutzelle und das Sammeln der Blütenprodukte aus. Sie sammelten zuerst Blütenöl, um ihre Brutzellen damit auszukleiden und anschließend Öl und Pollen für das Larvenbrot. Nach Fertigstellung und Verproviantierung der Zelle folgte die Eiablage. Bei gutem Wetter war es den Weibchen möglich, zwei Brutzellen pro Tag fertig zu stellen und zu verproviantieren. Für eine komplette Brutzelle haben die Bienen ungefähr 460 Blüten (10 Blütenstände) besucht. Um eine Population von 50 bis 500 Bienen zu erhalten sind somit 20 000 bis 200 000 Blüten (400-4 000 Blütenstände) der Wirtspflanze notwendig.

Die relative Bedeutung von Duft und olfaktorischen Signalen für die Wirtspflanzenfindung hängt vom Geschlecht der Biene und von ihrer Wirtspflanzenerfahrung ab. Die Männchen orientierten sich vorwiegend optisch. Naive weibliche Bienen orientierten sich eher am Duft im Vergleich zu visuellen Signalen, erfahrenen nutzten Duft und visuelle Signale etwa im selben Ausmaß zur Wirtspflanzenfindung.

Im Duft von 17 verschiedenen *Lysimachia*-Arten konnte ich 63 spezifische Duftstoffe der Blüten und 62 Duftstoffe der vegetativen Pflanzenteile ermitteln. Sowohl der Blüten- als auch der vegetative Duft waren spezifisch für die jeweilige Art. Sechs blütenspezifische Duftstoffe der Ölarten

stehen mit der Ölproduktion im Zusammenhang. Die Blütenblätter der Ölarten erscheinen den Bienen grün, die der nicht-Öl produzierenden Arten UV-blau, UV, UV-grün, oder blau-grün, jedoch nie grün. Das „bienen-grün" korreliert mit der Sekretion von Blütenölen. Die Duft- und Farbsignale, die mit der Ölproduktion korrelieren, wurden möglicherweise von *Macropis* selektiert und sind bei der Wirtsfindung dieser Bienen von Bedeutung.

Diacetin ist einer der Duftstoffe aus den Blüten von *L. punctata* und diesen Stoff habe ich auch in den meisten anderen untersuchten Öl produzierenden Arten nachgewiesen. Diese Substanz wurde bislang nicht als Blütenduftstoff beschrieben. *Macropis fulvipes* und die südafrikanische Ölbiene *Rediviva neliana*, nicht aber die nicht-Ölbienen *Melitta haemorrhoidalis* und *Apis mellifera*, können diese Komponente detektieren. Diacetin lockte in Verhaltensexperimenten *Macropis* Bienen an und spielt eine Schlüsselrolle in der Beziehung zwischen *M. fulvipes* und *L. punctata*. Es ist nicht auszuschließen, dass dieser Naturstoff auch in anderen Ölbestäubungssystemen sehr wichtig ist. Strukturell ist Diacetin den Blütenölen sehr ähnlich. Dies lässt vermuten, dass das Öl und Diacetin auf gleichem metabolischem Weg produziert werden. Somit ist Diacetin ein verlässliches und ehrliches Signal für das Vorhandensein des Blütenöls.

Introduction

Plant-pollinator interactions and advertisement in flowers

Insects are important pollinators of flowering plants (Ollerton et al., 2011), and pollination is the most important ecosystem service performed by insects (Losey and Vaughan, 2006). Economic calculation of the value of insect pollination services varies widely, but is within the scope of billions at the global scale (Losey and Vaughan, 2006, Potts et al., 2010, Kremen, 2005). Nowadays, pollination systems are threatened in many ecosystems (Murray et al., 2009, Potts et al., 2010) by lack of sustainable managed pollinators (Kevan and Phillips, 2001) as well as by land-use change and habitat fragmentation, which threatens the habitat of native pollinators (Kremen et al., 2007, Kearns et al., 1998, Potts et al., 2010).

Flowering plants have evolved numerous specific traits in scent, colour, shape and size of the flowers, or reward composition for pollinator attraction (Galliot et al., 2006). Flowers pollinated by the same visitors or the same guild of visitors tend to have particular features in common, which are related to the size, behaviour and other biological characteristics of their pollinators. The pattern of common floral characters may converge in species of quite different evolutionary origins. Flowers with these common patterns are classified in the so called 'pollination syndromes' (e.g., moth-pollination syndrome, bat pollination syndrome), each of which are characterised by specific reward, colour, scent, phenological, and morphological characteristics (Faegri and van der Pijl, 1979, Proctor et al., 1996, Fenster et al., 2004). Several of these syndromes are reward-based, as food (nectar, pollen, floral oils) is offered for pollinators or organic material for nest consturction (resign and waxes) (Minckley and Roulston, 2006). A vast array of non-rewarding, deceptive syndromes also have evolved, in which flowers mimick food, a brood site opportunity, or a sexual partner (Dafni, 1984, Brodmann et al., 2008, Schiestl et al., 1999).

Most important for pollinator attraction are visual and olfactory flower characteristics (Chittka et al., 2001, Lunau and Maier, 1995). The interplay between them is complex but several studies have shown that olfactory and visual cues can work synergistically and form a multimodal combined stimulus to attract pollinators (e.g. Kunze and Gumbert, 2001, Raguso and Willis, 2005). Shape, colour, colour pattern, and size can serve to attract pollinators without floral scent (Lunau, 1996, Gaskett and Herberstein, 2010, Osche, 1979, Ellis and Johnson, 2009). Compared to the visual cues, however, the complexity of floral scent is markedly higher if one only considers the 1 700 different volatile organic compounds described from nearly 1 000 species (Knudsen et al., 2006). Scents therefore seem to be a more specific attractant as visual cues. In contrast to the huge number of identified volatiles, only a few scent compounds were identified and directly linked to the attraction of

a specific pollinator (Whitehead and Peakall, 2009). Besides floral scent, the scent from vegetative plant material may also contribute to pollinator attraction and can even take over the signalling function from the flowers (Dufaÿ et al., 2003). In many cases, however, flower visitors respond especially to flower-specific scent cues, whereas the importance of vegetative material for pollinator attraction seems typically to be small. Instead, volatiles released from vegetative tissues are well known to deter potential herbivores (Lin et al., 1987) and also to attract parasitoids of herbivores following herbivore damage of leaves (Azuma et al., 1999, Turlings et al., 1990, Pichersky and Gershenzon, 2002).

Several plants pollinated by only a few of the potential available pollinators evolved different mechanisms to attract only appropriate pollinators and therefore obtain specificity. Specificity in pollinator attraction can be achieved either by sensory 'private channels', i.e. unusual compounds which are well detected by intended but not by non-intended receivers, or by specific ratios of ubiquitous compounds (Raguso, 2008b, Raguso, 2008a). For example, a sexually deceptive *Chiloglottis* orchid emits the female sex pheromone of *Neozeleboria cryptoides* wasps, chiloglottone, to attract and deceive the males of this species (Schiestl and Peakall, 2005, Schiestl et al., 2003). Specific ratios of oxygenated carboxylic acids mediate the pollination system between *Ophrys speculum* and males of *Campsoscolia ciliata* wasps (Ayasse et al., 2003). In the nursery pollination system between the fig species *Ficus semicordata* and its wasp pollinator *Ceratosolen gravelyi* the attractive floral compound 4-methylanisol is suggested to act as a private channel (Chen et al., 2009). Though all these compounds may indeed be private channels, the second assumption for a private communication channel, that the uncommon compound is hardly detected by other species of the available flower-visiting fauna has not been tested explicitly in above mentioned studies and also not in any other pollination system suggested to be mediated by a private communication channel (Raguso, 2008b). In the case of perfume collecting male *Euglossine* bees, sensory differences in the olfactory system as well as differences in the attractiveness of an uncommon aromatic compound (2-hydroxy-6-nona-1,3-dienylbenzaldehyde) reveal a mechanism by which two different species detect this compound differently from their habitat (Eltz et al., 2008). Although the origin of this scent is suggested to be from fungi and the compound is not suggested to be a private communication channel, because there is probably no fitness gain for the fungi when attracting the bees (compound may not be under selection by the bee), this study shows for the first time that component-specific differences in antennal perception can even exist in closely related insect species. Such an evolutionary shift in the olfactory system was also demonstrated in closely related *Drosophila* fruit flies, where the overrepresentation of methyl hexanoate neurons in the olfactory system of specialised *Drosophila sechellia* compared to *D. melanogaster* drives the olfactory preference of *D. sechellia* to methyl hexanoate, emitted by fruits of the host plant *Morinda citrifolia* (Dekker et al., 2006). These studies show that, though such adaptations towards specific compounds of host plants are not demonstrated in any pollinator yet, insects, even when closely related, detect specific compounds differently.

Specificity can also be reached when inappropriate floral visitors like florivores are excluded via 'floral filters'. Such filters can be of chemical nature, of morphological barriers, or of cryptic flower colouration (Raguso, 2008b). Especially plants, in which flowers are not morphologically adapted, e.g. by long nectar tubes (Moré et al., 2006) or floral spurs (Johnson and Steiner, 1997), to exclude particular floral visitors, can achieve specialisation or at least reduce the visitor spectrum through non-morphological filters like chemical ones. Such chemical filters range from deterrent volatiles in pollen scent, as it is discussed for protoanemonin in *Ranunculus* species (Praz et al., 2008, Jürgens and Dötterl, 2004), to additives in nectar, which are deterrent, repellent, or unpalatable for inappropriate visitors but not for pollinators (Irwin et al., 2004, Shuttleworth and Johnson, 2009, Adler, 2000, Johnson et al., 2006). To increase specificity, different types of floral filters (e.g. unpalatable nectar and cryptic colouring of flowers) can be combined (Shuttleworth and Johnson, 2009) or a floral filter co-occurs with a private channel (Chen et al., 2009, Raguso, 2008b).

In reward based pollination systems floral cues typically signal the rewards honestly. The cues are represented by scents, colours, or other sensory stimuli that indicate the presence of a metabolic reward, like nectar or pollen (Raguso, 2003). Although pollinators rely on these signals to detect the rewarding flowers, these signals do mostly not have any direct link to sugar (nectar) and protein (pollen) rewards (Dudareva and Pichersky, 2006). Pollination systems, in which the signal for host location is the reward itself or biosynthetically very similar to the rewards are very rare, but can be found in systems involving male perfume collecting euglossine bees (Teichert et al., 2009) and male tephritid flies (Tan et al., 2006). Euglossine males are attracted by and collect these scent compounds and use them during courtship behaviour (Eltz and Lunau, 2005), whereas male flies collect and either convert these compounds into male sex pheromones (Tan and Nishida, 2000), or directly use collected compounds for mate attraction (Tan and Nishida, 2005).

Host plant finding in specialised bees

Bees are the most important animal pollinators (Michener, 2007). Most bee species live solitary; they construct their own nests and provide food for their offspring. Among them, pollen specialised bees, so called 'oligoleges', restrict pollen foraging to a single plant species, genus, tribe, or family (Cane and Sipes, 2006). For these specialised bees it is essential to find their host plants among the vast array of other potential hosts. Therefore host plants need to have reliable floral cues that allow the bees to find, to recognise, and to discriminate them from non-host plants.

The fact that floral scent is nearly unlimited in its scent complexity (qualitatively, quantitatively, ratio of compounds) while the olfactory system of bees can be equipped, as shown for the the honey bee, with as many as 163 receptors (Robertson and Wanner, 2006), makes the floral scent as a cue for pollinators more distinctive in comparison to the floral colours. Olfactory cues can exhibit a highly specific identity of flowers, and especially in plants associated with oligolectic bees specific flower volatiles are suggested to play a key role in host recognition (Dobson, 1987, Dobson and Bergström, 2000, Dötterl and Vereecken, 2010). However, until now we know only a few specific floral compounds that are used by bees to recognise its host plants: (*E*)-cinnamaldehyde is used by the squash bee *Peponapis pruinosa* (Andrews et al., 2007), 1,4-dimethoxybenzene by the willow bee *Andrena vaga* (Dötterl et al., 2005a), the pollen odour protoanemonine by the *Ranunculus* specialist *Chelostoma florisomne* (Dobson and Bergström, 2000), and 1,4-benzoquinone by the *Echium* specialist *Hoplitis adunca* (Burger et al., 2011).

Bees are able to discriminate colours (Chittka et al., 1997), while their eyes are typically equipped with three spectral receptor types: the UV, blue and green receptor (other than in human which have a green, blue and red receptor) (Peitsch et al., 1992). Innate preferences in generalist and specialist bees was shown for blue (+UV) and yellow colours (Giurfa et al., 1995, Dobson and Bergström, 2000), however, until now studies about the relative importance of colour in comparison to scent for host plant finding in specialised bees are scarce. In the above mentioned *Echium* specialist *Hoplitis adunca* the visual stimulus of the host plant is obligatory to find the host plants, however the bees use specific scent cues to recognise them (Burger et al., 2010). In the *Campanula* specialist *Chelostoma rapunculi* it was demonstrated that the bees use either colour, scent or both cues to discriminate host plant flowers from other co-occuring plants (Milet-Pinheiro et al., 2012). Although the visual cues alone appear to attract the bees more strongly than olfactory cues, the combination of both was most attractiveand elicited most landing behaviour. This indicates that *Chelostoma* can use the single cues for host plant location, but integrate both cues for host plant recognition.

The oil bee oil flower pollination system

Among the 18 000 described bees worldwide (Michener, 2007), ca. 400 species of a few genera in Melittidae and Apidae collect floral fatty oil instead of nectar (in a few cases in addition to nectar) for their offspring. In some oil bee species the floral oil is additionally used to line the cell walls in the nest (Cane, 1983, Vogel, 1986, Alves-Dos-Santos et al., 2002, Aguiar and Garófalo, 2004). This floral oil is secreted by ca. 1 800 plant species in 11 different families (Renner and Schaefer, 2010) in specific organs (elaiophores). Oil secreting plants are almost exclusively pollinated by oil bees. Depending on the species, the oil is secreted in various parts of the flower, either from localised clusters of trichome elaiophores or from epithelial elaiophores (Vogel, 1974). This fascinating pollination system between oil secreting plants and oil collecting bees was discovered by Stefan Vogel in the 60ies (Vogel, 1969). He was the first who interpreted the oily substance in the flowers as a reward for oil collecting bees. This mutualistic interaction between oil secreting flowers and their bees is best developed, both in numbers of species and in diversity, in the Neotropic region and South Africa, but it is also found in Holarctic and Palaeotropical regions (Vogel, 1990, Vogel, 1986, Vogel, 1974). The oldest plant clades with oil secreting species are the Neotropical Malpighiaceae family (75 - 64 Myr), the Palaeotropical Cucurbitaceae (57 - 42 Myr), and the Holarctic *Lysimachia* L. (Primulaceae) (52 – 28 Myr). The latter system even is assumed to have coevolved from the onset, based on temporal coincidence of oil secreting *Lysimachia* and oil collecting *Macropis* bees, the only oil bee pollinators of these plants. (Renner and Schaefer, 2010).

Oil bees use the floral oil to provide their larvae. The oil is mixed with pollen in most cases from non-oil hosts (Machado, 2004). However, *Macropis* in Holarctic as well as *Ctenoplectra* oil bees in Palaeotropical regions collect both oil and pollen from their oil host. *Ctenoplectra* additionally collects nectar from the male oil flowers (females only produce oil) (Vogel, 1990). The major components of the non-volatile floral oils are similar across unrelated plant families and include mono-, di- and triglycerides with long chain (C16–C20) saturated or unsaturated fatty acids with an acetoxy or hydroxy group at the beta carbon, which is exceptionally rare in nature (Cane, 1983, Neff and Simpson, 2005, Vinson and Frankie, 1988, Vogel, 1974, Vogel, 1986, Vogel, 1990).

The Holarctic oil-bee oil-flower pollination system

The single genus in the Holarctic floristic region with oil secreting species is *Lysimachia* (Primulaceae). Among the 191 described species, 75 secrete floral oil and only these are involved in the highly specialised pollination system with *Macropis* (Melittidae; Melittinae) oil bees (Vogel, 1986). Non-oil secreting *Lysimachia* species offer nectar/pollen as reward and were suggested to be pollinated by generalist bees or, in the case of a single cleistogamous species (*L. minoricensis*), reproductive success was expected to be independent of pollinators (Vogel, 1986). *Macropis* comprise 16 species, all of which collect oil and pollen exclusively from *Lysimachia* oil species (Fig. 1) (Michez and Patiny, 2005). These two plant products are the only food for their larvae. Additionally, they need the oil to line their brood cells in the ground (Vogel, 1976, Vogel, 1986, Cane, 1983, Buchmann, 1987). Male bees patrol *L. punctata* patches to find females for mating. For their own food supply adult *Macropis* females and males visit a variety of other plants for nectar (Vogel 1986).

Figure 1:

Macropis fulvipes female collecting oil and pollen in a flower of *Lysimachia punctata*

Aims of the research

In the present study I investigated the bionomics of the oil bee *Macropis fulvipes* in detail. I also determined whether bees use visual, olfactory, or both cues to find and recognise their host plants. Further, I studied the importance of the pollinator mode on the evolution of plant cues in the genus *Lysimachia* and analysed floral scent compounds used by *Macropis fulvipes* for host plant finding.

The specific questions of my research are

What is the emergence pattern, sex ratio, nesting and foraging behaviour of a *M. fulvipes* population?

How many flowers need to be visited by *M. fulvipes* females for constructing and provisioning one cell?

What is the importance of visual compared to olfactory cues for host plant recognition in *M. fulvipes* and do bees learn floral cues during foraging? How do male bees find these plants?

Is there a clear difference between the floral scents of oil compared to non-oil secreting species in the genus *Lysimachia*? Are scent/colour cues evolved through pollinator mediated selection?

Which *L. punctata* compounds are responsible for host plant finding in *M. fulvipes*? Are compounds used by *Macropis* also emitted in other *Lysimachia* and non-*Lysimachia* oil secreting species?

Is there an indication of olfactory adaptation in oil bees compared to non-oil bees towards specific compounds of oil secreting plants?

Synopsis

Materials and methods:

The flight cage – study side (publication 1, 2, 4)

A population of *M. fulvipes* was successfully established in a 22 m^2 flight cage (Fig. 2) that was placed in a greenhouse in the Ecological Botanical Garden (EBG) of the University of Bayreuth (for more details see publication 1, and (Dötterl and Schäffler, 2007)). We offered the bees *Lysimachia punctata* plants as pollen and oil source, and *Geranium sanguineum* L. and *Origanum vulgare* L. as nectar sources. The bees were additionally provided with a sugar solution (30%, a 1:1 mixture of glucose and fructose) given to *Geranium* flowers or an artificial feeder.

Figure 2: Flight cage for data collection

Bees emerged from nesting sites or were introduced. In both cases they were marked individually with plastic discs commonly used for marking of honey bee queens (Opalith number plates, 1-99, in five colours).

1)   Emergence of female and male bees was studied during four years and compared with air temperature data, while we assume that the hatching depend on the temperature regime. Female bees were recorded during foraging. We found that females collect either oil only for cell lining or oil+pollen for brood cell provisioning. We determined, depending on the reward they collect, the a) duration of a collecting trip (time from leaving the nest until re-entering the nest with floral rewards),

b) number of flower visits per trip, c) duration of a single flower visit, d) duration of a nest stay (the time from entering the nest after a trip until leaving the nest for another trip), and e) number of trips required to complete one cell. Further, to determine whether the two types of foraging trips occurred at different times of the day, the number of females making each type of trip was recorded once per hour over a period of eleven days. To estimate the oil+pollen load per bee ten females were weighed before and after one foraging trip.

2) For testing the relative importance of olfactory and visual cues in *Lysimachia*-inexperienced (naive)/experienced *M. fulvipes* bees (females and males), two choice bioassays were performed with flower shoots of *L. punctata* in quartz glass cylinders (Fig. 3); for negative controls empty cylinders were offered. The cylinders were a) transparent and closed (visual treatment), b) black and with holes (olfactory treatment), or c) transparent with holes (visual+olfactory treatment). Host plants were introduced after finishing the tests with the inexperienced bees, henceforth these bees were able to forage. When bees showed provisioning behaviour after a few days (experienced) we continued with the same tests as conducted with the naive bees to test whether behaviour of bees is influenced by foraging experience on host flowers. Generally, during the experiments the host plants were removed. Bees approaching to within 5-10 cm of a cylinder were counted and caught with an insect net to assure that an individual bee is counted only once in a specific test.
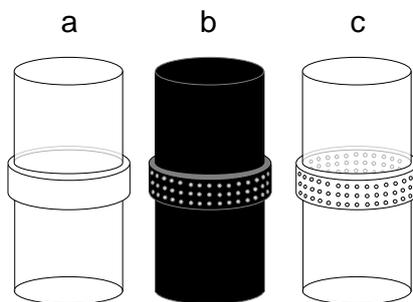
Figure 3: Quarz glass cylinders for testing the relative importance of visual and olfactory cues for host plant finding in *Macropis fulvipes*

4) To determine the importance of specific floral scent compounds of *L. punctata* for host plant finding in *M. fulvipes* bees we performed two choice bioassays by offering the test substances on a glass surface (bottom of a reversed beaker).

Volatile collection and chemical analyses of floral scent (publication 3, 4)

3) For comparison of scent within oil and non-oil as well as between oil and non-oil secreting *Lysimachia* species we collected dynamic headspace scent samples from 'flowers' (inflorescences in situ, cut inflorescences or individual cut flowers), and for comparative purposes from leaves (non-floral plant parts). Floral or vegetative parts were enclosed within polyester oven bags, and the emitted volatiles were trapped on a filter (in a quartz vials) using a membrane pump (headspace method: HS).

Ambient controls were collected from empty bags. The HS samples were analysed on a Varian Saturn 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector and a Chromatoprobe kit (GC-MS). Component identification was carried out using specific mass spectral data bases (NIST 08, Wiley 7, Adams, 2007), and confirmed by comparison of retention times with published data. Identification of individual components was confirmed by comparison of both mass spectrum and GC retention time with those of authentic standards (Dötterl et al., 2005b, Dötterl and Jürgens, 2005).

4) Flower scent samples from 50 oil and 8 non-oil secreting species from four florsitic regions were collected by elution to find potential similar compounds emitted by these species. The flowers were removed from the plants using clean forceps and extracted in 2-3 ml pentane for one minute. Floral compounds were identified using the GC-MS system as described before.

Colour analysis, hexagon colour space, and hexagon distances (publication 3)

Diffuse reflectance spectra were taken using a Varian Cary 5 spectrophotometer (Varian Inc., USA) equipped with a praying mantis accessory (Harrick Scientific Products, Inc., Pleasantville, NY, USA) to determine the floral colour of oil and non-oil secreting species in the genus *Lysimachia*. The mean reflections of petals and of leaves were used to determine the loci of petal colours in the hexagon colour space (Chittka, 1992). The positions of the colour loci show how bees perceive the colours with their ultraviolet, blue and green photoreceptors. We used the spectral sensitivity functions described for the honeybees as representative approximation for *Macropis* bees (Chittka and Kevan, 2005). To determine if bees can discriminate petal colour and its background as well as different petal colours of different *Lysimachia* species, the pairwise hexagon distances of colour loci among species, as well as the distance of each colour locus to its background (green leaves) was calculated (Chittka and Kevan, 2005).

Electrophysiology – GC-EAD (Gas chromatography-Electroantennographic detection) and EAG (Electroantennography) (publication 4)

Electrophysiological experiments were performed on a GC equipped with a flame ionisation detector (FID) and an EAD setup. In these experiments antennae of *Macropis fulvipes* were tested on flower extract samples of its host plant *Lysimachia punctata* and three other oil secreting species. For measurements the insect antenna was cut at the basis and the top and mounted between glass micropipette electrodes filled with insect ringer. The electrodes were connected to silver wires.

Dose-response curves (EAG) were measured for diacetin, which was the key compound in the *Macropis-Lysimachia* system and occurred in most of the oil secreting species. EAG tests were conducted with antennae of (i) *M. fulvipes* oil bees, (ii) *Rediviva neliana*, a South African oil bee ,closely related to *Macropis*, (iii) *Melitta haemorrhoidalis*, a Holarctic non-oil bee specialised on

Campanula species and closely related to both oil bees, and (iiii) honey bees (*Apis mellifera*), which are non-oil bees and generalised flower visitors.

Testing for correlated evolution and phylogenetic signals in floral scent and colour (publication 3)

We applied phylogenetically controlled correlations between pollination type (oil bee pollination vs non-oil bee pollination) and presence/absence of single scent compounds to determine the importance of pollinators and phylogeny on the evolution of floral and vegative scent in *Lysimachia*. The same method (presence/absence of the specific colour in oil secreting species) was used to determine the importance of pollinators and phylogeny on the evolution of floral colour.

The 'phylogenetic signals' that affected each compound (we used the ones tested for correlated evolution) as well as the presence of bee green colour (yellow coloured oil secreting flower) were assessed with independent Abouheif's test.

Results and Discussion

Phenology, foraging, and nesting behaviour of a *Macropis fulvipes* population (publication 1)

The flowering time of natural *Lysimachia punctata* plants and the emergence of *Macropis* bees in the flight cage coincided. The bees started to hatch when a specific temperature sum was reached. Especially the temperature profile in the last five weeks before emergence influenced the emergence pattern. Both the phenology of insects and that of plants is regulated by environmental cues (Reeves and Coupland, 2000, Mouradov et al., 2002), which might help to maintain synchronisation between insects and their host plants, especially if they use the same indicators.

In general, male bees emerged earlier than female bees (protandry) in the flight cage population and the sex ratio was mostly balanced. Protandry is described from numerous other European wild bee species (Westrich 1990) and seems to be a general rule in the life history of solitary bees (Stephen, 1969). This phenomenon guarantees the presence of males when females emerge and maximises the male/female reproductive success (Linsley, 1958, Wiklund and Fagerstrom, 1977).

After emergence, adults of both *M. fulvipes* sexes fed on pollen of *Lysimachia punctata* plants, which was not described before in *Macropis*. Pollen feeding is known from females of several other bee species and pollen is the principal protein source for bees (Michener, 2007). It is assumed to contribute to oogenesis because female bees were shown to use proteins from pollen to synthesise egg proteins (Hoover et al., 2006, Schäfer et al., 2006). In *M. fulvipes* it seems that pollen feeding triggers the nesting and provisioning behaviour in females, as they were searching for nests in the soil only when they fed on pollen before. Little is known about the importance of pollen feeding in male bees in general, but it is assumed that it influences their fitness (Colonello and Hartfelder, 2005).

Macropis female bees collected either floral oils only for cell lining or both floral products to provide their offspring on host flowers. Female bees visited on average 16 flowers in one oil collection trip but 56 flowers in an oil+pollen collection trip. They spent about 45 min to collect oil and prepare the cell lining, and about 90 min for collecting and storing the provisions for one larva. This larval bred had a weight of about 74 mg. On average the bees carried oil+pollen loads (11mg) of about a quarter of their body weight (42 mg). The floral products for one larva are collected from 460 flowers and in the flight cage it was possible for *Macropis* females to finish the provision for two larvae in one day at good weather conditions. Floral requirements for some non-oil bees has been estimated to range, depending on species, minimally from 1 to 1 100 flowers (Schlindwein et al., 2005, Müller et al., 2006). The minimal number of flowers needed is therefore highly variable and depends not only on the size of the bees but also on the amount of pollen available per flower as well as on other factors, such as the protein content of pollen (Müller et al., 2006). Consequently, it is difficult to compare the demand of flowers among bees if they use different host plants. In *M. fulvipes*, 20 000 – 200 000

flowers are needed to sustain a viable population of 50 – 500 individuals based on our data. Overall, our studies on the binomy of *M. fulvipes* provide new insights into the bionomics of bees.

Innate and learned responses to floral olfactory and visual cues in *M. fulvipes* bees (publication 2)

Lysimachia-inexperienced (naive) *Macropis* females preferred olfactory over visual *L. punctata* host plant cues, though visual cues increased the attractiveness of olfactory ones. In experienced females, the importance of visual cues was increased. Generally, bees that respond to visual cues of its host plant in the absence of olfactory ones may forage highly efficient under field conditions and the change of the relative weighting of visual and olfactory cues during learning (as observed in *Macropis*) seems to be an adaptive response. When revisiting host plant patches, e.g. by relying on their navigational memory (Reinhard et al. 2004; Von Frisch 1965), bees may see the plants but do not smell them e.g. due to wind blowing in the flight direction of the bees. When knowing the location of the host plant patches, visual cues therefore seem to be more reliable than olfactory ones (see also Kriston 1973).

In contrast to the female bees, both *Lysimachia*-naive and -experienced male bees relied more on visual cues. In inexperienced males, visual and olfactory cues had the same attractiveness, whereas experienced males mainly relied on visual cues for host plant location. Though inexperienced males strongly responded to visual cues when offered solely, we do not think that these cues allow identification of *Lysimachia* or discrimination from other plants. In the flight cage, male bees also patrolled other flowering and even non-flowering plants in the absence of *Lysimachia*. The innate responses towards the visual display of *Lysimachia* may have been a more generalised and not *Lysimachia*-specific response. To identify *Lysimachia*, they still may need the olfactory cues (see also Burger et al. 2010b). However, the visually guided female detection on flowers by males is a likely functional explanation for the differences in the weighting of visual and olfactory cues between the sexes, though final recognition of female mates is typically a matter of olfactory cues (Ayasse et al., 2001).

Overall, the visual (e.g. display size, shape, colour) as well as the olfactory (e.g. quality and quantity of floral scents) advertisement of flowers strongly differs among plants and may elicit more or less strong or specific responses in the visual or olfactory circuit of the pollinators, the sensitivity of which can differ among (Prokopy and Owens, 1983, Dekker et al., 2006) and within (Goyret et al., 2009) species. Depending on which cues are more reliable to locate the host plants or are more effectively detected, the pollinators seem to rely either more on visual or on olfactory cues (see also Burger et al., 2010, Milet-Pinheiro et al., 2012).

Scent and colour cues in oil and non-oil *Lysimachia* species and evolution of specific traits (publication 3)

We detected altogether 63 flower specific scent compounds in 15 different *Lysimachia* species and identified 50 of them. Most of the compounds are widespread floral scent constituents (Knudsen et al., 2006). However, we found also compounds like acetylated glycerides (1-monoacetin, 1,3-diacetin, 1,2-diacetin, triacetin) described for the first time as naturally occurring compounds. The floral scent was species specific and the variability in floral scent within oil species was lower than the variability in non-oil species. This may indicate that *Macropis* exerts a stabilising selective pressure on floral scent in oil-secreting *Lysimachia* species (Cresswell, 1998). We did not find overall differences in scent composition between oil and non-oil species, nor did we find any compound that was common in all oil species and which could be used by *Macropis* bees to discriminate oil from non-oil *Lysimachia* species. However, correlated evolution was found between oil-bee pollination and the pattern of occurrence of certain floral compounds, which were likely selected by pollinators. Among these compounds are linalool, 1-monoacetin, and 1,3-diacetin, that occurred in sympatric but distantly related oil species *L. punctata* and *L. vulgaris*. Linalool is among the most widespread floral scent compounds (Knudsen et al., 2006). It occurs in many species pollinated by specialised or generalised bees (Dobson, 2006) and is known as an attractant for social as well as solitary bee species (Dötterl and Vereecken, 2010). It also might be involved in host plant finding of European *Macropis* bees, though it might not be useful for *Macropis* to discriminate *Lysimachia* oil plants from other co-occurring plants. Better canditates for host plant recognition would be the acetylated glycerides 1-monoacetin and 1,3-diacetin (together with 1,2-diacetin and triacetin), which occurred with the exception of 1,2-diacetin, only in oil species. These glycerides resemble in their chemical structure the "non-volatile" floral oils (Vogel, 1986, Seipold, 2004, Dumri, 2008), and similar biosynthetic pathways are possible. These glycerides could indicate the presence of floral oils and *Macropis* bees could use these compounds as an ideal signal to recognise the oil secreting flowers (see publication 4). (*E*)-Cinnamaldehyde occurs in oil secreting species from three different clades and three different continents. It could have been selected by *M. fulvipes* and *M. europaea*, which can detect this compound (Schäffler and Dötterl, unpublished), and it could be important for host plant finding in *Macropis*, as it was shown that it attraceted specialized non-bees in another pollination system (Andrews et al., 2007).

In the vegetative scent there was no obvious pattern comparing oil and non-oil species. No compound occurred in more than one oil species and at the same time was absent from non-oil species. Our analyses therefore do not reveal a vegetative scent compound which seems to be under pollinator mediated selection and involved in attraction of *Macropis*.

Four of the five studied yellow coloured oil secreting species appear bee green to bees though they belong to three different clades. Bee green is not found in flowers of non-oil species (non-oil

flowers have UV, UV-blue, blue-green, or UV-green colours), though it is known to be attractive for generalist bees (Giurfa et al., 1995), the suggested pollinators of these species. Bee green may be attractive to *Macropis* in general (see publication 2), and there is evidence for correlated evolution between bee green and oil secretion. Five of the six studied white coloured non-oil species are blue-green for bees, and this similarity in colour can be explained by the close relatedness of the plants (all members of a single clade). Only one red coloured species of this clade evolved an UV-blue colour. Generally, these colours are known to elicit behavioural responses in bees (Menzel, 1985, Giurfa et al., 1995) and may be involved in attracting generalist bee pollinators in these species.

Chemical mediators in the oil flower oil bee pollination system (publication 4)

We found diacetin in the flower scent of *L. punctata* and in many other oil secreting species. This compound was not known as a natural compound before this study. It occurs as a floral scent compound in most (82%) of the studied Holarctic, Neotropic, and South African oil secreting plant species from quite different lineages (Asparagales; Malpighiales, Ericales, Lamiales). Due to the fact that the floral oils resemble diacetin in its chemical structure we assume that metabolic pathways proceed similarly in floral oil and diacetin production, while similar/same enzymes must be involved in esterification of the fatty acids with the hydroxy groups of glycerol (Yu et al., 2006). Based on these similarities between "non-volatile" floral oils and diacetin, we expect that diacetin is present in all oils that consist of a glycerol backbone and additionally have one or more acetyl group(s), whereas it may not be present in oils made up of other classes of compounds (e.g. free fatty acids). Indeed, we found diacetin in all plants having oils congruent with these criteria with the exception of two species, whereas we did not find diacetin in a species, the oils of which do not consist of acetylated glycerols (Seipold, 2004, Dumri, 2008). The suggested same metabolic pathway of the floral oils and diacetin makes diacetin an ideal and honest volatile signal for bees looking for floral oils.

Few compounds (heptanoic acid, geranic acid, (*E*)-2-dodecenal, 2-tridecanone) in the flower extract of *Lysimachia punctata* elicited antennal responses in *M. fulvipes* bees, among them, diacetin. Similar to *Macropis*, the South African oil bee *Rediviva neliana* responded to diacetin, in contrast, neither *Melitta haemorrhoidalis* non-oil bees, nor the honey bee responded in electroantennographic measurements to this compound. This difference in antennal response to diacetin between oil and non-oil bees demonstrates that the oil bees have specific olfactory adaptations (e.g. on the level of the ligand affinity of a specific olfactory binding protein (Stensmyr et al., 2003, Eltz et al., 2008, Hansson and Stensmyr, 2011) in the periphery of the olfactory circuit to detect diacetin. Such adaptations towards specific compounds of host plants were not demonstrated in any pollinator before.

The bioassays with *M. fulvipes* and EAD-active *L. punctata* scent compounds point towards a key function of diacetin in host location. Diacetin was capable in attracting *Macropis* bees, but less than a natural flower extract. A synthetic scent mixture containing diacetin and four other EAD-active

compounds was attractive as the natural extract. Further experiments revealed that two of the added compounds (2-tridecanone, geranic acid) are not involved in bees' attraction, but two other compounds (heptanoic acid, (*E*)-2-dodecenal) are. A mixture without diacetin did not attract any bee compared to a synthetic mixture with all the compounds.

Interestingly, while diacetin is very widespread and common in many oil secreting plants, the plants additionally emit other compounds, several of which are not that widespread and occur only in one or a few of the species, such as (*E*)-2-dodecenal (Kaiser, 2011, Steiner et al., 2011) and such compounds may also be involved in oil bee attraction. There is high overall variation in floral scent among oil plants, which is true for species within floristic regions and even for species pollinated by the same oil bee (Holarctic: publication 3, South Africa: Steiner et al., 2011) as well as among floristic regions. These findings led us to speculate that diacetin is a reliable volatile marker for 'non-volatile' fatty oils around the world, whereas the emission of other compounds may be important for allowing bees to discriminate among other co-blooming species.

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Publication 1

A day in the life of an oil bee: phenology, nesting, and foraging behavior.

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Original article

A day in the life of an oil bee: phenology, nesting, and foraging behavior

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Abstract – Little is known about the bionomics of solitary, ground-nesting bees. We established a population of the oil bee, *Macropis fulvipes*, in a flight cage and recorded the emergence phenology, sex ratio, nesting behavior, and foraging behavior of individually marked bees. The population was protandrous and the sex ratio was balanced in three of the four observation years. The date of first emergence varied even though the sum of temperatures before emergence was similar across years. Adults of both sexes fed on the pollen of *Lysimachia punctata* host plants. Females additionally visited flowers to collect oil for the nest-cell lining, as well as oil and pollen for larval provisions. Duration of collecting trips, flower visits, and nest stays were influenced by the reward collected. Bees required 12 collecting trips and 460 visitations to flowers to complete a single cell. Therefore, to sustain a viable population of 50–500 individuals, 20,000–200,000 flowers are required. Our study shows that observations in a closed system can provide new insights into the bionomics of bees.

Macropis fulvipes / oil bee / sum of temperature / solitary bee / *Lysimachia* / host plant requirement / nesting behavior / provisioning behavior

1. INTRODUCTION

Pollination is a process that occurs in almost all terrestrial ecosystems, and which is responsible for the seed set of many plant species as well as the genetic diversity of plant populations. Currently, pollination systems are threatened in many ecosystems (Murray et al. 2009) by lack of sustainably managed pollinators (Kevan and Phillips 2001) as well as by changes in the land use, which may threaten some native pollinators (Kearns and Inouye 1997). The main animal pollinators of wild plants and crops are managed honey bees (Kevan 1999) and native bees (Batra 1995; Klein et al. 2007). However, managed (Cox-Foster et al. 2007) as well as

unmanaged wild bee populations (Steffan-Dewenter et al. 2005; Biesmeijer et al. 2006; Murray et al. 2009) are subject to high rates of localized extinction. Understanding the biology of bees (e.g., host plant requirement, nesting biology, and phenology), especially of native wild bees, is important if we wish to protect and preserve these species, which would be one step towards ensuring pollination of wild and cultivated plants.

Pollinating bees typically use pollen and nectar as larval food. Quite a few bee species (360–370), however, use floral fatty oils together with or in lieu of nectar. 1,500–1,800 plant species produce this oil in specific floral organs, called elaiophores (Vogel 1974; Simpson and Neff 1981; Neff and Simpson 2005; Renner and Schäfer 2010). Some of the specialized oil bees additionally use the oil in the process of nest construction to line the cell

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wall (Cane et al. 1983b; Vogel 1986; Jesus and Garófalo 2000; Alves-Dos-Santos et al. 2002; Aguiar and Garófalo 2004).

Oil producing flowers and oil collecting bees are most widespread in the Neotropics, but these systems also occur in the Palaeotropics, Holarctic, and Southern Africa (Cape) (Neff and Simpson 2005). Oil collecting bees of the Neotropics and South Africa typically collect pollen from a variety of non-oil plants, whereas oil bees of the Palaeotropics and Holarctic collect pollen exclusively from their oil hosts. The only oil flowers in the Holarctic are 75 *Lysimachia* species (Myrsinaceae), which are tightly associated with the Holarctic *Macropis* bees (Melittidae; Melittinae). These bees collect oil and pollen from *Lysimachia* as the only food for their larvae and additionally use the oil to line their brood cells (Vogel 1976, 1986; Cane et al. 1983a; Buchmann 1987). *Lysimachia* flowers do not produce nectar, and adult *Macropis* visit a variety of other plants for nectar (Vogel 1986).

Macropis fulvipes (Fabricius 1804) is a widespread oil bee in Europe (Michez and Patiny 2005). It is a univoltine, solitary, and gregarious ground nester (Malyshev 1929). For nesting, *M. fulvipes* prefers banks or sloping ground and builds its cells near the surface (depth about 2.5 cm). The nests consist of a main tunnel with two or three lateral branches (each about 5 to 8 cm long) situated near the distal end of the main tube. Each branch has at most two cells situated end to end (Vogel 1986 and Celary 2004). *Lysimachia punctata* L. is an important host plant, and the interaction between *M. fulvipes* and *L. punctata* was studied in a pioneering study by Vogel (1986). He described the floral structure of *Lysimachia* and that of their elaiophores, analyzed the composition of the floral oil, and studied the morphological structures used by the bee to collect and transport the oil from *Lysimachia* to the nest. He also described the behavior of bees (in natural habitats) during harvesting of pollen and oil, and characterized the cell lining built with oil (see also, Cane et al. 1983a). Vogel (1986) also quantified the behavior of *Macropis* (e.g.,

number of flower visits and time needed for one collecting trip, number of collecting trips to build one cell), although he did not observe the bees during entire collecting trips or from the beginning to the end of cell provisioning. It was not possible to observe individual bees in the field at their nests and at host plant sites; therefore, several of these quantitative measurements—such as their floral requirements—are not conclusive. In *Macropis*, as in other specialized bees, the number of flowers needed to collect the reward for one offspring is especially important to know. Such data are necessary to determine the size of a plant population needed to sustain a viable bee population for conservation purposes. So far, however, the floral demand has been estimated only for a small number of bee species (Müller et al. 2006, and references therein).

We established a population of *M. fulvipes* in a flight cage and studied their emergence phenology, foraging and nesting behaviors, sex ratio, floral requirements (i.e., number of flowers needed) for constructing and provisioning one cell, and the weight of oil and pollen loads relative to the fresh weight of the bees. Our observations also revealed the factors that promote initiation of nesting behavior in *Macropis*.

2. MATERIAL AND METHODS

2.1. Study site

A population of *M. fulvipes*, nesting in the soil adhering to the bottom side of an uprooted beech stump, was caged in a greenhouse in the Ecological Botanical Garden (EBG) of the University of Bayreuth in spring 2006 (flight cage, $7.2 \times 3.6 \times 2.2$ m; wood-framed mesh gauze; Dötterl and Schäffler 2007). The side windows (2×15 m^2) and the roof lights (2×20 m^2) of the greenhouse were opened to avoid excessive temperatures. We offered the bees, which eclosed from the beech stump, *L. punctata* as a pollen and oil source, and *Geranium sanguineum* L. and *Origanum vulgare* L. as nectar sources. Bees additionally were provided with a sugar solution (30%, a 1:1 mixture of glucose and

fructose) that was added to the *Geranium* flowers or to an artificial feeder. Soil was arranged in a mound to offer additional nesting sites. To increase the population size of *Macropis* in the flight cage, free flying bees were caught in the EBG and introduced in the cage. Each bee that emerged from the stump nests or was introduced from outside the flight cage was marked with a plastic disc commonly used for marking honey bee queens (Opalith number plates, 1–99, in five colors). Because *M. fulvipes* is smaller than a honey bee queen, each label was trimmed to fit its smaller thorax. Before tagging, each bee was cooled on ice for several minutes. A few minutes after releasing, the tagged bees behaved normally.

2.2. Emergence phenology

The emergence phenology of *M. fulvipes* was recorded in 2006, 2007, 2008, and 2009. Newly eclosed bees were regularly marked (every 1–3 days) throughout the emergence periods. To test whether males and females emerged synchronously, the datasets of eclosed bees were compared separately for each year using a logistic model (StatSoft, Inc. 2004). The sex of the bees was coded as a dependent variable, and emergence day was used as a continuous predictor variable. In order to make the emergence dates of all 4 years comparable in one graphical representation, the days of emergence were aligned based on the first day of emergence. Total frequencies of emerged males and females each year were used to test whether the sex ratio was balanced (chi-square observed versus expected test, StatSoft, Inc. 2004).

2.3. Calculation of temperature sums as a trigger for emergence

Temperature sums are often used to forecast bud break or onset of flowering in plants (e.g., Galán et al. 2001). Because the phenology of *Macropis* had to be synchronized with the flowering period of the *Lysimachia* host plants, we applied temperature summing to forecast emergence of *M. fulvipes*. All daily average temperatures above an a priori defined temperature threshold and from an a priori defined date were cumulatively summed. To determine the temperature (measured at 2 m altitude) threshold and

the date that most precisely forecasted the emergence of *M. fulvipes*, we first tested different thermal thresholds (from 0 to 15°C, by steps of 1°C), and different dates (from 1st January to 1st April, by monthly steps) until the date where the first bee hatched. We performed these calculations for all 4 years (2006–2009) and compared the outcome among the years in order to calculate (depending on temperature threshold and date) the coefficient of variation (CV=SD/mean). The CV was lowest when the temperature threshold was 4°C, and when temperatures were summed beginning on 1st January (data not shown) indicating that these parameters were most appropriate to forecast the emergence of *M. fulvipes* (see Laaidi 2001, and Galán et al. 2001). We also determined, again separately for the four different years, the temperature sum of the 1–10 weeks prior to emergence, not to forecast, but to find the period before emergence that mostly influenced and triggered the emergence. By comparing the outcome among the years, we found that the CV was lowest when summing up the temperatures above 4°C during the last 5 weeks prior to eclosion (data not shown).

2.4. Observation of bees provisioning cells

In 2006, the behavior of female bees during provisioning of cells was investigated during good and comparable weather conditions. Preliminary observations revealed that *Macropis* bees visit their oil and pollen host *Lysimachia* for two purposes and in two distinct collecting trips: (1) to collect only floral oil and (2) to collect oil together with pollen during one flower visit (oil+pollen). To determine whether the two types of foraging trips occurred at different times of day, the number of females making each type of trip was recorded once per hour over a period of 11 days. Because of changing weather conditions during these days, however, it was not possible to monitor the number and type of foraging trips continuously from morning to evening. The number of replicates (days) ranged from one to 11. At a specific census, the percentage of bees that collected oil+pollen was calculated. We further used the total number of bees observed at a specific time of day that collected either oil or oil+pollen (dependent categorical variable) to test whether time of day (continuous predictor)

explained the reward collected by using a logistic model (Wald test; StatSoft, Inc. 2004).

As individually marked bees could be observed during the whole day, we further characterized the behavior of female bees during cell provisioning in detail. The following parameters were determined separately for oil and oil+pollen collecting trips: (a) duration of a collecting trip (time from leaving the nest until re-entering the nest with floral rewards), (b) number of flower visits per trip, (c) duration of a single flower visit, (d) time of a nest stay (the time from entering the nest after a trip until leaving the nest for another trip), and (e) number of trips required to complete one cell. Based on (b) and (e) we calculated the floral requirement. In cases where one parameter was recorded more than once for a specific individual, we calculated the mean and used this value for further calculations as parameters may be specific for each individual. Mann–Whitney U tests (StatSoft, Inc. 2004) were used to compare the different parameters between oil and oil+pollen collecting trips.

To understand how floral oil was used by a bee after returning to the nest, a flexible endoscope (flexible fiber Uretero-Renoscope ø 3 mm, type: 7,331.001 with a light cable ø 1.6 mm, type: 8,061.16; Richard Wolf GmbH, Germany) was inserted into the nest to observe bee behavior.

To determine the mass of oil+pollen collected during one foraging trip, we weighed 10 females on an electronic balance (Sartorius 1409, Sartorius AG, Göttingen, Germany) both before (after leaving the nest) and after (before entering the nest) the trip. To facilitate weighing, bees were cooled on ice.

3. RESULTS

3.1. Phenology

In 2006, the emergence period began in the middle of June, whereas in the following years bees eclosed approximately 3 weeks earlier (Table I). The duration of the emergence period was 18 days in 2006, and between 30 and 40 days in the following years (Table I; Figure 1). The number of bees emerging in the different seasons varied from 32 to 65 for males and 42 to 70 for females. The population was protandrous in 2006, 2007, and 2009 (males left their nests earlier than females), whereas emergence of females and males was synchronous in 2008 (Figure 1). More females than males eclosed from the nesting sites in the flight cage in all 4 years, however, only in 2008 was this difference significant (Table I).

3.2. Temperature sum

Across years, the temperature sum from 1st January until hatching of the first bee individual was between 463 and 525 K, and it varied between 299 and 307 K during the last 5 weeks before hatching (Table II).

3.3. Eating pollen, nest initiation

After emergence, both female and male bees visited *Lysimachia* flowers to feed on pollen. They facilitated this process by manipulating the anthers with their mandibles (Figure 2a, b).

Table I. Period of emergence of *Macropis fulvipes* males and females, number of emerged bees in the four observation years, and results of an observed versus expected chi-square test (test for a balanced sex ratio).

Year	Period of emergence		Number of emerged bees		$\chi2$	P
	♂♂	♀♀	♂♂	♀♀		
2006	13–27 June	17–30 June	32	42	1.351	0.24
2007	20 May–18 June	21 May–25 June	65	70	1.185	0.66
2008	29 May–22 June	27 May–25 June	41	66	5.841	<0.015
2009	22 May–25 June	23 May–30 June	42	50	0.695	0.40
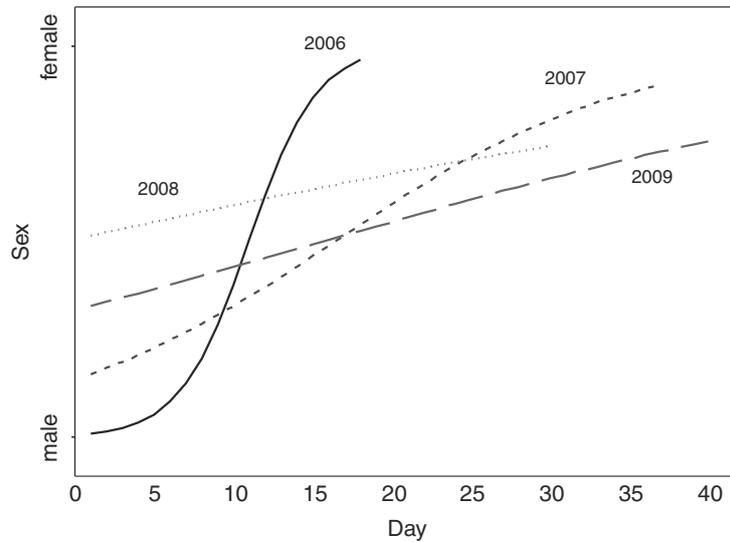
Figure 1. Fitted curves from logistic regressions on the sex of emerged bees in relation to the day of emergence (the day when the first bee emerged is given as day 1 in each year) in 2006–2009. In 2006, 2007, and 2009, the males emerged earlier than females (protandry); in 2008, both sexes emerged synchronously. Logistic regression—2006: $\chi^2=39.57$, $P<0.01$; 2007: $\chi^2=17.81$, $P<0.01$; 2008: $\chi^2=1.65$, $P=0.19$; 2009: $\chi^2=4.98$, $P=0.02$.

Females did not show any collecting behavior for the subsequent few days. During this time, we observed them regularly resting (at night or during bad weather conditions) together with males in aggregations on *Lysimachia* or *Geranium* flowers (Figure 2c). Following pollen feeding, females often flew near potential nesting sites (rootstock and soil hill), hovered from time to time at specific sites, and finally selected a place for nesting. Most females built new nests, but some females used pre-existing nests left from the previous year(s).

3.4. Collecting behavior

Two to three days after eating pollen, females started to collect floral rewards for their larvae. The observation of the bees during foraging bouts revealed that they visit *Lysimachia* flowers to collect only oil, or to collect oil together with pollen. To collect oil, they touched the staminal tube as well as the base of the petals, where most of the oil glands are situated, with the tarsal pads of the middle and front legs. Thereby, oil was taken up by capillary action. During this collecting behavior, the abdomen was in line with the head and thorax, and typically did not contact the anthers. Observations of a bee inside the nest after oil collecting trips using a flexible endoscope demonstrated that she used the oil collected during the previous trip for the lining of the cell, and not as larval food. Inside the cell the female brushed the oil onto the cell wall with the hind legs (scopae, in which oil is transported), and thereby coated the cell wall with this oil.

Table II. Heat sum in K of the four observation years from the 1st January until emergence of the first bee individual, and during the last 5 weeks before emerging, respectively.

	2006	2007	2008	2009	Mean±SD	CV
1st January until emergence	508.8	524.7	470.4	462.8	491.7±26	0.05
5 weeks before emergence	303.8	299.0	307.2	303.9	303.5±2.9	0.01

SD standard deviation, *CV* coefficient of variation
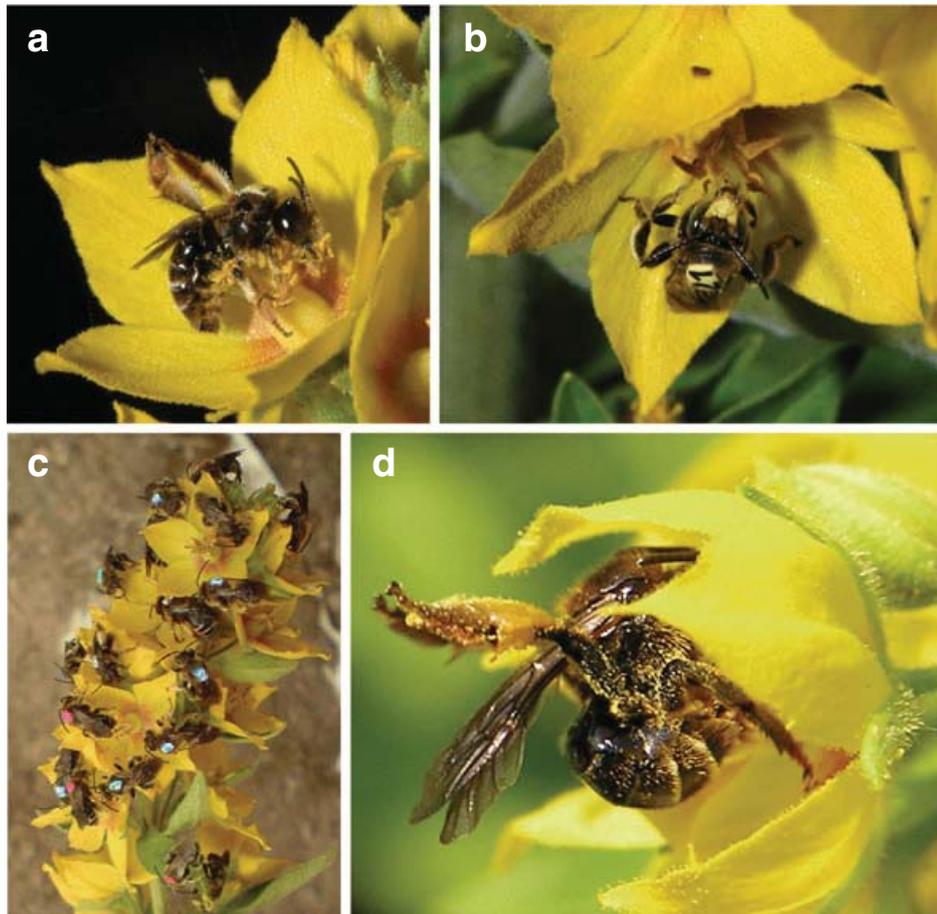
Figure 2. A newly emerged female (**a**) and male (**b**) of *Macropis fulvipes* feeding on pollen of *Lysimachia punctata*; individuals of both sexes resting on an inflorescence (**c**), and a female collecting oil+pollen (**d**). (**d**) was taken by Kathrin Milchreit.

Afterward, the bee licked the surface of the oil-containing cell wall.

Bees collecting oil+pollen handled the flowers quite differently from bees that collected only oil. To collect pollen, females pressed the ventral side of the abdomen (by bending) against the anthers (Figure 2d). Pollen grains thereby adhered to the sternal hairs, and subsequently were groomed and packed into the scopae of the hind legs to be carried into the nest. In the process of collecting pollen, bees thereby often manipulated the anthers with their mandibles, most likely to make more pollen available, and perhaps also to feed on pollen. While collecting pollen, they simultaneously collected oil with the front and middle legs as described above. The oil was also transferred to the scopae of the hind legs, where it was transported together with pollen.

The ethogram shows the sequence of collecting behavior by one female and reveals that the first time the female leaves her nest (either in the afternoon or in the morning) to start foraging, she begins with four oil collecting trips (Figure 3). The bee used the oil collected during these trips to line a new cell. After finishing the cell lining, she collected oil+pollen as food for the larvae. Provisioning of the cells was conducted in eight collecting trips during the afternoon and six during the morning. During four oil and pollen collecting trips in the afternoon, the bee visited a few *Geranium* flowers for nectar. Observations of other individuals during nest provisioning confirmed the sequence of behaviors: after excavation of a cell, the bees collected floral oil for the cell lining, and after finishing the cell lining, provided the cell with the larval food. They subsequently laid an egg, probably closed the cell
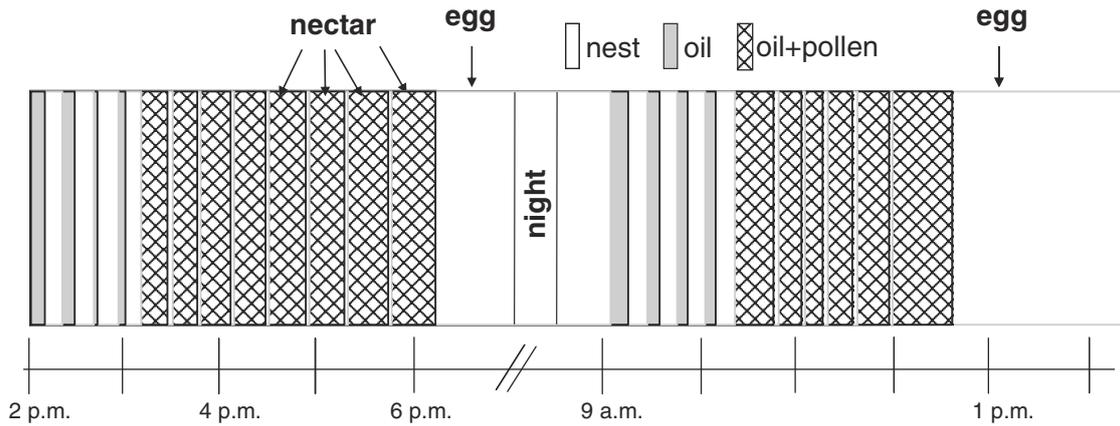
Figure 3. Ethogram showing the behavior of a *Macropis fulvipes* female during one afternoon and the following morning when provisioning cells. We recorded nest stays, as well as oil and oil+pollen collecting trips. During four oil+pollen collecting trips the bee visited few *Geranium* flowers each for nectaring and laid an egg in the evening after the last oil+pollen collection trip of the first day, and another one after the 6th oil+pollen collecting trip of the second day.

and built a new cell as indicated by burrowing behavior, which we observed in the main tunnel.

The collecting behavior of the female bees in the flight cage was nearly synchronous, and consequently the different kinds of collecting trips (oil versus oil+pollen) were not equally distributed throughout a day (logistic regres-

sion, Wald test: χ^2 (1; 10)=24.71, $P<0.01$; see also Figure 4). There was a diurnal pattern in cell preparation among the bees. Most bees collected oil from 8–9 a.m. and only a few collected oil+pollen at this time (median=0). The percentage of bees collecting oil+pollen increased until noon, decreased from noon to
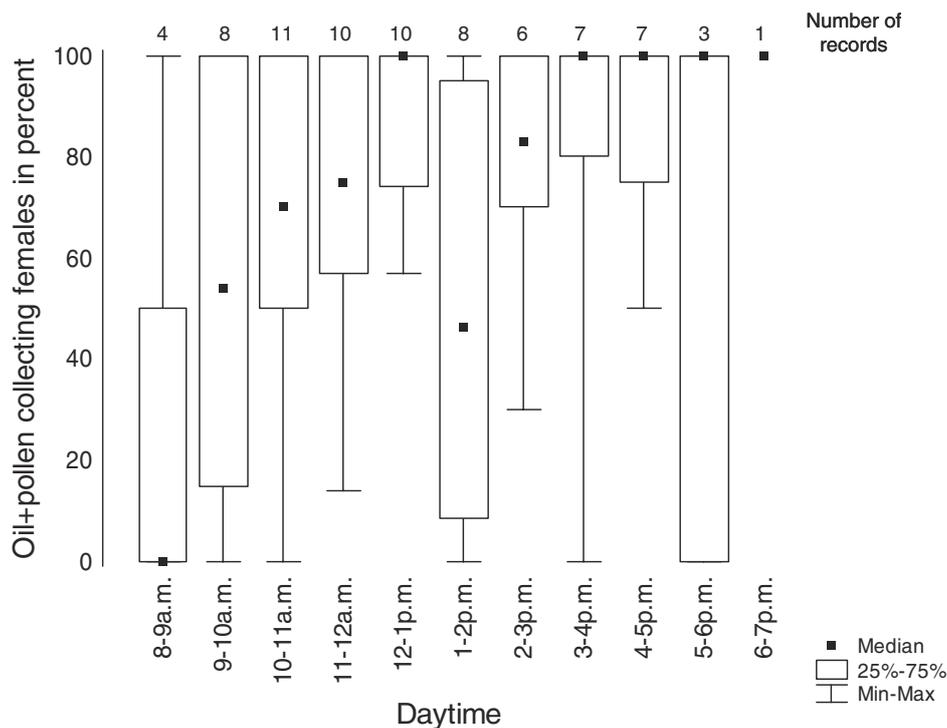
Figure 4. Percentage of females collecting oil+pollen and not only oil in the course of a day. Number of records gives the number of observations in a specific hour (see section 2 for more details).

early afternoon, and then increased again to 100% (median) in the afternoon and evening.

3.5. Quantitative data of collecting behavior

The duration of a collecting trip and the number of flower visits during one collecting trip differed between the two types of trips (Figure 5a, b). An oil collecting trip lasted 5 min (median), during which 16 flower visits were recorded, whereas an oil+pollen collecting trip lasted 10 min (median) during which 56 flower visits were recorded (U test oil vs. oil+pollen, collecting trip duration: $Z=-2.87$; $P<0.01$, $df=15$; number of flower visits: $Z=-3.09$; $P<0.01$, $df=12$). When collecting only oil bees visited a flower for twice as long (11 s) as they did when collecting oil+pollen (5 s; U test oil vs. oil+pollen, $Z=2.34$; $P=0.02$, $df=8$; Figure 5c). The bees stayed longer in the nest after an oil collecting flight (8 min) compared
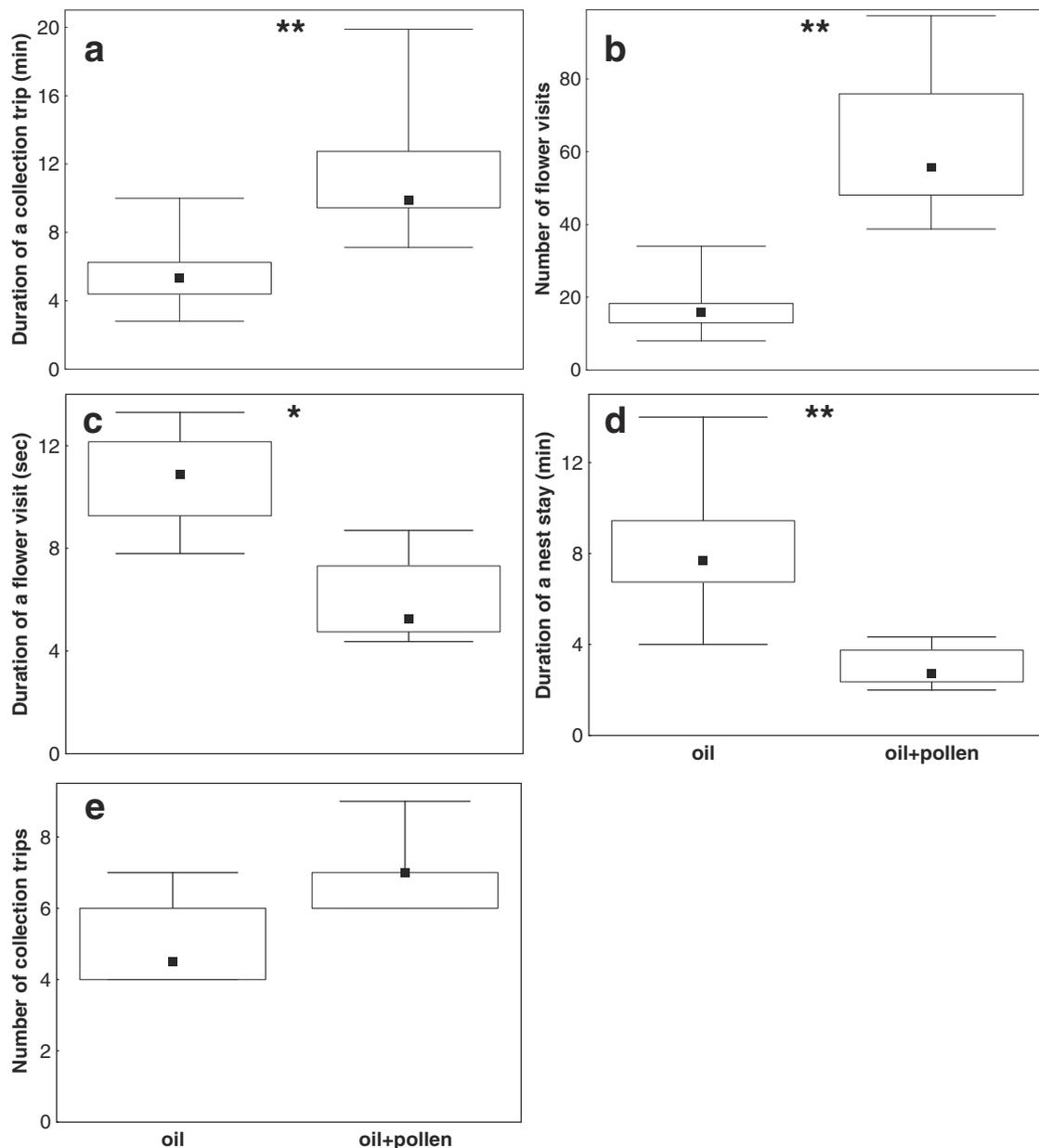
Figure 5. Duration of a collecting trip (**a**), number of flower visits per collecting trip (**b**), duration of a flower visit (**c**), and a nest stay (**d**), and number of collecting trips to complete one cell (**e**), depending each on the floral rewards collected (oil vs oil+pollen). The median, lower and upper quartile, and minimum-maximum are given. U test, *$0.05>P\geq0.01$; **$0.01>P\geq0.001$.

with an oil+pollen collecting flight (3 min; *U* test oil vs. oil+pollen, *Z*=3.09; *P*<0.01, *df*= 14; Figure 5d). The oil removal and lining of the cell took more time than the unloading of an oil+pollen packet. In total, females need in the median five oil and seven oil+pollen collecting trips to complete one cell (*U* test oil vs. oil+pollen: *Z*=−1.88; *P*=0.06, *df*=8; Figure 5e).

Overall, females spent about 1 to 1.5 h completing the provisions for a larva. The floral products for one larva are collected from 460 flowers (cell lining, 70 flowers and larval provision, 390 flowers; calculated from data presented in Figure 5b, e).

3.6. Oil+pollen load per bee

On average, the bees had a fresh body weight of 42 mg (min–max, 33–50 mg), and carried loads of 11 mg (min–max, 7–16 mg), which is approximately a quarter of their body weight (min–max, 18–33%).

4. DISCUSSION

4.1. Emergence phenology

In the 4 years of observation, the start of the flight period in both the *M. fulvipes* flight cage population and the free-living population in the EBG was very similar (Schäffler, unpublished data) and generally well timed with the blooming period of the *L. punctata* host plants. Flight activity periods of oligolectic bees generally correspond to the seasonal blooming period of their host plants (Linsley 1958). The phenology of insects as well as that of plants is regulated by environmental cues (Reeves and Coupland 2000; Mouradov et al. 2002), which might help to maintain synchronization between insects and their host plants especially if they use the same indicators. Photoperiod and temperature strongly influence the rate of diapause development in animals (Danks 2007), and temperature also influences plants' development and leaf-bud breaks in the spring (Wielgolaski 1999). Because

M. fulvipes nests in the ground, temperature rather than photoperiod is more likely to be the trigger for emergence. The initial time of eclosion differed among the four observation years. In the first year, the bees started to emerge in mid-June, whereas emergence in the following years started about 3 weeks earlier, during the second half of May. In plants, onset of flowering correlates with increasing air temperature, especially in spring (in temperate climates), and it was found that the start of flowering could be predicted by using temperature accumulation (heat sum) methods (Galán et al. 2001). By applying these methods to *M. fulvipes*, which is synchronized with the flowering phenology of *L. punctata*, we found that we could predict the date of emergence of these bees. *Macropis* bees start to emerge from the nests at a heat sum of 492 K (from 1st January until emergence of the first individual). This temperature accumulation method, which was originally designed for plants, has not previously been applied to bees (insects), and we therefore cannot compare our data with that for other insects. However, by using other methods (e.g., degree days), it has been shown that developmental temperature thresholds can effectively predict the date of insect emergence (Nietschke et al. 2007) and that the temperature regime during larval/pupal stages strongly influences emergence phenology of bees (Stephen 1965; Bosch and Kemp 2004). Our results also suggest that the temperature during the 5 weeks before emergence may be especially important for *M. fulvipes*, as there was only a very small difference in the heat sum during this period among the years (8 K, Table II). Overall, differences in the emergence phenology of *Macropis* are due to different temperature regimes among the different years.

In three of the four observation years, emergence phenology differed between the sexes and the *M. fulvipes* population was protandrous (Figure 1). Consistent with this finding, Celary (2004), who observed seven aggregated nests of *M. fulvipes*, noticed that males eclosed earlier than females. Protandry is described from numerous other European wild bee species (Westrich 1990) and seems to be a general rule in the life history of solitary bees

(Stephen et al. 1969). It guarantees the presence of males when females emerge and maximizes the male reproductive success (Linsley 1958; Stephen et al. 1969; Wiklund and Fagerström 1977). Why females and males eclosed simultaneously in 1 year of our observations is so far unclear.

4.2. Behavior after emergence

Macropis females visit *Lysimachia* flowers for three purposes: (a) to feed on pollen, (b) to collect oil for the cell lining, and (c) to collect oil+pollen as provision for the larvae.

a. Pollen feeding has not previously been described for *Macropis*, and this may be because it is obvious only when bees visit *Lysimachia* flowers for the first time. However, we cannot exclude that bees also feed on pollen when manipulating the anthers during the process of collecting oil and oil+pollen. Pollen feeding is known from females of several other bee species. Pollen is the principal protein source of female bees (Michener 2007), and is assumed to contribute to oogenesis because bees use proteins from pollen to synthesize egg proteins (Hoover et al. 2006; Schäfer et al. 2006; Minckley et al. 1994; Minckley and Roulston 2006). After feeding on pollen, *Macropis* females searched for an appropriate nesting site, constructed nests, and started to collect floral rewards (2–3 days after pollen feeding). Interestingly, we also found males of *Macropis* feeding on *Lysimachia* pollen. So far, little is known about the importance of pollen feeding in male bees in general, and whether it influences its fitness (e.g., through increasing production of sperm or proteinaceous accessory gland secretions; Colonello and Hartfelder 2005).

b. Our observations demonstrated the existence of oil collecting flights in *Macropis*. Though Vogel (1986) found females carrying only oil while foraging on *Lysimachia*, he could not observe the bees during a whole foraging trip in the field and was not sure whether the bees collected pollen subsequent to oil on a single foraging trip or transported the oil without pollen to the nest. Cane (1983a) noticed *Macropis nuda* females, returning to their nest, carrying liquid on their scopae and assumed that this was used for the cell lining, but he was not sure whether the liquid collected was actually oil. Similar oil collecting flights are, however, known for neotropical oil bees, and females of all these species seem to use oil to line the brood cells (Jesus and Garófalo 2000; Aguiar and Garófalo 2004; Alves-dos-Santos et al. 2002, 2006). In *Macropis*, Vogel (1976) assumed that floral oil is involved in the cell lining, which was confirmed later on by chemical analyses (Cane et al. 1983a). Cell linings are widespread in oil bees and more generally in other bees. They are hydrophobic, maintain the proper humidity in the cell, and protect the larval provision as well as the immature stages from inundation by water or attack by microorganisms such as fungi (e.g., Stephen et al 1969; Hefetz and Fales 1979; Albans et al. 1980; Cane 1981, Vinson and Frankie 1988; Rozen and Buchmann 1990). Non-oil bees however use resins or Dufour's gland secretions instead of oil to build the cell lining (Albans et al. 1980, Cane 1981). Interestingly, the Dufour's gland in oil bees (*Macropis* sp. and *Tetrapedia* sp.) is reduced, and its size is much smaller than in bees using Dufour's secretion for the cell lining (Cane et al. 1983b; Alves-dos-Santos et al. 2006).

Macropis makes 4–5 oil collecting trips to construct the nest cell lining, and this number is similar to that of neotropical oil bees, for example *Tetrapedia* (Camillo 2004) and *Centris* (Jesus and Garófalo 2000; Aguiar and Gaglianone 2003; Aguiar and Garófalo 2004). Differences are evident in the time invested in one oil collecting trip among oil bees. *M. fulvipes* needs 5 min, whereas *Tetrapedia* and *Centris* species require about 20 min. A scattered distribution of host plants in the habitat could be responsible

for the longer time needed in the tropical oil bees, whereas for *Macropis* oil was available ad libitum close to the nesting sites in the flight cage so that the time may also be increased in free flying *Macropis*. Additionally, the amount of oil per flower, the scopae absorption capacity of the legs, or the oil collecting behavior (Buchmann 1987) may lead to different times among bees. Beyond that, temporal variations are apparent during the activities of removing the oil and of lining the cells; it takes 8 min in *M. fulvipes* (after one trip), much less than in *Tetrapedia* (mean: 25 min; Alves-dos-Santos et al. 2002; Camillo 2004), but longer than in *Centris analis* (mean, 3.5 min; Jesus and Garófalo 2000). Additional comparative studies focusing on the behavior of the bees inside the nest are needed to be able to understand the differences among diverse oil bees in the time spent to remove the oil from the scopae and to perform the cell lining.

c. The collection of both oil and pollen during a single foraging trip is known for other *Macropis* species (Cane et al. 1983a) and for neotropical (Sérsic 2004; Cocucci 1991; Cocucci and Vogel 2001) as well as in South African oil bees (Dötterl, unpublished data). However, *Centris* and *Tapinotaspis* species are also known to collect oil and pollen during separate foraging trips (Alves-Dos-Santos et al. 2002; Aguiar and Gaglianone 2003; Aguiar and Garófalo 2004), and in such cases the bees collect oil for the cell lining (see above), to add it to the pollen mass already present in a partially provisioned cell, or both (Aguiar and Garófalo 2004; Camillo 2004).

Besides collecting oil+pollen in one bout, *Macropis* even collects oil and pollen simultaneously during a single flower visit. Simultaneous collection of oil and pollen is only known for some neotropical *Tapinotaspis* bees that collect oil and pollen on *Nierembergia* (Cocucci 1991), and, for one *Chalepogenus* species that collects the oil and pollen from *Sisyrinchium* flowers (Cocucci and Vogel 2001). Other neotropical

bees collect oil and pollen sequentially on a single flower (e.g., *Tapinotaspis* sp. on *N. browalloides*; Cocucci 1991), whereas the Palaeotropical *Ctenoplectra* collects pollen, oil, and even nectar sequentially from its *cucurbitaceous* host plants (*Momordica* and *Thladiantha*) during one flower visit (Vogel 1989). But in most cases, oil bees collect either oil or pollen from a specific host plant.

In our investigation, a female required, in the median, seven collecting trips (Figure 5e) to provision a single cell. This finding is similar to the five to eight collection trips suggested for *M. fulvipes* by Vogel (1986) and also similar to the number of trips required by *Centris* females (Aguiar and Gaglianone 2003; Aguiar and Garófalo 2004). *Tetrapedia* females need greater than 35 trips to supply their larvae (Camillo 2004), and accordingly it takes much longer to provision a cell (about 40 h, based on Camillo 2004).

Interestingly, *M. fulvipes* females invest less time per flower visit when collecting both oil and pollen, compared with oil alone. Therefore, it appears that *Macropis* harvests considerably more oil from a flower when restricting its visits to oil collection. Females may collect a smaller amount of oil when collecting both plant products together in order to maintain an optimal oil/pollen ratio for the larval food supply. This oil/pollen ratio may differ from the oil/pollen ratio offered by the flower. A flower seems to have too much oil relative to the pollen (or vice versa: too little pollen relative to the oil). It is unknown how bees determine the optimal proportion of liquid to solids during food provisioning trips (Neff 2008).

Vogel (1986) assumed that *Macropis* bees collecting both oil and pollen on a foraging trip gather oil actively, and pollen only passively. Our observations, however, demonstrate that pollen is also actively collected. Bees only pressed their abdomen against the anthers when collecting both pollen and oil, while the abdomen is not bent towards the anthers when bees collect only oil. Furthermore, bees scraped the anthers with their mandibles in order to

harvest more pollen only during trips when both oil and pollen were collected.

4.3. Number of cells per day

Under good weather conditions, a *M. fulvipes* female in the flight cage completed one cell in the afternoon and another cell during the following morning (Figure 2). This individual bee collected oil for the cell lining of one cell in the morning and for another cell in the afternoon. Interestingly, an observation of several additional bees during the day indicated that this pattern generally held for the entire population (Figure 4), suggesting a kind of synchronicity in the behavior of females. Females in the flight cage typically complete two cells per day, one in the morning and one in the afternoon. Under field conditions, however, *M. fulvipes* seemed to complete only one cell per day (Vogel 1986). The reduction in cell production under field conditions may be constrained by the availability of floral resources or proximity of nectar plants. The tropical and ground nesting oil bee *Centris aenea* is able to complete two cells per day under field conditions, whereas trap nesting *Centris* and *Tetrapedia* need at least 2 days (up to 5 days) for one cell (Jesus and Garófalo 2000; Aguiar and Gaglianone 2003; Aguiar and Garófalo 2004). Most non-oil solitary bee species complete one cell per day, but there are also very productive species, such as *Calliopsis persimilis* that can complete six cells per day (Danforth 1990). The rate of offspring production depends on the opportunity to gather large amounts of moderate quality pollen or smaller amounts of high quality pollen over a given time frame (Danforth 1990; Kim 1999). One might also expect that oil bees are more productive than non-oil bees, as oil has a higher energy content compared to nectar (Vogel 1974). However, the limiting factor for producing offspring not only depends on food availability but is also related to physiological constraints of egg maturation rates (Bosch 2008). Many different parameters influence the number of cells that a bee completes per day and this makes comparisons of fecundity among oil bees and between oil and non-oil bees difficult to interpret.

4.4. Number of flowers visited to complete one cell

We provide the first estimation of the floral visits required to produce an oil bee by counting the number of flower visits by marked individuals of female *M. fulvipes* bees. We found that approximately 70 visits for oil are needed to collect sufficient oil for the nest cell lining and approximately 390 oil+pollen visits are required to fully provision the nest cell, making a total of 460 floral visits necessary to produce one bee. Several thousand flowers were available simultaneously in the flight cage so it would not have been necessary for bees to visit a single flower more than once. However, we cannot exclude the possibility that flowers were visited more than once, and that the number of flowers required to produce one bee is actually somewhat lower than the total floral visits. Floral requirements for some non-oil bees has been estimated to range from 1 to 1,100 (Schlindwein et al. 2005; Müller et al. 2006). The (minimal) number of flowers needed according to such estimations is highly variable and depends not only on the size of the bees but also on the amount of pollen available per flower and other factors such as the protein content of pollen. It is therefore difficult to compare the demand of flowers among bees if they use different host plants.

Habitat loss, fragmentation, and degradation may lead to a shortage in food (and nesting sites) and is assumed to be responsible for the decline of many bee species (Kearns, et al. 1998; Müller et al. 2006). To conserve populations of bees, knowledge about their floral requirements is therefore crucial (Müller et al. 2006). The minimum size of a viable, isolated population of bees is regarded to range from 50 to 500, in order to maintain sufficient genetic variability for adaptation to changing environmental conditions (Shaffer 1981). Using these values and the data of the flower requirement determined in the present study, we can estimate the number of flowers needed to line the cells and produce enough food for a viable population of *M. fulvipes* at approximately 20,000 for a population of 50 individuals, and

200,000 for a population of 500 individuals. Because a flowering shoot of *L. punctata* produces ca. 50 flowers, 400–4,000 flowering shoots are needed to support a viable population of *M. fulvipes*. These values represent the lower limits required, as bees may need to produce an excess of cells to maintain a given populations size in order to account for losses (e.g., due to larval mortality). This high floral requirement in *M. fulvipes* explains why large populations of this species are found in Botanical Gardens with large populations of *Lysimachia* host plants (Westrich 1990).

4.5. Transport capacity and weight of larval provision

M. fulvipes females (8–10 mm) transport on average 25% (10.6 mg) of their body weight in every oil+pollen trip. One female makes an average of seven trips to provision a single cell (Figure 5e), and the larva therefore gets 74 mg of food (see also, Vogel 1986). So far, no data about the weight of scopal loads or larval provisions are available for other oil bees, but they are available for non-oil bees where weights of pollen loads as well as larval bread varies. Pollen loads of the similarly sized *Dieunomia triangulifera* (Michener 2007) are 18 mg and the larval bread is 70 mg (Minckley et al. 1994). While the weight of the larval bread is similar in *M. fulvipes*, the capacity to carry pollen is nearly double that of *D. triangulifera*. Large scopae make it possible for *Dasypoda hirtipes* (12–15 mm) to transport about 40 mg (larval supply, 290 mg; Westrich 1990) or approximately four times the amount of *M. fulvipes*. Pollen load weight depends not only on the size of the bee but also on its weight, and the morphology of its scopa. Furthermore, factors such as the type of load carried (dry or wet) as well as pollen texture make comparisons among species difficult (Neff 2008). Overall, Neff (2008) suggests that a female has to collect two to three times its fresh weight to provide a larva. From this perspective, a *Macropis* provision ball should weigh 80–120 mg. The actual weight is slightly lower than the lower limit,

which could be because floral lipids have higher energy content compared to nectar, and oil bees collect a smaller amount of oil than non-oil bees nectar (Vogel 1974; Rasmussen 1999).

4.6. Concluding remarks

In summary, we studied the bionomics of a solitary, ground nesting bee species in a flight cage. While some of the data observed in our closed system translate to a situation under natural conditions in the field (e.g., sequence of cell construction and provisioning, floral requirement, number of collection trips), others may differ (e.g., duration of a collection trip, and duration to complete one cell). Overall, our study gives new insights into the behavior of an oil bee, and generally shows that observations of solitary bees in a closed system are useful to learn more about their bionomics.

Une journée dans la vie d'une abeille récolteuse d'huile: phénologie, comportement de nidification et d'approvisionnement.

Macropis fulvipes / **somme des températures / abeille solitaire** / *Lysimachia* / **plante hôte / comportement de ponte / comportement d'approvisionnement**

Zusammenfassung – Ein Tag im Leben einer Ölbiene: Phänologie, Nestbau- und Verproviantierungsverhalten. Bienen sind die wichtigsten Bestäuber vieler Kultur- sowie Wildpflanzen (Klein et al. 2007; Michener 2007). Der Verlust von Lebensräu-

men bedingt durch die Veränderung von Landschaften führte zu einer Abnahme der Bienen in den letzten Jahrzehnten (Kearns and Inouye 1997; Murray et al. 2009). Um Bienen langfristig schützen zu können, ist es wichtig, deren Biologie und besonders deren Habitatansprüche (z. B. Anzahl benötigter Blüten, Niststandorte) zu kennen. Die vorliegende Arbeit befasst sich mit der Bionomie der im Boden nistenden und solitär lebenden Schenkelbiene *Macropis fulvipes*, die wir vier Jahre lang in einem Flugkäfig untersuchten. *M. fulvipes* ist auf das Blütenöl und den Pollen des Gilbweiderichs (*Lysimachia* spp.) spezialisiert. Das Blütenöl wird benutzt, um einzelne Brutzellen auszukleiden und zusammen mit Pollen dient es als Larvenfutter. Im ersten Beobachtungsjahr schlüpften die Bienen zwischen Mitte und Ende Juni, und damit ungefähr einen Monat später als in den darauffolgenden Jahren (Tab. I), wobei die Schlüpfzeit immer mit der Blütezeit der Wirtspflanze korrelierte. Möglicherweise waren Temperaturunterschiede zwischen den Jahren für diese Unterschiede in der Phänologie verantwortlich (Galán et al. 2001). In drei der vier Jahre erschienen die Männchen früher als die Weibchen. Bis auf ein Jahr, in dem mehr Weibchen als Männchen schlüpften, war das Geschlechterverhältnis ausgeglichen (Abb. 1; Tab. I). Nach dem Schlüpfen besuchten männliche sowie weibliche Bienen Blüten von *L. punctata*, um Pollen zu fressen (Abb. 2a, b). Proteine sind bei weiblichen Bienen für das Reifen der Eier wichtig (z.B. Hoover et al. 2006) und auch bei Männchen könnten sie einen Einfluss auf die Fertilität haben (Colonello and Hartfelder 2005). Die Weibchen begannen zwei bis drei Tage nach dem Fressen von Pollen und dem Graben eines Nestes, Blütenprodukte zu sammeln. Zuerst wurde Öl von *L. punctata* Blüten gesammelt (hauptsächlich morgens und am frühen Nachmittag, Abb. 3 and 4), um damit die Brutzellen auszukleiden. Danach besuchten sie die Blüten, um gleichzeitig Öl und Pollen als Larvenfutter zu sammeln. Einzelne Öl-Sammelflüge dauerten 5 Minuten, für einen Öl+Pollen-Sammelflug benötigten die Weibchen die doppelte Zeit (Abb. 5a). Während eines Öl-Sammelflugs besuchten sie 16 Blüten. Vier bis fünf solcher Flüge waren für die Auskleidung einer Brutzelle nötig. Während eines Öl+Pollen-Sammelfluges besuchten die Weibchen 66 Blüten. Sieben solcher Sammelflüge waren nötig, um eine Brutzelle zu verproviantieren. Das Sammeln von Öl dauerte elf Sekunden pro Blütenbesuch, das Sammeln von Öl+Pollen nur die Hälfte dieser Zeit. Daraus lässt sich folgern, dass die Blüten verhältnismäßig viel Öl im Vergleich zu Pollen produzieren und das Öl zu Pollen Verhältnis des Larvenfutters nicht dem Öl

zu Pollen Verhältnis in einer Blüte gleicht. Nach einem Öl-Sammelflug verblieben die Weibchen für sieben Minuten im Nest, nach einem Öl+Pollen-Sammelflug drei Minuten. Die Bienen benötigten 1 h, um Öl zu sammeln und die Zelle auszukleiden, 1,5 h, um Öl+ Pollen zu sammeln und die Brutzelle zu verproviantieren. Sie besuchten ca. 460 Blüten, um eine Zelle fertig zu stellen. Bei guten Witterungsbedingungen stellten die Bienen zwei Zellen pro Tag fertig. Zum einen gibt unsere Studie einen neuen Einblick in das Verhalten einer Ölbiene und zum anderen zeigt sie, dass Beobachtungen von solitären Bienen in einem Flugkäfig hilfreich sind, um mehr über deren Lebensweise und deren Ansprüche zu erfahren (Goubara and Takasaki 2003; Schindler 2004).

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Publication 2

Behavioural plasticity and sex differences in host finding of a specialized bee species.

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ORIGINAL PAPER

Behavioural plasticity and sex differences in host finding of a specialized bee species

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Abstract Many animals feed on flowers, and visual as well as olfactory cues are considered as most important mediators in animal–plant interactions. However, the relative importance of these cues is not well understood. Bees are the most important animal pollinators worldwide and here, we determined the importance of decoupled and combined visual and olfactory cues of *Lysimachia punctata* (Primulaceae) for host plant location in both sexes of the specialized, solitary bee, *Macropis fulvipes* (Melittidae). *Lysimachia*-inexperienced female bees preferred olfactory over visual cues though visual cues increased the attractiveness of olfactory ones. In experienced females, the importance of visual cues was increased. Both *Lysimachia*-naive and -experienced males relied more on visual cues as compared to females. This study demonstrates that the relative weighting of cues used for host plant finding depends on the sex and experience of *M. fulvipes*. The latter finding reveals the presence of learning-induced behavioural plasticity in host plant finding for a bee species. It may allow the bee to forage highly efficient. Visually guided female detection on flowers by males is a likely functional explanation for the differences in the weighting of visual and olfactory cues between the sexes.

Keywords Bioassay · Learning ·
Oil-flower oil-bee pollination system ·
Host location · Visual and olfactory cues

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Introduction

Almost 90% of the flowering plants are suggested to be pollinated by animals (Ollerton et al. 2011), and the visual and olfactory advertisement of flowers is most important for attracting pollinators. Little is known about the relative importance of these cues and there are very few studies available in which the attractiveness of natural visual cues is compared with the attractiveness of natural olfactory cues of host pants (e.g., Burger et al. 2010b). However, it is a general belief that olfactory cues are most important for attracting pollinators at night, when visual cues are inefficient (Brantjes 1978; Gottsberger and Silberbauer-Gottsberger 1991, but see Raguso and Willis 2002), whereas either visual or olfactory cues seem to be more important or have a similar attractiveness in diurnal pollination systems (Naumann et al. 1991; Andersson and Dobson 2003; Balkenius et al. 2006). Bees are the most important diurnal pollinators worldwide, and several species of bees restrict their diet to a limited group of flower species (oligolectic bees) (Michener 2007). For these oligoleges, it is essential that they find their specific host plants among the vast array of other potential host plants. The plant cues therefore need to be specific to allow bees to detect their host plants from co-occurring plants. After hatching, foraging-naive bees need to rely on the innate search image (see also Menzel 1985) for host plant location and this search image may be altered subsequently through learning. Bees are well-known for their excellent capabilities to learn many different kinds of plant cues (Menzel and Müller 1996). So far, however, little is known about the innate as well as the experienced search image in bees in general, and especially in specialized species (Dötterl and Vereecken 2010).

In the specialized bee species studied so far, olfactory and/or visual cues were involved somehow in the process

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of host plant location or host plant recognition (Dobson 1987; Dobson and Bergström 2000; Dötterl et al. 2005; Dötterl and Schäffler 2007; Andrews et al. 2007; Dötterl and Vereecken 2010; Burger et al. 2010b), but the relative importance of the visual in comparison to the olfactory cues for foraging-naïve as well as foraging-experienced female bees remains poorly understood. In a recent study with *Hoplitis adunca*, a bee specialized on *Echium/Pontechium*, it was shown that foraging-naïve as well as -experienced females are only attracted to the visual and not olfactory cues of *E. vulgare* when tested alone. However, olfactory cues increased the attractiveness of visual cues and were essential for discrimination of a host from a closely related non-host plant, a task that could not be performed by visual cues alone (Burger et al. 2010b). The authors concluded that *H. adunca* bees are alerted of potential host plants and approach them using their vision, and subsequently use their olfaction to finally recognize the host plants. *E. vulgare* inflorescences have a large visual display, but emit only a small amount of scent (Burger et al. 2010a), which may, in contrast to the visual display, just be perceived by the bees when they are already close to the inflorescences. This may explain why bees were not attracted to olfactory cues alone. Bees specialized on plants with stronger scents may behave differently.

Though in most bee species (excluding euglossines and parasitic species) females visit rewarding flowers more frequently than males, flowers and flowering patches are also essential for males. First, it is essential also for males to visit flowers for nectar (Michener 2007), and second, flower patches are the most common encounter/mating sites for bees (Eickwort and Ginsberg 1980). However, the relative importance of visual and olfactory cues for finding nectar and patrolling sites has not been studied so far in males, and it remains to be tested whether males and females use the same cues to find specific plants.

Here, we determine the relative importance of visual and olfactory cues for host plant location of an oligolectic *Macropis* oil-bee (Melittidae). *Macropis* does not, as most of the c. 20000 bee species (Michener 2007), collect nectar and pollen as food for its larvae, but rather collects fatty floral oil in addition to pollen (Vogel 1986). The only oil and pollen host plants are a few species within the genus *Lysimachia* (Primulaceae). Recently, we have shown that inflorescences of *L. punctata* L. oil plants emit quite strong scents, and that flower-experienced *M. fulvipes* (Fab.) females strongly respond to floral extracts of this host plant even without visual cues (Dötterl and Schäffler 2007). Responses to visual floral cues of *Lysimachia* alone and of inexperienced bees were not studied. *Lysimachia* plants are not only visited by female *Macropis* bees but also by males, which feed on pollen as newly hatched individuals when they encounter *L. punctata* for the first time

(Schäffler and Dötterl 2011) and patrol at patches of *Lysimachia* in search for females during their life (Vogel 1986). The responses of males to visual and olfactory cues have not been studied so far. We therefore offered combined (olfactory + visual cues) and the decoupled cues of *L. punctata* to *Lysimachia*-inexperienced and -experienced *M. fulvipes* bees of both sexes. This is a powerful technique to explore the relative contribution of a specific sensory modality for host plant finding of a pollinator (Raguso 2006; Burger et al. 2010b) and our approach also reveals whether the cues used by the bee depend on its sex and foraging experience.

All oil-producing *Lysimachia* species have yellow (to the human eye), bowl-shaped radial flowers (size in *L. punctata* c. 2 cm). In one species, *L. vulgaris*, the colour was quantitatively determined, and it was found that light is mainly reflected from 520 to 700 nm (Arnold et al. 2010). We found a very similar reflectance spectrum for *L. punctata* by spectrophotometry (Schäffler I, unpublished data). This yellow colour is very widespread and among the most common floral colours (Chittka et al. 1994). One might predict, therefore, that the colour of *L. punctata* alone might not be a useful specific host plant cue for *M. fulvipes*. Other visual cues relative to the structure of a flower (e.g. shape, radial symmetry) also seem unlikely to provide host plant specific cues to pollinators as they are also very common in other plant groups. In contrast, its variety of volatile compounds (Dötterl and Schäffler 2007) may provide a greater potential for specific host recognition cues. We therefore hypothesize that both sexes of *Lysimachia*-inexperienced as well as -experienced bees rely mainly on olfactory rather than visual cues of *L. punctata* for host plant location, though they might use visual cues of flowers/inflorescences to navigate to them after getting already attracted by olfactory ones (see also Streinzer et al. 2009).

Materials and methods

Study site

To test the relative importance of visual and olfactory cues to *Lysimachia*-inexperienced versus *Lysimachia*-experienced *M. fulvipes* bees in locating its *L. punctata* host plants, we conducted in 2007 and 2008 several two-choice bioassays in May (2007 only) and June in a flight cage (7.20 × 3.60 × 2.20 m), which was set up in a greenhouse (Dötterl and Schäffler 2007). During the experiments, the side (2×15 m^2) and overhead (2×20 m^2) windows of the greenhouse were opened to allow the natural light (without being filtered through the glass) entering.

The bees

All the bees used for the experiments were from a population established 2006 in the flight cage and before the experiments with experienced bees we also inserted bees caught in the adjacent grounds of the Ecological Botanical Garden of the University of Bayreuth (where a large population exists). All bees were individually marked after hatching or before introducing them in the cage with plastic labels from commercially available sets of sequential numbered tags (1–99) in five colours, such as commonly used for marking honey bee queens. Bees were free flying in the cage.

In both years, about 70 females and 40 (2008) to 65 (2007) males hatched in the flight cage. Before hatching, we excluded *Lysimachia* host plants; hence *Macropis* bees remained inexperienced with *Lysimachia* plants until the first bioassays (see below). When we finished the assays with the naive bees, we offered them potted and cut *L. punctata* plants and flowering shoots, respectively. Both sexes visited the flowers initially to feed on pollen. Thereafter males mainly patrolled the plants in search for females, and females regularly collected pollen and oil as larval provision after a few days (Schäffler and Dötterl 2011). These bees were then used for the two-choice bioassays as *Lysimachia*-experienced individuals. 1–2 h before the experiments started, however, *Lysimachia* plants were removed from the flight cage. For bees' energy supply, flowering *Geranium pratense* L. plants, which are often used by free-flying bees as a nectar source (Westrich 1989), and additionally a sugar solution (30%, a 1:1 mixture of glucose and fructose, v/v), which was given to *Geranium* flowers or provided by an artificial feeder, were offered all the time. If *Macropis* bees had been hungry during the experiments we would have expected them to search not only for *Lysimachia* but also for nectar plants as well. Because bees were well fed during our experiments the responses observed were unlikely to have been influenced by a need for nectar or by the experience of bees with *Geranium* flowers/the artificial feeder.

Bioassays

Five different two-choice bioassays were performed with naive as well as experienced female and male *M. fulvipes* to determine the relative contribution of visual and olfactory cues for location of host plants (Table 1). We tested visual and olfactory cues against a negative control, visual versus olfactory cues, and a combination of both cues versus visual or olfactory cues.

For the bioassays, six flowering shoots of *L. punctata* (not for the negative controls, see below) were offered to the bees in quartz glass cylinders (i.e., six shoots per

Table 1 Two-choice tests conducted in 2007 and 2008 with *Lysimachia*-inexperienced and -experienced *Macropis fulvipes* bees and flowering shoots of *Lysimachia punctata*. Separate olfactory and visual cues, both cues together, and negative controls (empty cylinders) were tested. Numbers in brackets give the number of replicates performed each year

	Inexperienced	Experienced
Olfactory vs. control	2007 (2) + 2008 (1)	2007 (1) + 2008 (2)
Visual vs. control	2007 (1) + 2008 (1)	2007 (1) + 2008 (2)
Olfactory vs. visual	2007 (1) + 2008 (2)	2007 (3) + 2008 (1)
Olfactory + Visual vs. visual	2008 (2)	2007 (2) + 2008 (1)
Olfactory + Visual vs. olfactory	2008 (2)	2008 (2)
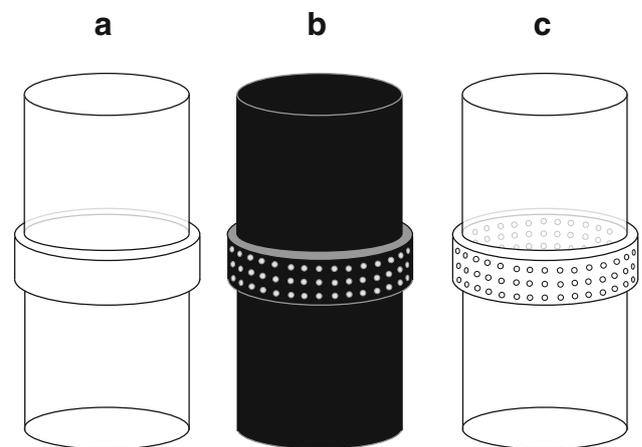
Fig. 1 Diagrams of quartz glass cylinders used to test the attractiveness of visual (**a**), olfactory (**b**), or a combination of both cues (**c**) of *Lysimachia punctata* inflorescences in behavioural experiments with *Macropis fulvipes*

cylinder). If both cylinders that were tested against each other contained flowering shoots, we picked 12 shoots, randomly selected six for one of the cylinders, and used the remaining six for the other. Quartz glass was used because UV can pass this kind of glass. UV is known to be an important component of the visible spectrum for many animals, among them bees (Lunau and Maier 1995). Three different types of cylinders (outer diameter of all was 10 cm, height 29 cm) were used to offer the bees either the combined visual and the olfactory cues of *L. punctata*, or to decouple the visual from the olfactory cues and offer the bees these cues separately (Fig. 1). Cylinders were attached to a wooden table, and offered the bees at a height of 10 cm. The cylinders were a) transparent and closed, such that the bees could see the flowers, but not smell them (visual treatment) b) painted black and had 60 holes (2 mm diameter; arranged in three horizontal lines in the centre of the cylinder), through which scent of *Lysimachia* was

pumped out (1 L min^{-1}) by a membrane pump (G12/01EB, ASF Thomas, Inc.) (olfactory treatment) or c) transparent and with the same number and arrangement of holes as above (olfactory + visual treatment). For the negative controls, we used corresponding empty cylinders (transparent and closed, or black with holes).

To test whether bees are attracted to non-flowering shoots, we offered inexperienced females and males, the combined visual and olfactory cues of six non-flowering shoots, and for comparison an empty transparent cylinder with holes. No female and no male responded to the combined cues and the negative control.

For each bioassay, the two test cylinders were set out 1 m apart from each other. Each test was conducted for 30 min, and 15 min after beginning the test, the position of the cylinders was exchanged. Bees approaching to within 5–10 cm of a cylinder were counted and caught (mostly during hovering in front of a cylinder; we did not allow the bees to land on the cylinders) with an insect net to assure that an individual bee is counted only once in a specific test. However, ten and four inexperienced, and six and nine experienced females and males, respectively, have participated in different two-choice tests, and two females and four males thereof also in tests with naive and experienced bees. Most of the five tests with flower-inexperienced and flower-experienced bees, respectively, were conducted once or twice in 2007 as well as 2008 (Table 1), and the data of the 2 years as well as the replicates per year were pooled. Bees that approached in two replicates of a specific test per year where only counted once (first response). We tested at first decoupled cues versus controls, then the decoupled cues against each other, and finally decoupled cues versus the combined cues. Only one experiment with single cues (experienced bees) in 2007 (one replicate of visual versus olfactory cues) and one in 2008 (one replicate of olfactory cues versus a control) were conducted after already one test with combined cues have been performed. It is very unlikely that the test sequence influenced the results as we do not have any evidence that bees which attended more than one bioassay behaved differently in a specific assay as compared to bees which attended only one bioassay.

All experiments were performed only on sunny days between 9 a.m. and 5 p.m., when flight-activity of bees was high. Depending on weather conditions, 1–3 two-choice tests were performed per day.

Statistics

One-tailed exact binomial tests were used to test the null hypothesis that both visual and olfactory cues attract less or the same number of bees than the controls and that combined cues attract less or the same number of bees than the

decoupled cues. A one-tailed layout was used as it is very unlikely that visual and olfactory cues of *L. punctata* have repellent properties for *M. fulvipes*. Two-tailed exact binomial tests were calculated to test the hypothesis that visual and olfactory cues attract the same number of bees. The tests were calculated using the spreadsheet provided by http://udel.edu/~mcdonald/statexactbin.html (accessed 2011, August 8; see also McDonald 2009). Fisher's exact tests were used to compare the responses of naïve bees with responses of experienced ones (McDonald 2009).

Results

Females

Olfactory cues attracted a high number of *Lysimachia*-inexperienced female bees, but the negative control attracted no individuals (Fig. 2). In contrast, when giving the bees a choice between visual cues and a control, only few inexperienced females were attracted to the visual cues, and few bees also approached the control cylinder. All bees with the exception of one approached the olfactory treatment when tested against the visual one. Both cues together were more attractive than either cue alone (Fig. 2).

Lysimachia-experienced females were significantly attracted to both cues (no bees approached the negative
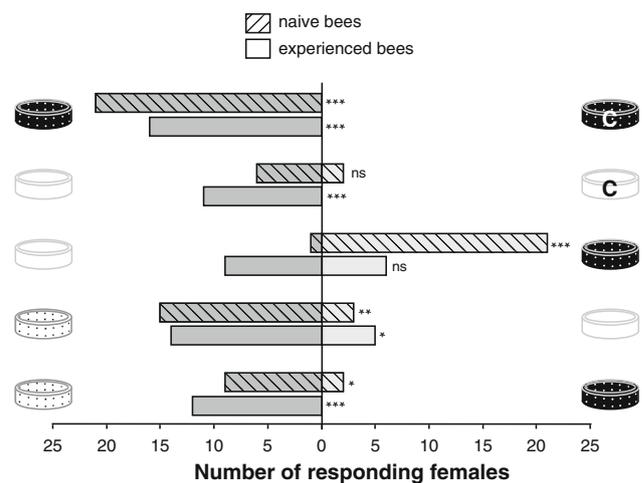
Fig. 2 Behavioural responses of *Lysimachia*-inexperienced (*fasciated bars*) and -experienced (*plain bars*) female *M. fulvipes* bees to visual (*transparent and closed cylinders*) and olfactory (*black cylinders with holes*) or a combination of both cues (*transparent cylinders with holes*) of *L. punctata* inflorescences. *Empty cylinders* were used as negative controls (*C*). The central parts of the cylinders used for the different bioassays are plotted. Data were analysed with a exact binomial test. ns: there was a non-significant ($P > 0.05$) test outcome. The test outcome was significant with $0.01 < *P < 0.05$. The test outcome was significant with $0.001 < **P < 0.01$. The test outcome was significant with $***P < 0.001$

controls), and the visual cues were equally as attractive as the olfactory cues. As in naive female bees, both cues together were more attractive than either cue alone (Fig. 2).

A comparison of responses of inexperienced and experienced bees revealed no significant differences in most of the two-choice bioassays (Fisher's exact tests: $p > 0.05$), however, the attractiveness of visual cues was increased in experienced bees when testing visual and olfactory cues against each other (Fisher's exact test: $p < 0.001$).

Males

Lysimachia-inexperienced males were significantly more attracted to visual as well as olfactory cues than to their respective negative controls (Fig. 3). Both cues, when tested against each other, were equally attractive to the bees. Olfactory + visual cues were more attractive than either one alone (Fig. 3).

Lysimachia-experienced male bees were, similarly as inexperienced ones, significantly more attracted to both cues than to the negative controls. They strongly preferred visual over olfactory cues when the two cues were tested against each other. Further, visual cues were as attractive as olfactory + visual cues, but no male was attracted to olfactory cues when tested against olfactory + visual cues (Fig. 3).

In three of the five different two-choice assays, inexperienced and experienced males responded similarly (Fisher's exact tests: $p > 0.05$), whereas in two assays

(visual versus olfactory cues; visual versus olfactory + visual cues) the responses differed between the inexperienced and experienced males (Fisher's exact tests: $p < 0.01$). In experienced males, the importance of the olfactory cues was decreased relative to naive ones.

Discussion

This study demonstrates that the relative importance of visual and olfactory cues for locating flowering *Lysimachia punctata* shoots depends both on sex and experience of oligolectic *Macropis fulvipes* bees. Consistent with our hypothesis, we found that *Lysimachia*-inexperienced females are more effectively attracted by olfactory as compared to visual cues. In inexperienced males, contrary to our prediction, visual and olfactory cues have the same attractiveness. The same is true for experienced females, and experienced males mainly rely on visual cues for host plant location.

Host plant finding in female bees

Inexperienced *M. fulvipes* females respond strongly to the decoupled olfactory cues and prefer olfactory over visual cues. We did not find a significant effect of visual cues when tested against a control, which also may have to do with the small number of bees attracted in this experiment, however, visual cues at least increase the attractiveness of olfactory ones and a combination of both cues is more attractive than olfactory cues alone. When searching for *L. punctata* plants initially, they seem to rely primarily on olfactory cues which may be more specific than visual cues (see Introduction) though also visual cues are of importance indicating that the bees integrate the input of different modalities (see also Burger et al. 2010b and references therein).

Dobson (1987) and Dobson and Bergström (2000) studied the importance of floral and especially pollen odours for host plant recognition altogether in four specialized bee species. Three of four species only responded to olfactory plant cues in the presence of visual ones (coloured paper), and only one species responded innately, similar to *Macropis*, to olfactory cues also in the absence of visual ones. Nevertheless, different methods were used in the present work as compared to these studies, e.g. size of the cage (large flight cage vs. small tent), behavioural responses measured (approaches versus probing with mouthparts), and processes analysed (host plant location vs. host plant recognition) thus making a comparison of results difficult. However, the same methodological setup was used to determine the relative importance of visual and olfactory cues for *Hoplitis adunca*, a megachilid bee
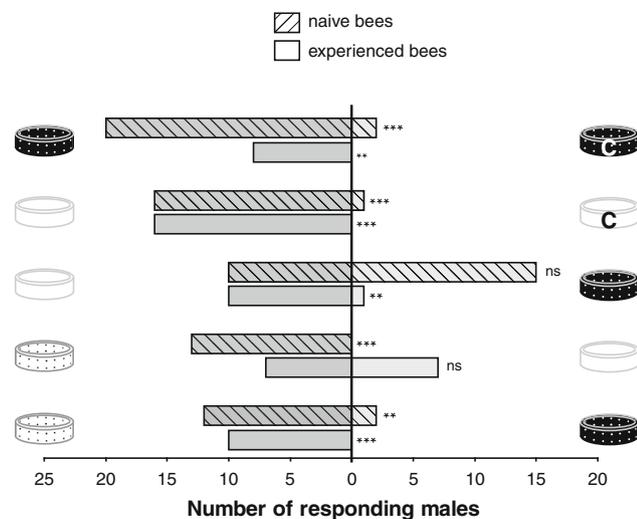
Fig. 3 Behavioural responses of *Lysimachia*-inexperienced (*fasciated bars*) and -experienced (*plain bars*) male *M. fulvipes* bees to visual (*transparent and closed cylinders*) and olfactory (*black cylinders with holes*) or a combination of both cues (*transparent cylinders with holes*) of *L. punctata* inflorescences. *Empty cylinders* were used as negative controls (*C*). The central parts of the cylinders used for the different bioassays are plotted. Abbreviations as in Fig. 2

specialized on *Echium/Pontechium* (Boraginaceae) (Burger et al. 2010b). Naïve *H. adunca* females behave quite differently as compared to *M. fulvipes* because they only pay attention to olfactory cues in the presence of visual ones for locating *Echium*.

Experienced *M. fulvipes* females behave differently as compared to inexperienced ones and do not prefer olfactory over visual cues. However, a combination of both cues is still more attractive than either one alone. The importance of visual cues is increased (rather than the olfactory decreased). An increase of the importance of the visual stimuli through learning was also found in a *Colletes fulgidus longiplumosus* bee population, which is specialized on *Grindelia stricta* (Asteraceae) (Dobson 1987). Generally, experienced bees that also respond to visual cues of its host plant in the absence of olfactory ones may forage highly efficient under field conditions and the change of the relative weighting of visual and olfactory cues during learning seems to be an adaptive response. When revisiting host plant patches, e.g. by relying on their navigational memory (Von Frisch 1965; Reinhard et al. 2004), it may well be that bees see the plants but do not smell them, e.g. due to wind blowing in the flight direction of the bees. When knowing the location of the host plant patches, visual cues therefore seem to be more reliable than olfactory ones (see also Kriston 1973).

Little is known about the relative importance of the natural visual and olfactory plant cues in non-specialized bees when in search for (specific) host plants. Nevertheless, research with artificial flowers indicates that naive as well as experienced non-specialized female bees are guided by both their vision and olfaction (e.g. Von Frisch 1919; Giurfa et al. 1995; Lehrer et al. 1995; Lunau and Maier 1995; Roy and Raguso 1997; Chittka and Raine 2006) and that there are synergistic/additive effects when both cues are offered simultaneously (Lunau 1992; Roy and Raguso 1997; Kunze and Gumbert 2001; Kulahci et al. 2008).

Host plant finding in male bees

Our data show that both decoupled visual and olfactory cues are attractive for inexperienced as well as experienced males (Fig. 3). However, the importance of olfactory cues is decreased during their life, and they seem to rely mostly on visual cues for selecting *Lysimachia* patches to search for females when experienced. This especially may be true if they revisit specific localities by relying on their navigational memory (see above). Though inexperienced males strongly responded to visual cues when offered alone, we do not think that these cues allow identification of *Lysimachia* or discrimination from other plants. In the flight cage, male bees also patrolled other flowering and even non-flowering plants in the absence of *Lysimachia*, and the

innate responses towards the visual display of *Lysimachia* may have been a more generalized and not *Lysimachia*-specific response. To identify *Lysimachia*, they still may need the olfactory cues (see also Burger et al. 2010b).

Host plant finding in flower visitors others than bees

For flower visitors others than bees, the relative importance of visual and olfactory floral cues for host finding has been studied in only a few Coleoptera, Lepidoptera, and Diptera. Both nocturnal beetles and moths rely for initiation of foraging behaviour innately (more) on olfactory cues of their host plants (e.g., Brantjes 1978; Pellmyr and Patt 1986; Gottsberger and Silberbauer-Gottsberger 1991; Balkenius et al. 2006). In the nocturnal hawkmoth *Manduca sexta*, however, either visual or olfactory cues are attractive (naive as well as experienced), but the combination of both cue modalities is needed to elicit feeding responses (Raguso and Willis 2002; Raguso and Willis 2005). Colours (Lunau 1988; Andersson and Dobson 2003; Ômura and Honda 2005; Balkenius et al. 2006) or odours (Andersson and Dobson 2003; Primante and Dötterl 2010) elicit strong innate and/or learned behaviour in diurnal Lepidoptera and Diptera pollinators, whereas the relative importance of the different cues not only differs among but also may differ within animal species, depending, e.g. on the "quality" of the single cues which can differ among various host plant species used by the same animal species (Ômura and Honda 2005).

Behavioural plasticity and sex differences in host finding

We found that the cues used by *M. fulvipes* for host plant finding depend on its sex and experience. The latter finding reveals the presence of learning-induced behavioural plasticity in host plant finding for a bee species. This finding is in agreement with other studies showing that foraging experience (learning) influences the behaviour of generalized as well as specialized bees (e.g., Dobson 1987; Dobson 1994). Our study determined for the first time the relative importance of visual and olfactory cues for finding food sources or patrolling sites in male bees, and shows that the relative weighting of the cues is different as compared to the females. Males rely more on visual cues, which may have to do with the mate finding behaviour. Though final recognition of female mates is typically a matter of olfactory cues in bees (Ayasse et al. 2001), female detection (as first step) seems to be guided by visual cues in bees, and male bees of other species are well known to respond to visual cues of mates and even to unspecific visual cues when in search for females (Gerig 1971; Sugiura et al. 2007), which is also true for *Macropis*

(Dötterl S, unpublished). In the honey bee, drones even have enlarged eyes as compared to workers and the queen (there is no obvious difference in *Macropis* between the sexes), which has to do with the detection, fixation and approaching of queens during the mating flight (Praagh et al. 1980; Menzel et al. 1991). These findings indicate a kind of visual bias in male bees, and in *Macropis*, visually guided female detection on flowers may be a functional explanation for the differences in the weighting of visual and olfactory cues between the sexes. Though between-sex variation in flower visiting behaviour is known for many pollinators (e.g., Alarcón et al. 2010 and references therein), it was not described before that the weighting of different cue modalities of a specific host plant differs between the sexes.

A comparison of results obtained in this study with results obtained from studies with other specialized and generalized insect pollinators demonstrates that the responses to visual and olfactory cues strongly differ among pollinator species. As an example, in contrast to this study, where olfactory cues of *L. punctata* were more attractive as compared to visual ones for inexperienced female *M. fulvipes* bees, *H. adunca* females were only attracted by visual and not olfactory cues alone of its *Echium* host. *Echium vulgare* inflorescences emits 60-fold less scent (total amount) as compared to *L. punctata* (Dötterl and Schäffler 2007; Burger et al. 2010a), and *H. adunca* bees may detect, in contrast to *M. fulvipes*, the scent of its host only in close proximity to the flowering plants when they can see them already.

Overall, the visual (e.g. display size, shape, colour) as well as the olfactory (e.g. quality and quantity of floral scents) advertisement of flowers strongly differs among plants and may elicit more or less strong or specific responses in the visual or olfactory circuit of the pollinators, the sensitivity of which can differ among (Prokopy and Owens 1983) and within (Goyret et al. 2009) species. Depending on which cues are more reliable to locate the host plants or are more effectively detected, the pollinators seem to rely either more on visual or on olfactory cues.

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Publication 3

Floral and vegetative cues in oil secreting and non-oil secreting *Lysimachia* species

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Floral and vegetative cues in oil-secreting and non-oil-secreting *Lysimachia* species

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• *Background and Aims* Unrelated plants pollinated by the same group or guild of animals typically evolve similar floral cues due to pollinator-mediated selection. Related plant species, however, may possess similar cues either as a result of pollinator-mediated selection or as a result of sharing a common ancestor that possessed the same cues or traits. In this study, visual and olfactory floral cues in *Lysimachia* species exhibiting different pollination strategies were analysed and compared, and the importance of pollinators and phylogeny on the evolution of these floral cues was determined. For comparison, cues of vegetative material were examined where pollinator selection would not be expected.
• *Methods* Floral and vegetative scents and colours in floral oil- and non-floral oil-secreting *Lysimachia* species were studied by chemical and spectrophotometric analyses, respectively, compared between oil- and non-oil-secreting species, and analysed by phylogenetically controlled methods.
• *Key Results* Vegetative and floral scent was species specific, and variability in floral but not vegetative scent was lower in oil compared with non-oil species. Overall, oil species did not differ in their floral or vegetative scent from non-oil species. However, a correlation was found between oil secretion and six floral scent constituents specific to oil species, whereas the presence of four other floral compounds can be explained by phylogeny. Four of the five analysed oil species had bee-green flowers and the pattern of occurrence of this colour correlated with oil secretion. Non-oil species had different floral colours. The colour of leaves was similar among all species studied.
• *Conclusions* Evidence was found for correlated evolution between secretion of floral oils and floral but not vegetative visual and olfactory cues. The cues correlating with oil secretion were probably selected by *Macropis* bees, the specialized pollinators of oil-secreting *Lysimachia* species, and may have evolved in order to attract these bees.

Key words: Colour hexagon, oil secretion, correlated evolution, flower and vegetative scent, headspace analysis, GC-MS, *Lysimachia*, multidimensional scaling, oil-bee *Macropis*, phylogeny, spectral photometry.

INTRODUCTION

Many flowering plant species rely on animal pollinators for their sexual reproduction, and adaptation of flowers to a specific guild of pollinators often promotes high efficiency in pollination (Baker and Hurd, 1968; Endress, 1994). Specific floral traits including size, shape, colour, scent and reward properties of plants pollinated by the same guild of animals may converge as a result of pollinator-mediated selection (Fenster *et al.*, 2004; Harder and Johnson, 2009). This is because pollinators within a guild are suggested to have similar sensory preferences, whereas different pollinators are suggested to have different sensory preferences (Schiestl and Dötterl, 2012). Groups of plants pollinated by, for example, moths, bats and carrion flies, respectively, are each well known for a particular suite of floral traits even where the individual plants within each group are not closely related (von Helversen *et al.*, 2000; Andersson *et al.*, 2002; Raguso, 2004). This convergence, though often most important, is, however, not the only factor that explains floral phenotypes, and should not be explained in isolation without considering

evolutionary relationships (Armbruster, 1997; Levin *et al.*, 2003; Raguso *et al.*, 2003; Theis and Lerdau, 2003; Steiner *et al.*, 2011). Independently of the type of pollinator, closely related plants often share specific traits (Levin *et al.*, 2003).

Visual (e.g. colour, shape) and olfactory (scent) floral cues play a central role in attracting pollinators and are often used by pollinators to discriminate between rewarding and non-rewarding plant species (Goulson *et al.*, 2001; Wright and Schiestl, 2009). The interplay of olfactory and visual cues is complex, but studies have shown that olfactory and visual cues are often additive/synergistic (e.g. Kunze and Gumbert, 2001; Raguso and Willis, 2005; Burger *et al.*, 2010), though pollinators may also/additionally be attracted by single floral cues (Dötterl and Schäffler, 2007; Dötterl *et al.*, 2011; Klahre *et al.*, 2011). In addition to floral traits, vegetative cues may also contribute to pollinator attraction or, in extreme cases, take over the signalling function from the flowers (Dufaÿ *et al.*, 2003). In most cases, however, flower visitors respond specifically to floral cues (Ayasse *et al.*, 2000; Plepys *et al.*, 2002; Huber *et al.*, 2005), whereas the importance of vegetative material for pollinator attraction seems

typically to be small. Instead, volatiles released from vegetative tissues are well known to deter potential herbivores (Lin *et al.*, 1987) and also to attract parasitoids of herbivores following herbivore damage of leaves (Turlings *et al.*, 1990; Pichersky and Gershenzon, 2002). Pollinator-mediated selection may therefore be of only minor importance in the evolution of olfactory and visual (colour) traits of vegetative plant parts, which is known to be the case for morphological traits (Conner and Sterling, 1996; Armbruster *et al.*, 1999). So far, however, no quantitative studies are available comparing the influence of pollinator-mediated selection on both olfactory and visual traits between floral and vegetative organs. Variation in scent within and among species has not yet been compared explicitly between vegetative and floral tissue. Instead, in studies focusing on pollination, vegetative volatiles are typically used as a control for floral scents (Raguso and Pichersky, 1995; Levin *et al.*, 2003) or floral and vegetative scents are pooled and analysed as one data set (e.g. Honda *et al.*, 1998; Füssel *et al.*, 2007; Jhumur *et al.*, 2008).

Lysimachia is a good model to study the importance of pollinators and phylogeny on olfactory and visual cues of floral as well as vegetative organs. The phylogeny of this genus is well known, and species of this genus exhibit different pollination strategies. About 40 % of the species of a few clades secrete floral fatty oils, and such species/clades are intermingled with species/clades of non-oil-secreting species (Hao *et al.*, 2004; Anderberg *et al.*, 2007). Oil species are involved in a highly specialized pollination system with *Macropis* oil bees (Vogel, 1986). These bees collect floral rewards, i.e. oil and also pollen, for their offspring only from *Lysimachia* species, and *Lysimachia* oil species are only/mainly pollinated by these bees. Non-oil-secreting *Lysimachia* species offer nectar/pollen as reward and were suggested to be pollinated by generalist bees or, in the case of a single cleistogamous species (*L. minoricensis*), reproductive success is expected to be independent of pollinators (Vogel, 1986). Species of the oil floral type have yellow (for *L. vulgaris*, see also Arnold *et al.*, 2010) flowers. Non-oil species have yellow, red, white or purple flowers (Vogel, 1986; Arnold *et al.*, 2010). Very little is known about olfactory floral cues in *Lysimachia*, and scent data are available for only one of the oil species (Dötterl and Schäffler, 2007). Recently, we demonstrated that both olfactory and visual cues of a *Lysimachia* species are involved in the attraction of a *Macropis* oil bee (Dötterl *et al.*, 2011).

Oil-secreting *Lysimachia* species and *Macropis* bees are distributed in the Holarctic region including North America and Europe, but the highest diversity occurs in China (Vogel, 1986). In a specific region, *Macropis* bees collect oil and pollen not only from their native *Lysimachia* hosts, which may be from the same or from different clades, but also from introduced non-native *Lysimachia* oil flowers regardless of their clade membership. As an example, in Europe, both *M. fulvipes* and *M. europaea* visit *Lysimachia* species that occur natively in Europe (e.g. *L. punctata* and *L. vulgaris*), but are, according to Anderberg (2007), members of different clades (see also Fig. 4). Further, Vogel (1986) as well as Simpson and Neff (1983) mentioned that American *Macropis* bees collect floral rewards on introduced European

L. punctata and *L. nummularia*. Both species belong to a different clade compared with oil plants native to North America (e.g. *L. ciliata*; Table 1, Fig. 4). Similarly, the European *M. fulvipes* visits the Asian *L. congestiflora* as well as the American *L. ciliata* in a flight cage (I. Schäffler, unpubl. data), both of which also belong to different clades compared with the native host plants (Table 1, Fig. 4). It seems that *Macropis* bees are not specialized on a specific *Lysimachia* oil species; instead, they seem to be attracted by any *Lysimachia* oil species independent of the geographic origin and clade membership.

In the present study, we characterized qualitative and (semi-) quantitative floral and vegetative odours in *Lysimachia* oil and non-oil species. We also determined the flower and leaf colour in *Lysimachia* spp. by spectrophotometry and determined how the flower colours are perceived by bees, which have a different visual system to that of humans (e.g. instead of red they have an UV receptor; Backhaus, 1992).

Because specific *Macropis* bees visit *Lysimachia* oil plants belonging to different clades, we hypothesize that oil-secreting *Lysimachia* species evolved, independent of the phylogenetic relatedness, an oil-specific floral volatile compound or bouquet as well as a uniform bee colour. *Lysimachia* species that do not secrete floral oils are predicted to differ in their floral scent and colour from oil-secreting species. We expected correlated evolution between specific floral scent compounds/colours and secretion of floral oils.

In contrast to the visual and olfactory flower cues, potential leaf cues would be expected to vary independently of pollinator mode or floral type, and therefore we predicted that there would be no difference in leaf volatiles or spectral reflectance between oil and non-oil species.

MATERIALS AND METHODS

Plant material

Individual plants of five oil and 12 non-oil *Lysimachia* species (Table 1) were cultivated from seeds and plants obtained from Botanical Gardens or commercial suppliers, or collected in natural habitats (Supplementary Data Table S1). The classification of floral types follows Vogel (1986) and Klotz *et al.* (2002). Two of the species of our study, *L. maritima* and *L. arvensis*, have only recently been moved to *Lysimachia* from *Glaux* and *Anagallis*, respectively, based on molecular analyses (Banfi *et al.*, 2005; Manns and Anderberg, 2009).

Volatile collection

Dynamic headspace scent samples from 'flowers' were collected from inflorescences *in situ*, from cut inflorescences or from individual cut flowers, whereas vegetative scents were collected from leaves or other non-floral plant parts, i.e. non-flowering shoots (Table 1). All samples were collected on sunny days between 10:00 and 16:00 h. When using cut material (to get more concentrated samples), we collected scent immediately after cutting. In two species, *L. maritima* and *L. congestiflora*, we collected scent *in situ* as well as from cut flowering plant parts and found that cutting did not

TABLE 1. *Species studied, their abbreviations used throughout the text, clade membership, floral types (O, oil-secreting species; NO, non-oil-secreting species), human-perceived flower colour and geographic origin (native region: E, Europe; M, Mediterranean; NA, North America; C, China; H, Hawaii; J, Japan). The number of scent samples collected from flower and vegetative parts of the different species, the number of flowers used for colour measurements and the GenBank codes of sequences used for testing phylogenetic patterns and correlated evolution of scent compounds/colour and pollination by oil bees are given. Species are listed according to clade membership.*

| Species | Species abbreviation | Clade membership* | Floral type | Floral colour | Native region | Number of samples | | GenBank code |
						Scent: flower/ vegetative	Colour	
L. ciliata	*Lci*	Subgenus *Lysimachia* group A	O	Yellow	NA	5/5	6	AY839977
L. nummularia	*Lnu*	Subgenus *Lysimachia* group B	O	Yellow	E	5[†]/5[‡]	5	AY839988
L. punctata	*Lpu*	Subgenus *Lysimachia* group B	O	Yellow	E	5[‡]/4[‡]	3	AY839987
L. nemorum	*Lne*	Subgenus *Lysimachia* group C	NO	Yellow	E	4[†]/5[‡]	4	AF213747
L. vulgaris	*Lvu*	Subgenus *Lysimachia* group E	O	Yellow	E	6[‡]/5[‡]	3	AY839960
L. congestiflora	*Lco*	Subgenus *Lysimachia* group F	O	Yellow	C	6[†]/5[‡]	4	AY839963
L. atropurpurea	*Lat*	Subgenus *Palladia* + *Lysimachiopsis*	NO	Purple	M	5/3	8	AY839954
L. clethroides	*Lcl*	Subgenus *Palladia* + *Lysimachiopsis*	NO	White	C	5/3	3	AY839955
L. decurrens	*Lde*	Subgenus *Palladia* + *Lysimachiopsis*	NO	White	C	5/5	–	
L. ephemerum	*Lep*	Subgenus *Palladia* + *Lysimachiopsis*	NO	White	M	5/5	3	AY839976
L. fortunei	*Lfo*	Subgenus*Palladia* + *Lysimachiopsis*	NO	White	C,J	5/5	3	
L. lichiangensis	*Lli*	Subgenus *Palladia*	NO	White	C	5/1	3	
L. mauritiana	*Lmau*	Subgenus *Palladia* + *Lysimachiopsis*	NO	White	H	5/5	–	AY839956
L. minoricensis	*Lmi*	Subgenus *Palladia* + *Lysimachiopsis*	NO	Off-white	M	5/5	3	AF213749
L. thyrsiflora	*Lth*	Subgenus *Naumburgia*	NO	Yellow	E	4/5	5	AY839959
L. arvensis	*Lar*	*Anagallis* s. str.	NO	Blue	E	1[†],2[‡]/2	1[§]	AF213735
L. maritima	*Lmar*	*Glaux*	NO	White-purple	E	1,4[‡]/1	–	AF213743

* According to Anderberg *et al.* (2007) or, in the case of Lde and Lfo, Hao *et al.* (2004), and in the case of Lli, Vogel (1986).
[†] Cut flowers.
[‡] Cut inflorescence or cut non-flowering stems.
[§] Data from Arnold *et al.* (2010).

influence floral scent emission when scent was collected immediately after cutting (I. Schäffler, unpubl. data). Floral or vegetative parts were enclosed within polyester oven bags (the size depending on the plant material; from 10×10 cm to 20×30 cm; Toppits®, Germany) for 10 min (flowers) and 60 min (vegetative parts), respectively, and the emitted volatiles were trapped on 1.5 mg of Tenax (mesh 60–80; Supelco, Bellefonte, PA, USA) and 1.5 mg of Carbotrap B (mesh 20–40, Supelco) in a quartz vial (Varian Inc.; length 15 mm, inner diameter 2 mm) for 2 min (4 min for vegetative parts: 2 min after 30 min and another 2 min after 60 min of enclosure) using a membrane pump (G12/01 EB, ASF Rietschle-Thomas, Puchheim, Germany). Although in all species the vegetative parts (especially the leaves) comprise a greater proportion of the plant than the corresponding floral material, the scent discharge from the vegetative material was low. For this reason we sampled the scent from vegetative parts for a longer period than for floral tissue. The flow rate was adjusted to 200 mL min^{-1} (Dötterl *et al.*, 2005). Ambient controls were collected from empty bags (10×10 cm) following the procedure as described above.

Analysis of scent compounds

The dynamic headspace samples were analysed on a Varian Saturn 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector. The quartz vials used for scent collections were inserted into the injector by the use of the ChromatoProbe kit (Dötterl *et al.*, 2005).

The injector split vent was opened and the injector heated to 40 °C to flush introduced air from the system. After 2 min, the split was closed and the injector heated to 200 °C at a rate of 200 °C min^{-1}. This temperature was then held for 4.2 min, after which the split vent opened and the injector cooled down. A ZB-5 column (5 % phenyl polysiloxane) was used for the separation of compounds (60 m long, inner diameter 0.25 mm, film thickness 0.25 μm; Phenomenex). Helium carrier gas flow was 1.0 mL min^{-1}. GC oven temperature was 40 °C for the first 7 min then increased by 6 °C min^{-1} to 250 °C and was held at the end temperature for 1 min. The MS-interface temperature was 260 °C and the ion trap worked at 175 °C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan s^{-1} from m/z 30 to 350. The GC-MS data were processed using the Saturn Software package $5.2.1$. Component identification was carried out using the NIST 08, Wiley 7 and Adams (Adams, 2007) mass spectral databases, or the database provided in MassFinder 3, and confirmed by comparison of retention times with published data (Adams, 2007). Identification of individual components was confirmed by comparison of both mass spectrum and GC retention time with those of authentic standards. Compounds found in ambient control samples were excluded from the analyses.

To identify flower-specific compounds, we compared the 'flower' scent samples with the vegetative samples within species. Only compounds that were found in 'flower' but not in vegetative scent samples were treated as flower specific. We estimated total scent emission (absolute amount) by

injecting known amounts of several standards compounds, and the mean response of these compounds (mean peak area) was used for quantification (Dötterl *et al.*, 2005).

Statistical analyses of scent samples

Scents of plants pollinated by one or a few closely related bee species, i.e. specialized pollination systems, are known to contain unique compounds or unique blends (relative amount) of relatively widespread compounds (Schiestl *et al.*, 1999; Burger *et al.*, 2012). We therefore analysed our scent data using both qualitative (presence/absence of compounds) and semi-quantitative (relative amount of compounds with respect to total peak area) approaches.

For analyses of qualitative differences in flower-specific as well as vegetative scent among species, we calculated the qualitative Sørensen index using Primer 6·1·6 (Clarke and Gorley, 2006) to determine pairwise qualitative similarities among the individual samples. Based on the obtained similarity matrices (individual based matrix), we performed analyses of similarities (ANOSIM, 10 000 permutations) in Primer 6·1·6 to assess differences in scent among species. ANOSIM is a commonly used multivariate procedure roughly analogous to ANOVA/MANOVA that operates directly on a (dis)similarity matrix. It yields a test statistic R that is a relative measure of separation among *a priori* defined groups. It is based on differences of mean ranks among and within groups. An R value of '0' indicates completely random grouping, whereas a value of '1' indicates that samples within groups are more similar to each other than to any sample from a different group (Clarke and Gorley, 2006).

To test for qualitative differences in scent between oil and non-oil species, we used the overall compounds found per species (one list of compounds per species), calculated the Sørensen index to determine pairwise qualitative similarities among the species and, based on the obtained similarity matrix (species-based matrix), performed an ANOSIM as described above, but instead of testing for a species effect, we tested for an effect of presence/absence of oil.

For analyses of semi-quantitative differences in scent among species, we calculated the Bray–Curtis (semi)-quantitative similarity index using Primer 6·1·6 to assess pairwise semi-quantitative similarities among the individual samples, and again performed an ANOSIM (10 000 permutations) based on the obtained similarity matrix (individual based matrix). Semi-quantitative instead of quantitative data were used because the total amount of trapped volatiles strongly varied among as well as within species.

To test for semi-quantitative differences in scent between the oil and non-oil species, we determined the mean relative amount of compounds per species, calculated the pairwise semi-quantitative similarities (Bray–Curtis) to obtain a species-based matrix, and again performed an ANOSIM (10 000 permutations).

PERMDISP (Anderson *et al.*, 2008) was used in Primer 6·1·6 to test for differences in within-group variability (dispersion) of vegetative and floral scent (based on qualitative as well as semi-quantitative species-based matrices) between oil and non-oil species (10 000 permutations).

Non-metric multidimensional scaling (NMDS), based on Bray–Curtis similarities, was used to display graphically the semi-quantitative differences in flower-specific as well as vegetative scents among species (based on the mean relative amount of compounds per species). Stress values indicate how well the two-dimensional plot represents relationships among samples in multidimensional space. Stress values <0·15 indicate a good fit (Smith, 2003).

To test if vegetative and flower-specific scents correlate (qualitatively and semi-quantitatively), i.e. plants have similar vegetative scents if they have similar flower scents, RELATE analyses (Mantel test) were performed in Primer 6·1·6 (Spearman; 10 000 permutations) using the qualitative as well as quantitative species-based similarity matrices.

Colour analysis, hexagon distance matrix and colour space

All *Lysimachia* species used for scent analyses, except *L. decurrens*, *L. maritima* and *L. mauritiana*, were also used to determine spectral reflection properties of the upper side of leaves and the apical petal parts (Table 1). Spectral reflection properties of red-coloured *L. arvensis* are from Arnold *et al.* (2010).

Diffuse reflectance spectra were taken every nanometre from 300 to 700 nm using a Varian Cary 5 spectrophotometer (Varian Inc., USA) equipped with a praying mantis accessory (Harrick Scientific Products, Inc., Pleasantville, NY, USA) using the same method as described by Burger *et al.* (2010). Barium sulfate was used as white standard and the disconnected beam as black reference.

The mean reflections of the petals and of leaves (built from the replicate samples per species) were used to determine the loci of petal colours in the hexagon colour space (Chittka, 1992). The standard illumination function (D65) and the spectral sensitivities of the honeybee's photoreceptors were used from Chittka and Kevan (2005). Typically, bees do not differ substantially in their sensory systems (Peitsch *et al.*, 1992) and therefore we used the spectral sensitivity functions described for the honeybees as representative approximation for *Macropis* bees (Chittka and Kevan, 2005). For comparison of the bee colours among the different *Lysimachia* species, the pairwise hexagon distances of colour loci among species, as well as the distance of each colour locus to its background (green leaves) was calculated (Chittka and Kevan, 2005). Behavioural experiments with bumble-bees trained to visit artificial flowers have demonstrated that colour distances <0·05 hexagon units are poorly discriminated, whereas distances >0·10 are easily discriminated by the bees (Dyer and Chittka, 2004).

Testing for correlated evolution

Phylogenetically controlled correlations between pollination type (oil-bee pollination vs. non-oil-bee pollination) and presence/absence of single scent compounds were analysed using correlated evolution of discrete binary traits implemented in the program BayesTraits (Pagel and Meade, 2006), using a consensus phylogeny obtained from MrBayes. Chloroplast *ndhF* gene sequences of 14 species (sequence data were not available for *L. decurrens*, *L. fortunei* and *L. lichiangensis*)

were downloaded from GenBank (Table 1). A consensus tree was constructed using Bayesian analyses under a GTR model of sequence evolution with gamma-distributed rate variation in MrBayes 3·1·2 (Ronquist and Huelsenbeck, 2003) (Fig. 4). We ran three independent runs of four Markov chains for 10 million generations, sampling every 500 generations. Adequacy of sampling and run convergence were assessed using the effective sample size diagnostic in TRACER 1·5 (Rambaut and Drummond, 2007). Trees from the first million generations were discarded based on an assessment of convergence.

BayesTraits tests for correlated evolution in two binary traits by comparing the fit of two models, one in which the traits are allowed to evolve independently of one another on a phylogenetic tree (scent compound does not correlate with oil-bee pollination) and one in which traits evolve in a correlated fashion (scent compound correlates with oil-bee pollination). The method applies reversible-jump Markov chain Monte Carlo (RJ MCMC), which samples the posterior probability distributions of the parameter values of the model of trait evolution. The independent and dependent models can be compared with Bayes factors (BFs; Kass and Raftery, 1995), with the marginal likelihood of each model approximated by the harmonic mean of the likelihoods in the Markov chain. For this comparison, a BF value greater than '2' and additionally a smaller harmonic mean for the independent model is taken as positive evidence that the dependent model is favoured (Pagel and Meade, 2006). For the analyses we used a uniform prior for the independent model and an exponential hyperprior (0 100) for the model of dependent evolution as suggested by Pagel and Meade (2006). The analyses were run for 11 000 000 iterations with burn-in at 1 000 000 iterations and sample frequency of 500 iterations. As harmonic means can be unstable, analyses for each model were repeated five times.

To test the correlated evolution of floral colours and pollinator mode (oil-bee vs. non-oil-bee pollination) we used a similar approach. We tested hexagon bee-green (the unique colour present in at least two oil species; see Fig. 3) vs. the rest of the colours. We use the same consensus phylogeny obtained from MrBayes. However, as colour data of *L. maritima* and *L. mauritiana* were not available, we marked this information as ambiguous in BayesTraits analysis.

Phylogenetic signal in scent compounds and colour

The 'phylogenetic signals' that affected each floral and vegetative compound (present in at least two oil species) and the presence of bee-green colour were assessed with independent Abouheif's tests (permutation: 1 000 000; adephylo package in R software; Abouheif, 1999; Jombart *et al.*, 2010). The Abouheif's *C* statistic tested the null hypothesis that compounds did not experience phylogenetic autocorrelation (based on the topology of the tree). The test statistic *C* ranges from '−1' to '1'. A value of '0' indicates a random phylogenetic pattern, values ' > 0' indicate phylogenetic autocorrelation, and values ' < 0' indicate negative phylogenetic autocorrelation (non-random alternation). We performed the Abouheif's test additionally on oil-bee pollination to evaluate

the phylogenetic constraint in the evolution of the pollination mode (oil-bee vs. non-oil-bee pollination).

RESULTS

Qualitative properties in flower specific and vegetative scents

We detected altogether 63 flower-specific scent compounds in the different species, 50 of which were (tentatively) identified (Table 2; for complete compound list see Supplementary Data Table S2). No flower-specific compounds were found in *L. decurrens* and *L. ephemerum*, but the other species emitted from one (*L. mauritiana* and *L. nemorum*) to 20 (*L. punctata*) flower-specific compounds. Aliphatics (26), aromatic compounds (17) and terpenoids (14) were the most common compounds present among the analysed species. Some of the species, such as *L. punctata*, emitted compounds from all three of these groups, whereas others had compounds from only two groups [e.g. aliphatics and aromatics (*L. nummularia*)], or one group [e.g. only aromatics (*L. ciliata*) or only terpenoids (*L. nemorum*)]. There was no single compound which occurred in all 15 species analysed, and different species emitted different sets of flower-specific compounds overall (ANOSIM: global $R_{14,58} = 0.995$; $P < 0.001$). Post-hoc comparisons among pairs of species revealed values of $R > 0.8$ and $P < 0.03$ indicating that there were differences in scent among all the species which contained at least one compound. Variability (dispersion) in qualitative scent composition was lower in oil compared with non-oil species (PERMDISP: $F_{1,13} = 16.5$; $P = 0.009$). Oil and non-oil species overall did not differ significantly in flower scent composition (ANOSIM: global $R_{1,13} = -0.053$; $P = 0.62$).

In the vegetative scent samples, we detected 62 compounds [34 (tentatively) identified], mainly terpenoids (41), aliphatics (10) and aromatics (5) (Table 3; for complete compound list see Supplementary Data Table S3). Some of these compounds (e.g. benzaldehyde, benzyl alcohol and 4-oxoisophorone) were also listed as flower-specific compounds (Table 2; Supplementary Data Table S2), indicating that in some species they were found in samples collected from vegetative material, whereas in others they were only found in flower samples. Analogous to our finding that flower scents were species specific, the scent of vegetative parts also differed among species and the set of compounds emitted was species specific (ANOSIM: global $R_{16,51} = 0.838$; $P < 0.001$). In contrast to flower-specific scents, however, vegetative scents did not differ among all species (27 % of the post-hoc tests with $P > 0.05$), which mainly has to do with the small number of vegetative samples ($n = 1$) in *L. arvensis*, *L. lichiangensis* and *L. maritima*. The only non-significant post-hoc tests between species pairs that did not contain at least one of these three species were between *L. clethroides* and *L. ephemerum*, and *L. clethroides* and *L. atropurpurea*. Analogous to the floral scents, there was no compound which occurred in vegetative samples of all the species. In contrast to floral scents, variability in vegetative scent composition did not differ between oil and non-oil species (PERMDISP: $F_{1,15} = 5.9$; $P = 0.07$). Species of the oil and the non-oil group were not characterized by a specific

TABLE 2. *Number of compounds, mean total absolute as well as percentage amount of flower-specific scent compounds (listed within classes according to Kovats retention index, RI)*

	RI	Lci	Lnu	Lpu	Lne	Lvu	Lco	Lat	Lcl	Lfo	Lli	Lmau	Lmi	Lth	Lar	Lmar
												Species				
No. of compounds		**7**	**9**	**20**	1	**8**	**4**	8	6	9	4	1	4	6	12	2
Amount of trapped scent, ng per flower per 12 min		**5**	**54**	**5**	Tr	**Tr**	**Tr**	3	7	8	40	Tr	Tr	Tr	1	Tr
Aliphatics																
Methyl hexanoate*	934	–	–	–	–	–	–	–	–	–	–	–	–	65	–	–
Methyl 2-methylhexanoate†	972	–	–	–	–	–	–	–	–	–	–	–	41	–	–	–
Hexyl acetate*	1008	–	–	–	–	–	–	–	–	–	–	–	–	–	30	–
m/z: 74, 43, 55, 41, 39, 101	1067	–	–	–	–	–	–	–	–	–	–	–	15	–	–	–
(Z)-3-Hexenyl propionate*	1092	–	–	–	–	–	–	–	–	–	–	–	–	–	22	–
1,3-Diacetin‡	1232	–	–	Tr	–	28	–	–	–	–	–	–	–	–	–	–
1,2-Diacetin‡	1236	–	–	Tr	–	17	–	–	–	–	–	–	–	tr	–	–
2-Undecanone‡	1281	–	6	Tr	–	–	67	–	–	–	–	–	–	–	1	–
2-Tridecanone‡	1484	–	15	1	–	–	–	–	–	–	–	–	–	–	–	–
Methyl dodecanoate*	1507	–	–	–	–	–	–	–	–	–	–	–	–	27	–	–
Aromatics																
Benzaldehyde‡	982	**89**	**12**	**26**	–	–	–	–	–	52	87	–	–	–	–	–
Benzyl alcohol‡	1050	**7**	**Tr**	–	–	33	24	–	67	24	4	–	–	–	–	–
Benzyl acetate‡	1104	**Tr**	–	–	–	–	–	–	–	1	–	–	–	–	–	70
1-Phenyl-1,2-propanedione‡	1171	–	**57**	**43**	–	11	–	–	–	–	–	–	–	–	–	–
3,5-Dimethoxytoluene*	1268	–	–	–	–	–	–	12	–	–	–	–	38	–	–	–
Terpenoids																
allo-Ocimene*	1135	–	–	–	–	–	–	–	–	–	–	–	–	–	15	–
4-Oxoisophorone‡	1142	–	–	–	–	–	–	–	18	14	–	100	–	–	–	–
m/z: 108, 93, 95, 67, 39, 79	1212	–	–	–	–	–	–	–	–	–	–	–	–	–	–	30
m/z: 189, 133, 105, 91, 147, 79	1484	–	–	–	100	–	–	–	–	–	–	–	–	–	–	–
N-containing compounds																
1-Nitro-2-phenylethane*	1313	–	–	–	–	–	–	–	–	–	–	–	–	–	10	–
Unknowns																
m/z: 56, 41, 39, 42, 43, 69	1168	–	–	–	–	–	–	67	–	–	–	–	–	–	–	–

Data for oil-secreting species are highlighted in bold. Tr, trace amount (percentage <0.5 % or total absolute amount <0.5 ng). For species abbreviations see Table 1. Only compounds that contributed at least 10 % in any species are shown. A table with all the compounds detected in the individual samples can be found as Supplementary Data Table S2.
* Identification based on mass spectrum and retention index.
† Identification based on mass spectrum.
‡ Identification based on authentic standards.

set of vegetative scent compounds (ANOSIM: global $R_{1,15} = -0.16$; $P = 0.87$).

Vegetative and flower scent correlated based on the Sørensen similarity matrices ($\rho_{13} = 0.443$; $P = 0.007$) indicating that species emitting a similar set of floral scent compounds also emitted a similar set of vegetative scents.

Quantitative and semi-quantitative properties in flower-specific and vegetative scents

The total amount of scent trapped per flower and per 12 min ranged from <0.5 ng (e.g. *L. vulgaris*) to 54 ng (*L. nummularia*) (Table 2). The species differed in their semi-quantitative floral scent composition (ANOSIM: global $R_{14,58} = 0.955$; $P < 0.001$) (Fig. 1), and comparisons among all pairs of species were significant ($R > 0.2$, $P < 0.04$). The most abundant compound in the floral scent of *L. punctata* and *L. nummularia* was 1-phenyl-1,2-propanedione, in *L. vulgaris* and *L. clethroides* benzyl alcohol, in *L. maritima* benzyl acetate, and in *L. arvensis* hexyl acetate. Benzaldehyde was the compound with the highest relative amount in floral scents of *L. ciliata*, *L. fortunei* and *L. lichiangensis*, 2-undecanone in

L. congestiflora, methyl hexanoate in *L. thyrsiflora*, 4-oxoisophorone in *L. mauritiana*, methyl 2-methylhexanoate in *L. minoricensis*, an unknown sesquiterpene in *L. nemorum*, and an unknown compound in *L. atropurpurea*. Variability in semi-quantitative floral scent composition was lower in oil compared with non-oil species (PERMDISP: $F_{1,13} = 11.5$; $P = 0.02$). Oil species did not differ overall in their semi-quantitative scent composition from non-oil species (ANOSIM: global $R_{1,13} = -0.057$; $P = 0.64$).

The total amount of scent trapped per leaf ranged from <0.5 ng (most of the species) to 3 ng (*L. clethroides*) per 12 min (Table 3). The species differed overall in their semi-quantitative vegetative scent composition (ANOSIM: global $R_{16,51} = 0.608$; $P < 0.001$) (Fig. 2); however, differences were not that prominent compared with the qualitative differences. More than 50 % of the post-hoc tests revealed non-significant values (data not shown). The most abundant compound in the vegetative scent samples of several species was (Z)-3-hexenyl acetate (Table 3, Fig. 2). (E)-4,8-Dimethyl-1,3,7-nonatriene was most abundant in *L. congestiflora* and *L. decurrens*, benzyl alcohol in *L. atropurpurea* and *L. mauritiana*, (Z)-3-hexenol in *L. ephemerum*, and (E)-β-ocimene in

TABLE 3. *Number of compounds, mean total absolute as well as percentage amount of vegetative scent compounds (listed within classes according to Kovats retention index, RI)*

	RI	Lci	Lnu	Lpu	Lne	Lvu	Lco	Lat	Lcl	Lde	Lep	Lfo	Lli	Lmau	Lmi	Lth	Lar	Lmar
																	Species	
No. of compounds		**10**	**9**	**10**	14	**23**	**9**	3	31	9	5	18	18	6	7	7	12	10
Amount of trapped scent, ng per leaf per 12 min		**2**	**Tr**	**Tr**	Tr	**Tr**	**Tr**	Tr	3	Tr	Tr	Tr	Tr	Tr	Tr	Tr	ND	ND
Aliphatics																		
(Z)-3-Hexenol*	850	**13**	**5**	**3**	12	**5**	**–**	–	4	–	39	3	–	18	–	–	1	28
(Z)-3-Hexenyl acetate*	1004	**61**	**68**	**41**	48	**72**	**7**	–	60	–	–	36	–	–	72	74	15	58
Methyl octanoate†	1123	**–**	**–**	**–**	–	**–**	**–**	–	–	–	–	–	–	–	–	10	–	–
m/z: 67, 57, 82, 41, 39, 83	1225	**–**	**1**	**3**	–	**2**	**–**	–	1	–	–	–	–	–	–	1	16	–
m/z: 67, 57, 82, 41, 39, 83	1230	**–**	**–**	**Tr**	–	**Tr**	**–**	–	1	–	20	3	–	–	–	3	20	–
Aromatics																		
Benzyl alcohol*	1049	**–**	**–**	**–**	–	**–**	**–**	49	–	–	–	–	–	72	–	–	–	–
Methyl benzoate*	1107	**–**	**–**	**–**	–	**–**	**–**	33	–	–	–	–	–	–	–	–	–	–
Methyl salicylate*	1208	**–**	**–**	**–**	–	**2**	**–**	–	2	–	16	–	–	–	–	–	–	–
p-Anisaldehyde*	1262	**–**	**–**	**–**	–	**–**	**–**	18	–	–	–	–	–	–	–	–	–	–
Terpenoids																		
Camphene†	946	**–**	**16**	**–**	–	**–**	**–**	–	–	–	–	–	–	–	–	–	–	–
(E)-β-Ocimene*	1044	**2**	**1**	**3**	3	**1**	**–**	–	1	2	–	10	43	–	–	–	30	2
(E)-4,8-Dimethyl-1,3,7-nonatriene*	1109	**3**	**–**	**–**	8	**4**	**46**	–	6	60	24	19	8	7	–	–	2	1
α-Copaene*	1377	**–**	**–**	**–**	3	**2**	**21**	–	5	–	–	3	5	–	–	–	–	–
β-Caryophyllene*	1440	**11**	**–**	**3**	13	**1**	**17**	–	5	15	Tr	9	13	–	–	–	1	–
(E, E)-α-Farnesene*	1496	**4**	**–**	**41**	3	**6**	**–**	–	Tr	13	–	2	1	–	–	1	–	–
Unknowns																		
m/z: 179, 69, 107, 39, 95, 40	1296	**–**	**–**	**–**	–	**–**	**–**	–	–	–	–	–	–	–	14	–	–	–

Data for oil-secreting species are highlighted in bold. Tr, trace amount (percentage <0·5 % or total absolute amount <0·5 ng). For species abbreviations see Table 1. Only compounds which contributed at least 10 % in any species are shown. A table with all the compounds detected in the individual samples can be found as Supplementary Data Table S3. ND, not determined.

* Identification based on authentic standards.
† Identification based on mass spectrum and retention index.

A

Most abundant compounds in species:
(1) Unknown compound RI: 1168
(2) Methyl 2-methylhexanoate
(3) Unknown terpenoid RI: 1484
(4) Methyl hexanoate
(5) 1,3-Diacetin
(6) 1-Phenyl-1,2-propanedione
(7) Hexyl acetate
(8) 2-Undecanone
(9) Benzyl alcohol
(10) Benzaldehyde
(11) 4-Oxoisophorone
(12) Benzyl acetate

● Oil-secreting species
○ Non-oil-secreting species

B
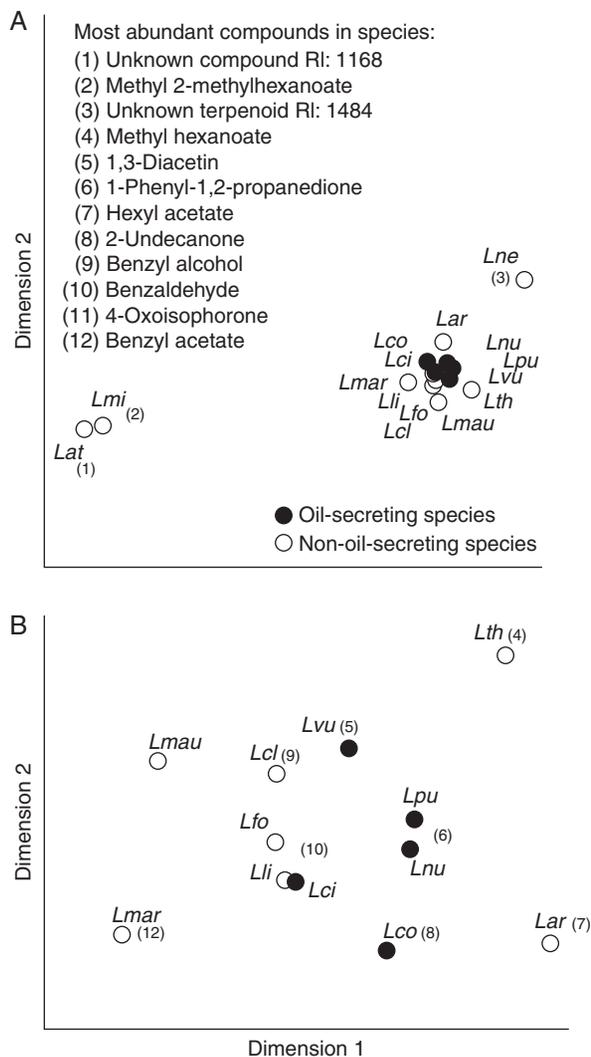
Fig. 1. (A) Comparison of floral scent bouquets among five oil- and ten non-oil-secreting *Lysimachia* species based on semi-quantitative Bray–Curtis similarities (stress value = 0·01; see text for details). See Table 1 for species' abbreviations. In (B), the species *L. atropurpurea* (*Lat*), *L. minoricensis* (*Lmi*) and *L. nemorum* (*Lne*), which were outliers in (A), were excluded from the non-metric multidimensional scaling (NMDS) analysis (stress value = 0·06). The most abundant compounds in floral scents of the different species are indicated.

Most abundant compounds in species:
(1) (Z)-3-Hexenyl acetate
(2) (E)-4,8-Dimethyl-1,3,7-nonatriene
(3) Benzyl alcohol
(4) (Z)-3-Hexenol
(5) (E)-β-Ocimene
(6) (E,E)-α-Farnesene

● Oil-secreting species
○ Non-oil-secreting species

Fig. 2. Comparison of vegetative scent bouquets among five oil and 12 non-oil *Lysimachia* species in a non-metric multidimensional scaling (NMDS) based on semi-quantitative Bray–Curtis similarities (stress value = 0·09. For species' abbreviations see Table 1. The most abundant compounds in scent of vegetative parts are indicated.

Leaf and floral colour properties

Leaves of oil and non-oil *Lysimachia* species had similar reflectance properties, and all leaves reflected most strongly at around 550 nm. One of the species, *L. atropurpurea*, also reflected to a lesser extent in the blue spectrum (450–500 nm; Supplementary Data Fig. S1). The yellow-coloured oil flowers reflected strongly at wavelengths of 500–700 nm, whereas *L. ciliata* additionally reflected strongly at 300–350 nm. Flowers of the non-oil species reflected at 400–700 nm (white coloured), 300–400 nm + 500–700 nm (yellow coloured), 300–400 nm + 600–700 nm (blue coloured) or 300–450 nm + 600–700 nm (purple coloured; see Supplementary Data Fig. S2).

Flowers that secrete oil appear bee-green or UV-green (only *L. ciliata*) to bees against their leaves as background and non-oil-secreting flowers appear blue-green, UV-blue, UV, UV-green or blue-green (Fig. 3). For details on flower reflectance properties, hexagon distances among colours and the distances of colour loci to the background, see Supplementary Data Fig. S2 and Table S4.

Phylogenetic signal and correlated evolution

Pollination systems showed a significant phylogenetic signal ($C = 1$; $P < 0.05$) and did not vary randomly on the tips of the phylogenetic tree. In addition, correlated evolution was found between oil-bee pollination and the pattern of occurrence of certain floral compounds (Supplementary Data Table S5). The aromatics 1-phenyl-1,2-propanedione and (E)-cinnamaldehyde, the aliphatics 2-nonanone, monoacetin and 1,3-diacetin, and the monoterpene linalool showed BF values >2 and

L. lichiangensis as well as *L. arvensis*. (E,E)-α-Farnesene was, besides (Z)-3-hexenyl acetate (see above), dominant in *L. punctata*. In contrast to floral scents, variability in vegetative scent composition did not differ between oil and non-oil species (PERMDISP: $F_{1,15} = 4·5$; $P = 0·11$). The oil and the non-oil species did not have different vegetative scents overall based on the relative amount of compounds (ANOSIM: global $R_{1,15} = -0·158$; $P = 0·90$).

Vegetative and floral scent were not correlated, based on the semi-quantitative scent matrices ($\rho_{13} = 0·161$; $P = 0·186$).

The total amount of volatiles emitted from flowers was higher (2- to 2000-fold) than from leaves in 12 species (Tables 2 and 3). Only for *L. vulgaris* was the total amount of scent emitted higher in leaves (20-fold).

FIG. 3. Petal colours of five oil and nine non-oil *Lysimachia* species displayed in a hexagon colour space. E_U, E_B, E_G: excitation of the UV, blue, and green receptor, respectively. For species' abbreviations see Table 1. The pairwise interspecific distances in colour loci and the distances to the centre of the single species can be found in Supplementary Data Table S4.

additionally a smaller harmonic mean for the independent model in all five replicates.

Floral compounds (*E*)-2-dodecenal, 2-tridecanone, (*E*)-citral and 1-hydroxy-1-phenyl-2-propanone exhibited a significant phylogenetic signal (Fig. 4; Supplementary Data Table S5).

The significant phylogenetic pattern of the vegetative compound terpinolene and the correlated evolution with oil-bee pollination of floral benzaldehyde and benzyl alcohol, and vegetative (*E*)-α-bergamotene is skewed due to missing sequence data from three non-oil species. Thus, it is not considered further here. These compounds occurred in at least one of the three non-oil species with no sequences available in GenBank (*L. decurrens*, *L. fortunei* and *L. lichiangensis*; Supplementary Data: compare Table S5 with Tables S2, S3).

There was correlated evolution between the petal colour bee-green and the oil-bee pollination system (harmonic means: correlated model −12·962, independent model −14·635; BF 3·346). Further, bee-green was not randomly distributed on the phylogenetic tree but showed a significant phylogenetic signal ($C = 0.309$, $P = 0.03$).

DISCUSSION

Contrary to our prediction, we found no specific compound or blend of compounds common in and unique to the floral scents of oil-secreting species. However, the occurrence of some compounds was correlated with oil secretion, suggesting that these compounds were selected by *Macropis*. As expected, we found no difference between the vegetative scents of plants

bearing oil flowers vs. those with flowers having other types of food rewards (e.g. pollen or nectar) or being cleistogamous. There was no clear evidence for correlation between volatiles of vegetative parts and oil secretion. Independent of the pollination mode, leaf colours were similar among all species. In contrast, flower colours varied strongly among species. Flowers of most of the oil-secreting species were bee-green, a colour not found in any non-oil species. As a consequence, bee-green flower colour correlated with oil secretion, and the preponderance of this colour in oil species is likely due to selective pressures exerted by *Macropis*.

Floral scent characteristics and its evolution in Lysimachia

Most of the 63 compounds found in our analyses are widespread floral scent constituents (Knudsen *et al.*, 2006), and several of the ones we found in oil-secreting species, such as benzaldehyde, benzyl alcohol and 2-tridecanone, are also known from other oil-secreting plants, i.e. South African/Neotropical orchids or a South African Iridaceae (Manning and Goldblatt, 2005; Kaiser, 2011; Steiner *et al.*, 2011). The oil-secreting plants of South Africa, which are pollinated by melittid bees (*Rediviva*) closely related to *Macropis*, are, similarly to oil *Lysimachia* spp., typically dominated by aliphatics or aromatics. In contrast, terpenoids are very rarely found in abundant amounts (Table 2; Supplementary Data Table S2, Fig. S3), and this compound class may not play an important role for attracting melittid oil bees though it may be important to attract other bees; terpenoids are very widespread in other bee-pollinated plants (Dobson, 2006). In *Lysimachia*, the variability in floral scent within the oil as well as non-oil species is quite high; however, variability in oil species is lower than variability in non-oil species. This may indicate that *Macropis* exerts a stabilizing selective pressure on floral scent in oil-secreting *Lysimachia* species (Cresswell, 1998). We did not find overall differences in scent composition between oil and non-oil species (Table 2, Fig. 1; Supplementary Data Table S2), nor did we find any compound that was common in all oil species. Generally, almost 70 % of the flower-specific volatiles occurred only in one of the studied species and floral scent was generally species specific, which may be important for reproductive isolation among sympatric species (Knudsen, 1999; Waelti *et al.*, 2008), such as *L. punctata*, *L. vulgaris* and *L. nummularia*. The remaining compounds occurred in at least two and up to seven species, and the pattern of occurrence of some of these compounds specific to plants with oil can be explained by phylogeny, whereas the presence of other compounds may be a result of pollinator-mediated selection.

Importance of phylogeny

Lysimachia punctata and *L. nummularia* are oil plants that are members of the subgenus *Lysimachia* group B (Table 1), and both species emitted a few compounds, such as (*E*)-2-dodecenal and 2-tridecanone, that we did not find in any other species. The common occurrence of these compounds in *L. punctata* and *L. nummularia* can be explained by a shared common ancestor (Supplementary Data Table S5; see also Fig. 4). (*E*)-2-Dodecenal and 2-tridecanone are

FIG. 4. Phylogenetic tree of 14 studied *Lysimachia* species (sequences available from GenBank) with the oil-secreting species shown in bold. The clade membership of the single species is as in Table 1. The pattern of occurrence of (*E*)-2-dodecenal, 2-tridecanone, (*E*)-citral and 1-hydroxy-1-phenyl-2-propanone (**a**) can be explained by phylogeny, and that of (*E*)-cinnamaldehyde (**b**) by correlated evolution with oil secretion/pollination by *Macropis* (Supplementary Data Table S5).

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known as floral compounds of non-oil plant species (Kaiser, 2006, 2011; Knudsen *et al.*, 2006; Balao *et al.*, 2011), and recently they were also found in oil-secreting South African orchids (Steiner *et al.*, 2011). (*E*)-2-Dodecenal occurred only in a few of these orchids, but 2-tridecanone was very widespread and in some of the species an abundant compound. Steiner *et al.* (2011) suggested that this compound may be biologically active for *Rediviva*, and electrophysiological analyses show that it is at least active in *Macropis*, since it elicited strong responses in antennae of *M. fulvipes* (Dötterl, 2008). This compound may therefore be involved in the attraction of Holarctic and South African oil bees. However, 2-tridecanone is well known as a feeding deterrent or repellent for insects (Williams *et al.*, 1980) including generalist bee pollinators (Dobson *et al.*, 1999), and may, therefore, prevent generalist pollinators from visiting oil plants. Plants secreting floral oil typically receive few, if any, visits from insects other than oil bees, even though some have flowers that offer large amounts of pollen (e.g. *Lysimachia*) as a pollinator reward (S. Dötterl, unpubl. res.). Taken together, 2-tridecanone may restrict the pollinator spectrum to the oil bees in *L. punctata*, *L. nummularia* and several South African orchids, and therefore act as a floral filter (Johnson *et al.*, 2006; Balao *et al.*, 2011).

Influence of pollinator-mediated selection

The apparent convergence of linalool, 1-monoacetin and 1,3-diacetin in sympatric, but distantly related oil species *L. punctata* and *L. vulgaris* (Table 1) suggests pollinator-mediated selection (Supplementary Data Table S5). These two oil species occur in Europe where they are visited by *M. fulvipes* and *M. europaea*. Linalool is among the most widespread floral scent compounds (Knudsen *et al.*, 2006), occurs in many species pollinated by specialized or generalist bees (Dobson, 2006) and is known as an attractant for social as well as solitary bee species (Dötterl and Vereecken, 2010). It also may be involved in host plant finding for European *Macropis* bees, though on its own it may not be useful for discriminating between *Lysimachia* oil plants and other co-occurring plants. The acetylated glycerides 1-monoacetin and 1,3-diacetin, together with 1,2-diacetin (which was detected in *L. vulgaris* and non-oil *L. thyrsiflora*) and triacetin (detected in the scent of *L. vulgaris*), are described here for the first time as floral scents. They are structurally related to the 'non-volatile' floral oils in *Lysimachia* and other oil plants (Vogel, 1986; Seipold, 2004; Dumri, 2008), which often consist of mono-, di- or triacylglycerides (Neff and Simpson, 2005). Mono-, di- and triacetin occur, with the exception of 1,2-diacetin, only in oil species, and their occurrence seems to have to do with the presence of oil. They may be produced by biosynthetic pathways similar to those of the non-volatile floral oils. We are currently investigating whether these volatile acetylated glycerides are involved in attracting *Macropis* bees to *Lysimachia* oil flowers. The occurrence of 1,2-diacetin in non-oil *L. thyrsiflora* may have to do with its close relatedness to oil-secreting *L. vulgaris*. We do not yet know whether *L. thyrsiflora* produces and secretes trace amounts of floral oils despite being regarded as a non-oil plant.

(*E*)-Cinnamaldehyde, which occurs in *Lysimachia* species from three different clades and three different continents, and 1-phenyl-1,2-propanedione, which occurs in two different clades and three different European species, are other examples of correlated evolution with oil secretion (Supplementary Data Table S5; see also Fig. 4). (*E*)-Cinnamaldehyde is known from Cucurbitaceae flowers and attractive for *Peponapis pruinosa*, a bee specialized on Cucurbitaceae (Andrews *et al.*, 2007), and we found in electroantennographic measurements that *M. fulvipes* as well as *M. europaea* can detect this compound (I. Schäffler and S. Dötterl, unpubl. res.).

1-Phenyl-1,2-propanedione was the most abundant compound in European *L. punctata* (see also Dötterl and Schäffler, 2007) and *L. nummularia*, both from the subgenus *Lysimachia* group B, and also was abundant in European *L. vulgaris* (subgenus *Lysimachia* group E) (Table 2). This aromatic compound is an uncommon floral volatile that is known to be emitted by only a few non-oil orchid species (Kaiser, 1993; Huber *et al.*, 2005). Its reduced form, 1-hydroxy-1-phenyl-2-propanone, also a very rare floral scent compound (Knudsen *et al.* 2006), is probably emitted from the same three *Lysimachia* species, and could be selected by pollinators as well, although we detected it only in *L. punctata* and *L. nummularia*. However, 1-hydroxy-1-phenyl-2-propanone partly rearranges to 1-phenyl-1,2-propanedione during GC-MS analyses (S. Dötterl, unpubl. res.), and the amount of 1-phenyl-1,2-propanedione in *L. vulgaris* was at least 100-fold smaller than in *L. punctata*. 1-Hydroxy-1-phenyl-2-propanone, though present, may have been below the detection threshold after rearrangement of a large proportion to the diketone. The fact that 1-hydroxy-1-phenyl-2-propanone elicited strong responses in electrophysiological measurements with antennae of *M. fulvipes* could support the idea of pollinator-mediated selection; however, it failed to attract bees in behavioural experiments (Dötterl and Vereecken, 2010).

Scent from vegetative parts in Lysimachia

Scents from the vegetative parts of *Lysimachia* are species specific and, in contrast to floral scents, variability (dispersion) among oil species is comparable with that among non-oil species. These vegetative scents are mostly dominated by aliphatics and/or terpenoids (Fig. 5; Supplementary Data Fig. S3), which are well-known and widespread vegetative scents in plants (Kessler and Baldwin, 2001; Pichersky and Gershenzon, 2002). Therefore, it is unlikely that vegetative scents have played a major part in pollinator attraction in *Lysimachia*. There was also no obvious pattern to the distribution of scent compounds between oil and non-oil species. No compound occurred in more than one oil species and, at the same time, was absent from non-oil species. Furthermore, there was no evidence of convergence on a specific vegetative scent that could be considered characteristic for oil species, nor did oil species lack specific compounds that were abundant in several non-oil species. Some of the compounds, especially a group of non-identified terpenoids, occurred as minor (trace) compounds in a few non-oil species only (Supplementary Data Table S3), but the occurrence of these compounds can be explained best by the close relationship of the plant species emitting these compounds. Our analyses therefore do not reveal a
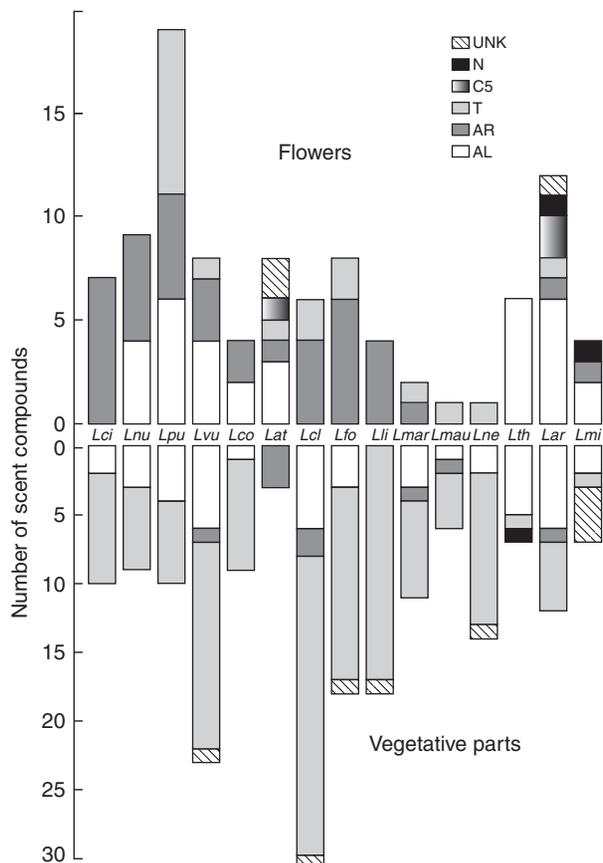
FIG. 5. Number of scent compounds per compound class in floral and vegetative scent of the plants studied. Species abbreviations' are as in Table 1; AL, aliphatics; AR, aromatics; T, terpenoids; C5, C5-branched chain compounds; N, N-containing compounds; UNK, unknown compounds.

vegetative scent compound which seems to be under pollinator-mediated selection and involved in attraction of *Macropis*.

Two of the non-oil species, *L. mauritiana* and *L. atropurpurea*, of the clade subgenus *Palladia + Lysimachiopsis*, differed in their vegetative scent pattern from the other species, and emitted only or mainly (relative amount) aromatics (Fig. 5; Supplementary Data Fig. S3). In both species, the most abundant compound was benzyl alcohol, but *L. atropurpurea* also emitted large amounts of methyl benzoate and *p*-anisaldehyde. These three compounds are known to elicit behavioural responses in generalist pollen-collecting bees and male fragrance-collecting euglossine bees (Dötterl and Vereecken, 2010), and it is possible that vegetative scents contribute to pollinator attraction in these generalist melittophilous *Lysimachia* species.

We found a correlation in qualitative scent pattern (set of compounds) between floral and vegetative scent data, i.e. species emitting a similar set of floral compounds also emitted a similar set of vegetative compounds, and species strongly differing in their floral scents also differed in their vegetative scents. One might predict that this correlation is due to shared biosynthetic pathways in floral and vegetative organs. However, several of the species emitted scents from different pathways in the different organs. As an example, some of the species emitted aliphatics only from vegetative parts (Fig. 5; Supplementary Data Fig. S3). Similarly, the

occurrence of aromatics differed strongly between vegetative and floral parts. The correlation between floral and vegetative scents can, therefore, only partially be explained by shared pathways. Several species emitted aromatics only from their flowers (but see above), suggesting that these compounds are important for pollinator attraction. Indeed, recent meta-analyses suggest that aromatics evolved in flower scents in order to attract pollinators (Junker and Blüthgen, 2010; Schiestl, 2010).

Floral colour evolution in *Lysimachia*

Though flowers of all oil species are yellow to the human eye, we found differences in the colours that bees perceive (i.e. bee-colour) among the different species (Fig. 3). Nevertheless, there is evidence for correlated evolution between bee-green (yellow to the human eye) and oil secretion. Most of studied oil species are bee-green, though they belong to three different clades (sub-genus *Lysimachia* group B, E and F, Table 1). Bee-green is not found in non-oil species, though it is known to be attractive for generalist bees (Giurfa *et al.*, 1995), the suggested pollinators of these species. It also may be attractive to *Macropis* in general and, in the case of *L. punctata*, bee-green colours may have been responsible for attracting *M. fulvipes* to visual cues of the inflorescences (see also Dötterl *et al.*, 2011). Nearly all species of the clade *Palladia + Lysimachiopsis* are bee blue-green, and this similarity in colour can be explained by the close relatedness of these species. Only one species of this clade, *L. atropurpurea*, evolved another colour and is bee UV-blue. Generally, these colours are known to elicit behavioural responses in bees (Menzel, 1985; Giurfa *et al.*, 1995) and seem to be involved in attracting bee pollinators in these species.

The flower colour of one of our study species, cleistogamous *L. minoricensis*, was very similar to its leaf colour (distance to centre <0·1 hexagon units; Supplementary Data Table S4), and bees may, therefore, have difficulties in discriminating flowers from leaves. In this species, where pollinators are not required for reproductive success, selection may have favoured the evolution of cryptic flowers to prevent the attraction of flor-ivores (Penet *et al.*, 2009).

Conclusions

Lysimachia species emit specific and highly variable floral and vegetative scents. Oil-secreting species have a lower variability in floral scents compared with non-oil species, but there is no corresponding difference in variability of vegetative scents between oil and non-oil species. Thus, as predicted, floral, but not vegetative, scent seems to be under stabilizing selection from *Macropis* pollinators. Overall vegetative and floral scent compositions do not differ between oil and non-oil species, and none of the compounds occurs in all oil or in all non-oil species. However, some floral compounds specific to a few oil species exhibit correlated evolution with oil bee pollination, and these compounds may be under selection by *Macropis*. Leaf colours are similar among all studied species, but flower colour differs among species. Most notable is the correlated evolution between the flower colour bee-green and oil secretion, even though most, but not all oil

flowers have this colour. This relationship is not surprising, however, because non-oil species are never bee-green. Overall, the data suggest that floral, but not vegetative, scents and colours are under selection by *Macropis* oil bees and that this is congruent with the study of Dötterl *et al.* (2011) showing that *M. fulvipes* bees use both visual and olfactory inflorescence cues for host location, whereas vegetative cues are not attractive. Oil species, though all pollinated by *Macropis* bees, do not share a signature volatile compound or bee-colour, and this suggests that different *Macropis* species are effectively attracted by different scents or colours or that flowers of the oil species emit a compound or compounds that are commonly used by *Macropis* for finding oil hosts, but that have not yet been detected by our methods.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following. Table S1: species used for the study and their origin. Table S2: percentage amount of flower-specific scent compounds in the individual samples. Table S3: percentage amount of vegetative scent compounds in the individual samples. Table S4: colour distance matrix in hexagon units. Table S5: phylogenetic signal and BayesTraits results of scent cues (floral, vegetative). Figure S1: mean spectral reflection of leaves in 12 *Lysimachia* species. Figure S2: mean spectral reflection of flowers in 14 *Lysimachia* species. Figure S3: relative amount of different compound classes in floral and vegetative scent of the study plants.

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SUPPLEMENTARY DATA

TABLE S1. Origin of plant material

Species	Plants/seeds origin
Lysimachia punctata	EBG (P)
L. vulgaris	EBG (P)
L. nummularia	Native habitat near Bayreuth (P)
L. ciliata	EBG (P)
L. congestiflora	Garden center Bayreuth (P)
L. nemorum	Native habitat near Bayreuth (P)
L. thyrsiflora	EBG (P)
L. clethroides	BG of University Hohenheim (S)
L. fortunei	Jardin Botanique, Nantes, France; wild plants Japan (S)
L. lichiangensis	National BG Glasnevin, Dublin (S)
L. decurrens	Jardin Botanique, Nantes, France (S)
L. ephemerum	österr. Gartenbaugesellschaft, Graz, Austria (S)
L. mauritiana	Jardin Botanique de la ville de Lyon, France (S)
L. minoricensis	Jardin Botanique Ville et Université, Besancon, France (S)
L. atropurpurea	EBG (S)
L. maritima	Natural habitat, Saxony-Anhalt, Germany (P)
L. arvensis	EBG (P)

P, plants; S, seeds. EBG, Ecological-Botanical Garden of the University of Bayreuth; BG, Botanical Garden.

[Tables S2 and S3 are provided separately in an Excel file]

TABLE S4. Distances (in hexagon units) between flower colour loci of 14 *Lysimachia* species (values below 0.1 units are in bold) and distances of each flower colour to the hexagon centre (in hexagon units)

	Lci	Lnu	Lpu	Lne	Lvu	Lco	Lat	Lcl	Lep	Lfo	Lli	Lmi	Lth	Distance to the centre
Lci														0.17
Lnu	0.15													0.15
Lpu	0.30	0.18												0.33
Lne	**0.09**	0.23	0.39											0.20
Lvu	0.22	0.11	**0.08**	0.33										0.25
Lco	0.30	0.19	**0.01**	0.39	**0.08**									0.33
Lat	0.29	0.37	0.55	0.23	0.48	0.56								0.24
Lcl	0.38	0.27	0.35	0.43	0.29	0.36	0.42							0.23
Lep	0.29	0.20	0.32	0.34	0.25	0.33	0.34	**0.09**						0.14
Lfo	0.42	0.31	0.37	0.47	0.32	0.37	0.46	**0.05**	0.13					0.27
Lli	0.34	0.24	0.35	0.38	0.28	0.35	0.37	**0.05**	**0.04**	0.10				0.18
Lmi	0.22	0.12	0.26	0.28	0.19	0.27	0.33	0.16	**0.08**	0.20	0.12			0.10
Lth	**0.06**	0.11	0.25	0.15	0.19	0.25	0.34	0.36	0.28	0.41	0.33	0.21		0.16
Lar	0.41	0.53	0.71	0.31	0.64	0.71	0.43	0.65	0.56	0.69	0.60	0.53	0.46	0.44

TABLE S5. Phylogenetic signal of floral and vegetative compounds present in at least two oil-species and BayesTraits results (one of five independent runs) for independent and correlated models of evolution with oil-bee pollination. Values are given in bold if there is a significant phylogenetic signal and/or a correlated evolution between the presence of specific compounds and secretion of floral oil

| Compound | Type | Phylogenetic signal | | Correlated evolution | | |
| | | | | Harmonic mean | | |
		C	P	Independent model	Dependent model	BF
2-Nonanone	F	–0.15	0.69	–15.308	–14.280	**2.055[a]**
1-Monoacetin/Linalool/1,3-Diacetin	F	–0.13	0.47	–15.636	–14.472	**2.328[a]**
1,2-Diacetin	F	0.22	0.11	–15.858	–15.600	0.517
2-Undecanone	F	0.14	0.13	–15.209	–15.536	0.653
(*E*)-2-Dodecenal/2-Tridecanone	F	**0.49**	**0.02**	–12.603	–12.481	0.246
Benzaldehyde[c]	F	**0.46**	**0.01**	–13.900	–12.759	**2.282[a]**
Benzyl alcohol[c]	F	–0.19	0.67	–18.357	–16.969	**2.776[a]**
1-Phenyl-1,2-propanedione	F	0.27	0.06	–14.538	–13.305	**2.466[a]**
Aromatic, RI: 1204 [c]	F	–0.21	0.88	–17.029	–17.696	1.333
(*E*)-Citral/1-Hydroxy-1-phenyl-2-propanone	F	**0.49**	**0.02**	–12.682	–12.212	0.940
(*E*)-Cinnamaldehyde	F	–0.08	0.32	–16.288	–14.159	**4.257[a]**
(*Z*)-3-Hexenol[c]	V	–0.09	0.45	–17.407	–18.445	2.077[b]
(Z)-3-Hexenyl acetate[c]	V	–0.06	0.3	–16.253	–16.284	0.062
FAD, RI: 1225	V	0.14	0.15	–17.289	–16.424	1.729
FAD, RI: 1230[c]	V	0.23	0.06	–17.634	–18.557	1.845
(*Z*)-β-Ocimene[c]	V	–0.16	0.56	–17.019	–16.824	0.392
(*E*)-β-Ocimene[c]	V	0.09	0.19	–16.743	–17.642	1.799
Terpinolene[c]	V	**0.49**	**0.02**	–12.781	–12.266	1.031
(*E*)-4,8-Dimethyl-1,3,7-nonatriene[c]	V	0.04	0.26	–17.700	–18.488	1.576
Camphor	V	–0.29	0.97	–16.665	–16.753	0.177
Borneol	V	–0.12	0.46	–16.767	–16.771	0.008
Bornyl acetate	V	–0.01	0.24	–16.026	–16.194	0.337
α-Ylangene[c]	V	–0.14	0.48	–16.149	–16.888	1.475
α-Copaene[c]	V	–0.37	0.95	–16.768	–17.678	1.820
β-Bourbonene[c]	V	–0.14	0.48	–16.091	–16.908	1.633
β-Caryophyllene[c]	V	–0.38	0.96	–19.123	–19.410	0.572
(*E*)-α-Bergamotene[c]	V	–0.13	0.47	–15.579	–14.370	**2.417[a]**
α-Farnesene[c]	V	–0.19	0.74	–17.765	–18.929	2.327[b]

[a] Evidence of correlated evolution (dependent evolution)
[b] Evidence of independent evolution
[c] Compounds occurred additionally in species, where gene sequences were not available (*L. decurrens*, *L. lichiangensis*, *L. fortunei*)

F$_{IG}$. S1. Mean spectral reflection of leaves in 12 *Lysimachia* species.
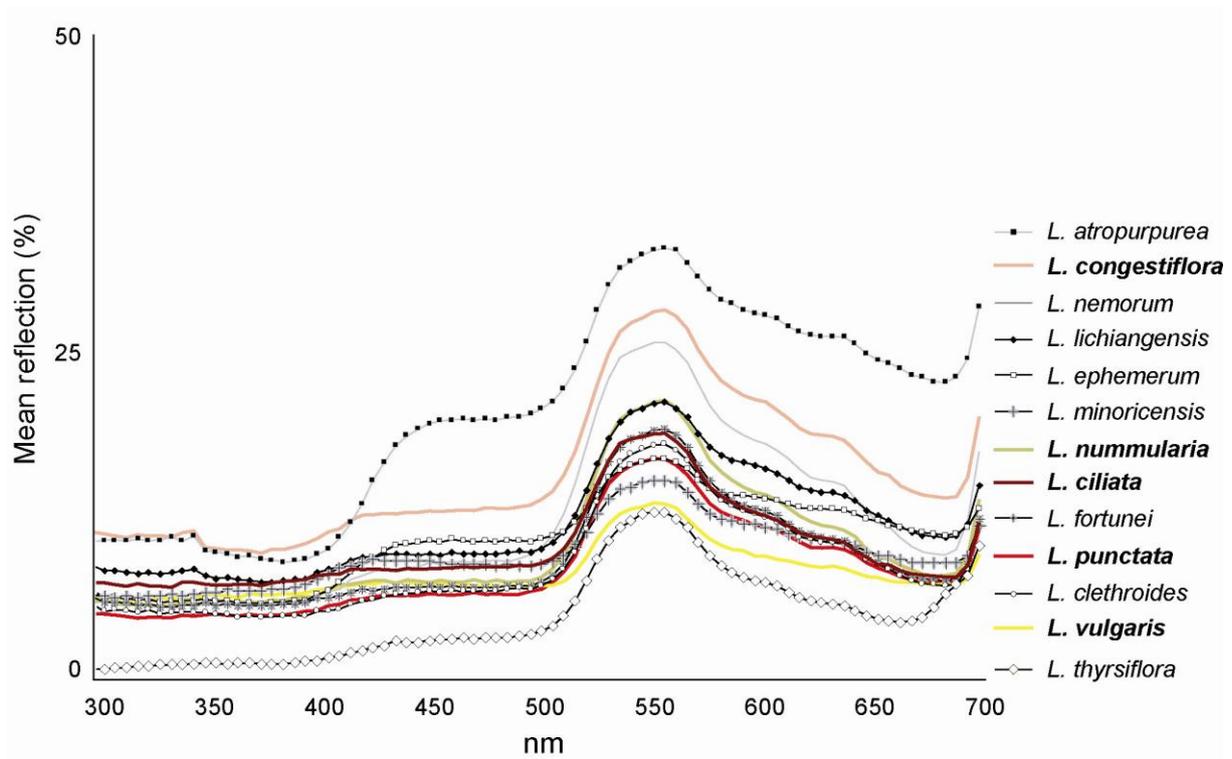
F<small>IG.</small> S2: Mean spectral reflection of flowers in 14 *Lysimachia* species.
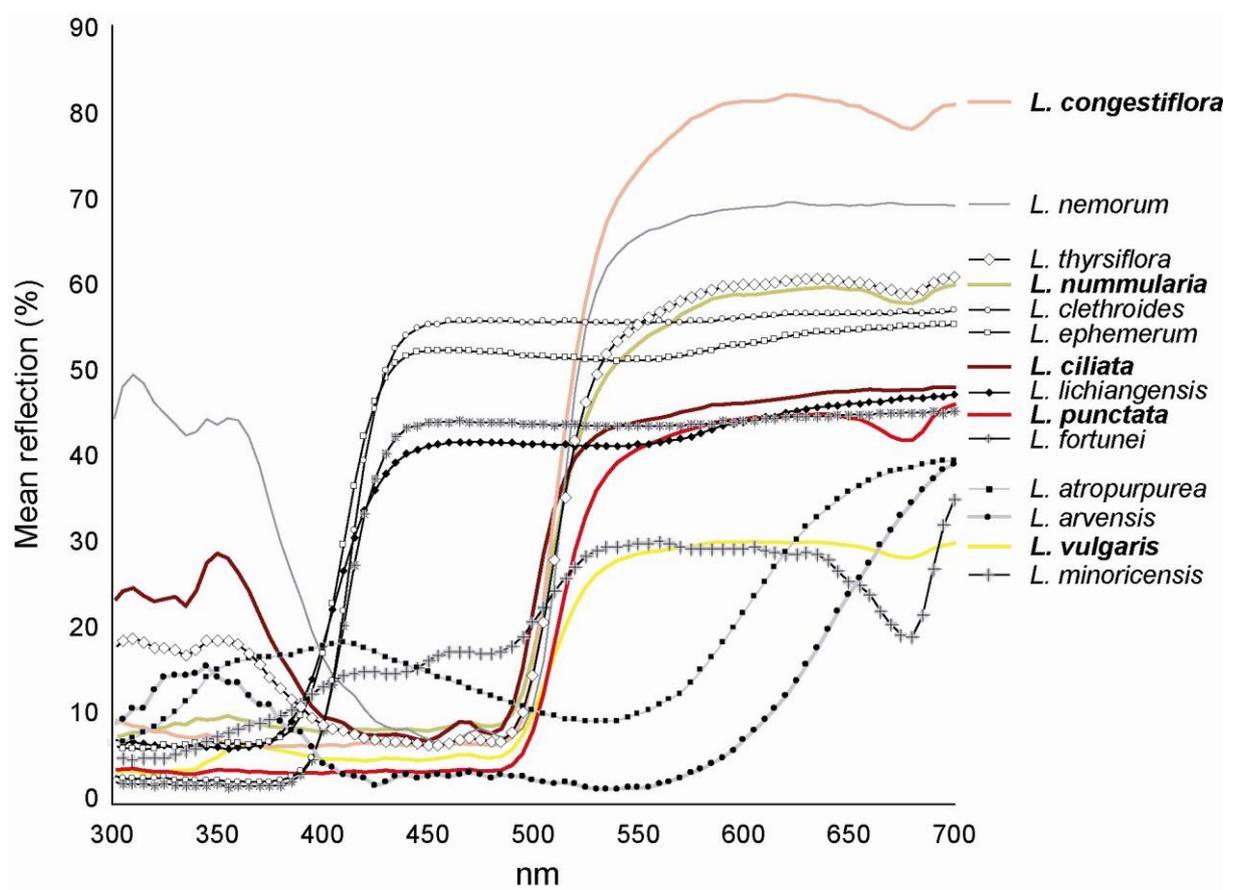
F<small>IG</small>. S3. Relative amount of different compound classes (AL, aliphatics;, AR, aromatics; T, terpenoids; C5, C5-branched chain compounds; N, N-containing compounds; UNK, unknown compounds) in floral and vegetative scent of the study plants. Species' abbreviations as in Table 1 in the text.
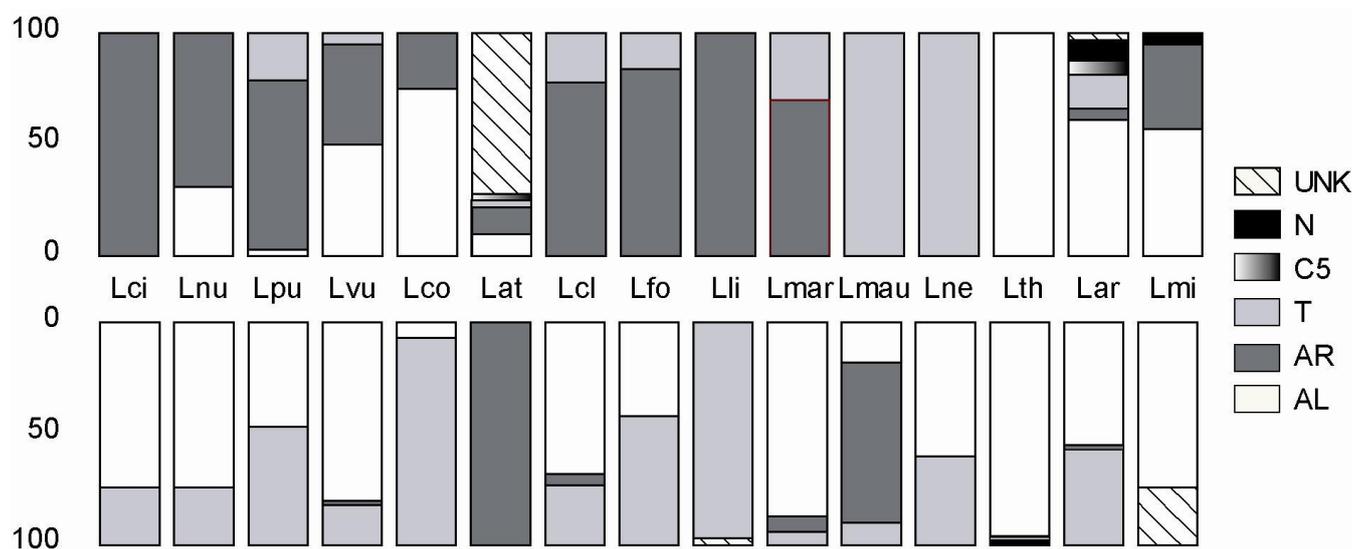
SUPPLEMENTARY DATA

Table S2. Percentage amount of flower specific scent compounds in the individual samples
(listed within classes according to Kovats retention index, RI). Oil producing species are
printed in bold.
For species abbreviations see Table 1.

(a) Identification based on authentic standards
(b) identification based on mass spectrum and retention index
(c) identification based on mass spectrum

	RI	Lci 1	Lci 2	Lci 3	Lci 4	Lci 5	Lnu 1	Lnu 2	Lnu 3	Lnu 4	Lnu 5	Lpu 1	Lpu 2	Lpu 3	Lpu 4	Lpu 5	Lne 1	Lne 2
Amount of trapped scent ng / flower * 12 minutes		1.8	0.9	1.6	0.5	0.3	0.1	0.0	0.2	42.0	11.7	0.7	1.6	0.9	0.9	1.0	0.1	0.0
Aliphatics	RI																	
Methyl hexanoate[b]	934	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methyl-2-hexen-4-one[c]	966	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 2-methylhexanoate[c]	972	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 4-methylhexanoate[c]	1004	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexyl acetate[b]	1008	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 4-methyl-2-hexenoate[c]	1032	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 87, 57, 43, 118, 102, 55	1047	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 74, 43, 55, 41, 39, 101	1067	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-2-Pentenyl butanoate[c]	1087	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Nonanone[a]	1088	-	-	-	-	-	-	37.5	3.0	0.1	0.3	-	-	-	-	-	-	-
1-Monoacetin[a]	1091	-	-	-	-	-	-	-	-	-	-	-	-	0.0	4.1	-	-	-
(*Z*)-3-Hexenyl propionate[b]	1092	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 68, 57, 39, 41, 40	1115	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,3-Diacetin[a]	1232	-	-	-	-	-	-	-	-	-	-	tr	tr	-	0.1	0.2	-	-
1,2-Diacetin[a]	1236	-	-	-	-	-	-	-	-	-	-	tr	tr	tr	tr	-	-	-
m/z: 67, 57, 82, 39, 41, 85	1271	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Undecanone[a]	1281	-	-	-	-	-	2.7	20.7	4.7	0.4	1.5	0.2	0.2	0.3	0.4	0.5	-	-
Triacetin[a]	1328	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 88, 55, 73, 101, 157, 61	1375	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl decanoate[b]	1382	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 41, 69, 39, 57, 71, 43	1385	-	-	-	-	-	-	-	-	-	-	0.8	0.4	0.7	0.5	0.0	-	-
(*E*)-2-Dodecenal[a]	1458	-	-	-	-	-	0.5	0.4	0.9	1.5	4.6	0.3	0.3	0.7	-	-	-	-
2-Tridecanone[a]	1484	-	-	-	-	-	10.8	41.4	13.5	1.1	6.1	1.3	1.0	1.1	2.3	1.3	-	-
Methyl dodecanoate[b]	1507	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aromatics																		
Benzaldehyde[a]	982	83.4	87.3	88.4	90.0	94.9	-	-	-	10.1	52.2	32.6	23.4	-	48.0	28.1	-	-
Benzyl alcohol[a]	1050	11.8	5.6	9.7	6.0	3.4	-	-	-	0.5	1.7	-	-	-	-	-	-	-
Methyl benzoate[a]	1104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate[a]	1104	0.1	0.1	0.3	0.2	0.0	-	-	-	-	-	-	-	-	-	-	-	-
1,4-Dimethoxybenzene [a]	1174	-	-	-	-	-	-	-	-	-	-	4.2	3.0	3.3	7.0	4.7	-	-
Benzenepropanal[b]	1162	0.1	0.5	0.3	0.2	0.0	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl benzoate[b]	1171	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione[a]	1171	-	-	-	-	-	85.9	-	77.8	86.2	33.2	40.0	43.8	69.0	13.8	50.4	-	-
m/z: 79, 80, 108, 43, 77, 80	1204	-	-	-	-	-	-	-	-	-	-	0.7	1.2	0.1	0.0	0.0	-	-

		Lci 1	Lci 2	Lci 3	Lci 4	Lci 5	Lnu 1	Lnu 2	Lnu 3	Lnu 4	Lnu 5	Lpu 1	Lpu 2	Lpu 3	Lpu 4	Lpu 5	Lne 1	Lne 2
Amount of trapped scent ng / flower * 12 minutes		1.8	0.9	1.6	0.5	0.3	0.1	0.0	0.2	42.0	11.7	0.7	1.6	0.9	0.9	1.0	0.1	0.0
m/z: 91, 43, 65, 162, 118, 93	1206	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Cinnamaldehyde[a]	1225	0.0	0.1	-	0.0	0.0	-	-	-	-	-	-	-	-	-	-	-	-
1-Hydroxy-1-phenyl-2-propanone[a]	1236	-	-	-	-	-	-	-	-	0.1	0.2	2.7	1.7	5.6	1.0	1.3	-	-
3,5-Dimethoxytoluene[b]	1268	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Cinnamaldehyde[a]	1287	4.4	6.2	1.2	3.5	1.6	-	-	-	0.1	0.2	-	-	-	-	-	-	-
(E)-Cinnamyl alcohol[a]	1305	0.2	0.2	0.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl valerate[b]	1392	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-3-Hexenyl benzoate	1586	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids																		
Camphene[b]	969	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Linalool[a]	1104	-	-	-	-	-	-	-	-	-	-	7.5	8.1	7.7	9.6	5.5	-	-
m/z: 91, 93, 77, 121, 79, 92	1117	-	-	-	-	-	-	-	-	-	-	0.6	0.6	1.0	0.2	1.1	-	-
allo-Ocimene[b]	1135	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone[a]	1142	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citronellal[a]	1151	-	-	-	-	-	-	-	-	-	-	0.8	1.0	1.9	1.3	1.1	-	-
Dihydrooxophorone[b]	1170	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Terpineol[a]	1190	-	-	-	-	-	-	-	-	-	-	0.8	0.6	0.5	0.0	0.4	-	-
m/z: 108, 93, 95, 67, 39, 79	1212	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neral[a]	1239	-	-	-	-	-	-	-	-	-	-	0.8	1.1	2.0	0.5	-	-	-
Nerol[a]	1248	-	-	-	-	-	-	-	-	-	-	1.3	2.0	3.6	-	-	-	-
(E)-Citral[a]	1264	-	-	-	-	-	0.0	-	-	-	-	5.4	10.9	1.0	9.8	5.5	-	-
Geranic acid[a]	1338	-	-	-	-	-	-	-	-	-	-	0.3	0.8	1.5	1.2	-	-	-
m/z: 189, 133, 105, 91, 147, 79	1484	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	100	100
C5-branched chain compound																		
Methyl tiglate[a]	< 900	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amyl/isoamyl butanoate[c]	1055	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methyl-2-butenyl butanoate[c]	1068	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N-containing compounds																		
2-Methoxy-3-isobutyl pyrazine[b]	1179	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Nitro-2-Phenylethane	1313	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknowns																		
unidentified m/z: 93, 91, 108, 107, 39, 41	1109	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unidentified m/z: 56, 41, 39, 42, 43, 69	1168	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unidentified m/z: 43, 71, 67, 95, 121, 138	1451	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Lne 3	Lne 4	**Lvu 1**	**Lvu 2**	**Lvu 3**	**Lvu 4**	**Lvu 5**	**Lvu 6**	**Lco 1**	**Lco 2**	**Lco 3**	**Lco 4**	**Lco 5**	**Lco 6**	Lat 1	Lat 2	Lat 3	Lat 4	Lat 5	Lcl 1
Amount of trapped scent ng / flower * 12 minutes	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.7	0.5	0.6	2.6	1.1
Aliphatics																				
Methyl hexanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methyl-2-hexen-4-one[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 2-methylhexanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 4-methylhexanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.5	0.7	2.4	-	-	-
Hexyl acetate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 4-methyl-2-hexenoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	18.1	6.0	11.2	-	-	-
m/z: 87, 57, 43, 118, 102, 55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.0	2.7	3.5	-	-	-
m/z: 74, 43, 55, 41, 39, 101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-2-Pentenyl butanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Nonanone[a]	-	-	-	-	-	-	-	-	39.6	-	10.8	-	4.9	-	-	-	-	-	-	-
1-Monoacetin[a]	-	-	-	0.3	-	-	7.5	-	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-3-Hexenyl propionate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 68, 57, 39, 41, 40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,3-Diacetin[a]	-	-	9.8	33.8	14.6	33.5	47.7	30.3	-	-	-	-	-	-	-	-	-	-	-	-
1,2-Diacetin[a]	-	-	4.9	21.7	7.0	23.6	36.5	10.2	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 57, 82, 39, 41, 85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Undecanone[a]	-	-	-	-	-	-	-	-	60.4	100.0	89.2	100.0	49.2	97.6	-	-	-	-	-	-
Triacetin[a]	-	-	2.1	5.2	5.0	5.5	3.7	0.3	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 88, 55, 73, 101, 157, 61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl decanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 41, 69, 39, 57, 71, 43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*E*)-2-Dodecenal[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Tridecanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl dodecanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aromatics																				
Benzaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol[a]	-	-	83.3	39.1	73.5	-	-	-	-	-	-	-	41.0	-	-	-	-	-	-	62.4
Methyl benzoate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,4-Dimethoxybenzene [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzenepropanal[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl benzoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione[a]	-	-	-	-	-	2.7	3.4	57.4	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 79, 80, 108, 43, 77, 80	-	-	-	-	-	0.3	1.3	1.7	-	-	-	-	-	-	-	-	-	-	-	4.1

	Lne 3	Lne 4	Lvu 1	Lvu 2	Lvu 3	Lvu 4	Lvu 5	Lvu 6	Lco 1	Lco 2	Lco 3	Lco 4	Lco 5	Lco 6	Lat 1	Lat 2	Lat 3	Lat 4	Lat 5	Lcl 1
Amount of trapped scent ng / flower * 12 minutes	0.0	0.1	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.2	0.0	0.1	0.1	0.7	0.5	0.6	2.6	1.1
m/z: 91, 43, 65, 162, 118, 93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0
(Z)-Cinnamaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Hydroxy-1-phenyl-2-propanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3,5-Dimethoxytoluene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.6	33.4	10.5	8.0	5.0	-
(E)-Cinnamaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	4.9	2.4	-	-	-	-	-	-
(E)-Cinnamyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl valerate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.7
(Z)-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids																				
Camphene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8.0	3.0	2.1	-	-	-
Linalool[a]	-	-	-	-	-	34.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 93, 77, 121, 79, 92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
allo-Ocimene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	27.4
Citronellal[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydrooxophorone[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.4
α-Terpineol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 108, 93, 95, 67, 39, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neral[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nerol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Citral[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geranic acid[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 189, 133, 105, 91, 147, 79	100	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5-branched chain compound																				
Methyl tiglate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.3	7.1	2.4	-	-	-
Amyl/isoamyl butanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methyl-2-butenyl butanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N-containing compounds																				
2-Methoxy-3-isobutyl pyrazine[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Nitro-2-Phenylethane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknowns																				
unidentified m/z: 93, 91, 108, 107, 39, 41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unidentified m/z: 56, 41, 39, 42, 43, 69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	54.8	39.8	62.9	84.9	91.2	-
unidentified m/z: 43, 71, 67, 95, 121, 138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.6	7.3	5.1	7.2	3.8	-

	Lcl 2	Lcl 3	Lcl 4	Lcl 5	Lfo 1	Lfo 2	Lfo 3	Lfo 4	Lfo 5	Lli 1	Lli 2	Lli 3	Lli 4	Lli 5	Lmau 1	Lmau 2	Lmau 3	Lmau 4	Lmau 5	Lmi 1
Amount of trapped scent ng / flower * 12 minutes	0.9	1.3	0.8	0.4	1.8	3.1	1.2	1.7	3.6	13.5	8.9	4.7	9.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1
Aliphatics																				
Methyl hexanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methyl-2-hexen-4-one[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 2-methylhexanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	29.2
Methyl 4-methylhexanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexyl acetate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 4-methyl-2-hexenoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 87, 57, 43, 118, 102, 55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 74, 43, 55, 41, 39, 101	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	21.3
(*Z*)-2-Pentenyl butanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Nonanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Monoacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-3-Hexenyl propionate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 68, 57, 39, 41, 40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,3-Diacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,2-Diacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 57, 82, 39, 41, 85	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Undecanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Triacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 88, 55, 73, 101, 157, 61	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl decanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 41, 69, 39, 57, 71, 43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*E*)-2-Dodecenal[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Tridecanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl dodecanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aromatics																				
Benzaldehyde[a]	-	-	-	-	48.7	28.3	68.5	44.2	68.2	92.6	93.1	74.5	99.4	73.5	-	-	-	-	-	-
Benzyl alcohol[a]	45.7	74.2	76.8	75.4	20.9	50.0	5.1	31.2	11.4	-	5.8	12.6	-	3.6	-	-	-	-	-	-
Methyl benzoate[a]	-	-	-	-	0.3	2.0	3.8	4.8	4.5	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate[a]	-	-	-	-	0.0	1.5	0.7	1.2	-	-	-	-	-	-	-	-	-	-	-	-
1,4-Dimethoxybenzene [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzenepropanal[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl benzoate[b]	-	-	-	-	1.8	3.0	3.2	1.0	1.1	-	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 79, 80, 108, 43, 77, 80	34.8	-	-	-	-	-	-	-	-	5.0	0.9	0.4	0.6	0.0	-	-	-	-	-	-

	Lcl 2	Lcl 3	Lcl 4	Lcl 5	Lfo 1	Lfo 2	Lfo 3	Lfo 4	Lfo 5	Lli 1	Lli 2	Lli 3	Lli 4	Lli 5	Lmau 1	Lmau 2	Lmau 3	Lmau 4	Lmau 5	Lmi 1
Amount of trapped scent ng / flower * 12 minutes	0.9	1.3	0.8	0.4	1.8	3.1	1.2	1.7	3.6	13.5	8.9	4.7	9.1	0.0	0.0	0.0	0.0	0.0	0.0	0.1
m/z: 91, 43, 65, 162, 118, 93	0.0	0.6	4.5	5.7	1.7	2.5	1.9	6.0	0.0	2.5	0.2	12.4	-	22.9	-	-	-	-	-	-
(Z)-Cinnamaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Hydroxy-1-phenyl-2-propanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3,5-Dimethoxytoluene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	45.8
(E)-Cinnamaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Cinnamyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl valerate[b]	0.2	2.5	1.2	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids																				
Camphene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Linalool[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 93, 77, 121, 79, 92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
allo-Ocimene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone[a]	17.5	18.8	13.0	15.8	24.8	9.7	13.6	9.8	13.6	-	-	-	-	-	100	100	100	100	100	-
Citronellal[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydrooxophorone[b]	1.8	3.9	4.6	3.0	1.8	3.0	3.2	1.9	1.1	-	-	-	-	-	-	-	-	-	-	-
α-Terpineol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 108, 93, 95, 67, 39, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Neral[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nerol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Citral[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geranic acid[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 189, 133, 105, 91, 147, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5-branched chain compound																				
Methyl tiglate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amyl/isoamyl butanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3-Methyl-2-butenyl butanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
N-containing compounds																				
2-Methoxy-3-isobutyl pyrazine[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.7
1-Nitro-2-Phenylethane	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknowns																				
unidentified m/z: 93, 91, 108, 107, 39, 41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unidentified m/z: 56, 41, 39, 42, 43, 69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unidentified m/z: 43, 71, 67, 95, 121, 138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Lmi 2	Lmi 3	Lmi 4	Lmi 5	Lth 1	Lth 2	Lth 3	Lth 4	Lar 1	Lar 2	Lar 3	Lmar 1	Lmar 2	Lmar 3	Lmar 4	Lmar 5
Amount of trapped scent ng / flower * 12 minutes	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.1	
Aliphatics																
Methyl hexanoate[b]	-	-	-	-	81.5	82.6	31.2	65.1	-	-	-	-	-	-	-	-
3-Methyl-2-hexen-4-one[c]	-	-	-	-	9.7	3.3	1.5	6.8	-	-	-	-	-	-	-	-
Methyl 2-methylhexanoate[c]	59.3	36.4	43.5	39.0	-	-	-	-	-	-	-	-	-	-	-	-
Methyl 4-methylhexanoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hexyl acetate[b]	-	-	-	-	-	-	-	-	34.0	30.9	25.0	-	-	-	-	-
Methyl 4-methyl-2-hexenoate[c]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 87, 57, 43, 118, 102, 55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 74, 43, 55, 41, 39, 101	10.3	18.2	7.9	16.6	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-2-Pentenyl butanoate[c]	-	-	-	-	-	-	-	-	-	1.9	-	-	-	-	-	-
2-Nonanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Monoacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*Z*)-3-Hexenyl propionate[b]	-	-	-	-	-	-	-	-	20.6	45.5	-	-	-	-	-	-
m/z: 67, 68, 57, 39, 41, 40	-	-	-	-	-	-	-	-	7.8	1.3	8.8	-	-	-	-	-
1,3-Diacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1,2-Diacetin[a]	-	-	-	-	-	1.0	-	-	-	-	-	-	-	-	-	-
m/z: 67, 57, 82, 39, 41, 85	-	-	-	-	-	-	-	-	1.4	0.4	-	-	-	-	-	-
2-Undecanone[a]	-	-	-	-	-	-	-	-	2.7	0.2	0.3	-	-	-	-	-
Triacetin[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 88, 55, 73, 101, 157, 61	-	-	-	-	-	1.5	1.5	0.8	-	-	-	-	-	-	-	-
Ethyl decanoate[b]	-	-	-	-	2.6	-	3.0	-	-	-	-	-	-	-	-	-
m/z: 41, 69, 39, 57, 71, 43	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(*E*)-2-Dodecenal[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Tridecanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl dodecanoate[b]	-	-	-	-	6.2	11.5	62.8	27.4	-	-	-	-	-	-	-	-
Aromatics																
Benzaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl benzoate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl acetate[a]	-	-	-	-	-	-	-	-	-	-	-	49.0	66.7	66.7	66.7	100
1,4-Dimethoxybenzene [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzenepropanal[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ethyl benzoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Phenyl-1,2-propanedione[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 79, 80, 108, 43, 77, 80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Lmi 2	Lmi 3	Lmi 4	Lmi 5	Lth 1	Lth 2	Lth 3	Lth 4	Lar 1	Lar 2	Lar 3	Lmar 1	Lmar 2	Lmar 3	Lmar 4	Lmar 5
Amount of trapped scent ng / flower * 12 minutes	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.1	
m/z: 91, 43, 65, 162, 118, 93	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Cinnamaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
1-Hydroxy-1-phenyl-2-propanone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3,5-Dimethoxytoluene[b]	25.2	36.2	44.6	40.4	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Cinnamaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Cinnamyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl valerate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-3-Hexenyl benzoate	-	-	-	-	-	-	-	-	12.0	0.0	1.5	-	-	-	-	-
Terpenoids																
Camphene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Linalool[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 93, 77, 121, 79, 92	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
allo-Ocimene[b]	-	-	-	-	-	-	-	-	9.1	0.1	36.6	-	-	-	-	-
4-Oxoisophorone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Citronellal[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dihydrooxophorone[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Terpineol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 108, 93, 95, 67, 39, 79	-	-	-	-	-	-	-	-	-	-	-	51.0	33.3	33.3	33.3	-
Neral[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nerol [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-Citral [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Geranic acid[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 189, 133, 105, 91, 147, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C5-branched chain compound																
Methyl tiglate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Amyl/isoamyl butanoate[c]	-	-	-	-	-	-	-	-	1.4	11.9	-	-	-	-	-	-
3-Methyl-2-butenyl butanoate[c]	-	-	-	-	-	-	-	-	-	6.8	-	-	-	-	-	-
N-containing compounds																
2-Methoxy-3-isobutyl pyrazine[b]	5.1	9.1	4.0	4.0	-	-	-	-	-	-	-	-	-	-	-	-
1-Nitro-2-Phenylethane	-	-	-	-	-	-	-	-	7.7	-	21.9	-	-	-	-	-
Unknowns																
unidentified m/z: 93, 91, 108, 107, 39, 41	-	-	-	-	-	-	-	-	3.4	0.9	5.9	-	-	-	-	-
unidentified m/z: 56, 41, 39, 42, 43, 69	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
unidentified m/z: 43, 71, 67, 95, 121, 138	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

SUPPLEMENTARY DATA

Table S3. Percentage amount of vegetative scent compounds in the individual samples (listed within classes according to Kovats retention index, RI). Oil producing species are printed in bold.

For species abbreviations see Table 1.

(a) Identification based on authentic standards

(b) identification based on mass spectrum and retention index

(c) identification based on mass spectrum

	RI	Lci 1	Lci 2	Lci 3	Lci 4	Lci 5	Lnu 1	Lnu 2	Lnu 3	Lnu 4	Lnu 5	Lpu 1	Lpu 2	Lpu 3	Lpu 4	Lne 1	Lne 2	Lne 3
Amount of trapped scent ng / leaf * 12 minutes		1.5	0.0	0.1	3.5	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.3
Aliphatics																		
(Z)-3-Hexenol[a]	850	26.1	5.6	1.7	16.6	17.0	-	-	13.5	8.2	1.5	1.4	-	3.4	8.4	4.0	-	45.7
(Z)-3-Hexenyl acetate[a]	1004	71.6	59.5	9.8	83.2	83.0	78.0	39.7	68.2	75.2	79.0	24.8	16.6	36.9	87.8	49.3	26.2	47.6
Methyl octanoate[b]	1123	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 41, 42, 40, 82, 55	1140	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 74, 43, 101, 69, 55, 141	1160	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 41, 42, 40, 82, 55	1180	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 57, 82, 41, 39, 83	1225	0.2	-	-	0.1	0.0	0.1	6.3	-	-	-	3.3	2.4	2.1	2.4	-	-	-
m/z: 67, 57, 82, 41, 39, 83	1230	0.1	-	-	0.0	0.0	-	0.3	-	-	-	-	-	-	0.9	-	-	-
m/z: 67, 55, 83, 82, 39, 41	1316	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl decanoate[b]	1323	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aromatics																		
Benzaldehyde[a]	982	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol[a]	1049	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl benzoate[a]	1107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl salicylate[a]	1208	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p-Anisaldehyde[a]	1262	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids																		
Camphene[b]	946	-	-	-	-	-	15.0	40.7	9.8	7.6	7.6	-	-	-	-	-	-	-
(Z)-β-Ocimene[a]	1032	0.1	0.7	-	-	-	-	-	-	-	-	-	1.9	4.9	-	-	-	-
α-Terpinene[b]	1033	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-β-Ocimene[a]	1044	0.3	-	9.8	-	-	-	-	-	0.9	-	2.6	7.6	3.1	-	0.3	8.4	5.5
γ-Terpinen[b]	1071	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Linalool-oxide[a]	1085	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpinolene[b]	1100	-	-	-	-	-	0.7	2.9	0.9	0.0	2.4	4.8	2.4	3.3	0.0	-	-	-
(E)-4,8-Dimethyl-1,3,7-nonatriene[a]	1109	0.3	8.1	8.2	0.0	0.0	-	-	-	-	-	-	-	-	-	3.5	22.3	0.9
1,3,8-p-Menthatriene[b]	1133	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone[a]	1145	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Camphor[b]	1149	-	-	-	-	-	2.6	5.1	5.0	6.9	5.7	-	-	-	-	4.6	2.2	0.1
Borneol[a]	1174	0.3	1.9	1.3	0.0	0.0	1.5	2.5	0.9	1.1	0.4	-	-	-	-	2.9	1.8	1.3
Verbenone[a]	1217	0.1	2.6	0.3	-	-	-	-	-	-	-	-	-	-	-	11.5	3.3	0.8
Methyl geranate[a]	1274	-	0.2	3.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	RI	Lci 1	Lci 2	Lci 3	Lci 4	Lci 5	Lnu 1	Lnu 2	Lnu 3	Lnu 4	Lnu 5	Lpu 1	Lpu 2	Lpu 3	Lpu 4	Lne 1	Lne 2	Lne 3
Amount of trapped scent ng / leaf * 12 minutes		1.5	0.0	0.1	3.5	4.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.3
Bornyl acetate[b]	1287	0.2	0.7	1.3	-	-	2.1	2.5	0.9	0.9	0.7	-	-	-	-	5.4	1.7	0.0
m/z: 105, 119, 161, 91, 81,120	1354	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Ylangene[b]	1376	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Copaene[a]	1377	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.9	6.8	0.6
m/z: 105, 91, 106, 204, 92, 107	1385	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 81, 79, 80, 123, 77, 39, 161	1387	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 161, 91, 133, 204, 119	1407	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Bourbonene[b]	1407	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 105, 133, 77, 93, 76	1413	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 120, 119, 79	1430	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 79, 119, 77, 93, 105	1431	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Caryophyllene[a]	1440	0.3	13.9	38.5	-	-	-	-	-	-	-	4.1	6.8	1.6	0.2	1.8	23.2	1.0
m/z: 161, 105, 91, 119, 133, 79	1442	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-β-Farnesene[b]	1451	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 91, 161, 119, 133, 79	1453	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 91, 119, 79, 78, 81	1457	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Caryophyllene[a]	1476	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.2	0.0
m/z: 161, 105, 91, 119, 204, 133	1476	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-α-Bergamotene[b]	1486	-	-	-	-	-	-	-	-	-	-	1.0	1.0	2.0	0.0	-	-	-
m/z: 161, 105, 91, 119, 133, 79	1489	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Germacrene D[a]	1492	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E,E)-α-Farnesene[a]	1496	0.3	3.9	17.9	-	-	-	-	-	-	-	53.1	65.8	45.8	-	6.5	3.7	1.8
m/z: 121, 105, 161, 136, 91, 204	1501	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 161, 91, 119, 79, 204	1501	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 93, 91, 119, 77, 105, 107	1506	0.1	3.0	8.0	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 79, 133	1521	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 119, 161, 105, 204, 91, 134	1529	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.2	1.9	0.1
N-containing compounds																		
Methyl nicotinate[b]	1145	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknowns																		
m/z: 43, 123, 95, 69, 101, 139	1113	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 153, 138, 128, 53, 81	1192	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 179, 69, 107, 39, 95, 40	1296	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43, 125, 107, 108, 126, 135	1327	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 159, 131, 129, 128, 41, 144	1528	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.2	0.0

	Lne 4	Lne 5	**Lvu 1**	**Lvu 2**	**Lvu 3**	**Lvu 4**	**Lvu 5**	Lco 1	Lco 2	Lco 3	Lco 4	Lco 5	Lat 1	Lat 2	Lat 3	Lcl 1	Lcl 2	Lcl 3	Lde 1	Lde 2
Amount of trapped scent ng / leaf * 12 minutes	0.0	0.0	0.3	0.5	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.5	7.3	2.5	0.0	0.0
Aliphatics																				
(Z)-3-Hexenol[a]	10.3	0.0	2.4	6.0	6.0	3.7	7.8	-	-	-	-	-	-	-	-	9.6	0.8	0.3	-	-
(Z)-3-Hexenyl acetate[a]	33.3	82.3	71.8	71.9	64.0	79.3	72.0	-	-	-	20.1	15.0	-	-	-	72.0	82.3	25.7	-	-
Methyl octanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 41, 42, 40, 82, 55	-	-	0.3	0.3	0.1	0.0	0.2	-	-	-	-	-	-	-	-	1.0	-	1.0	-	-
m/z: 74, 43, 101, 69, 55, 141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 41, 42, 40, 82, 55	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3.5	0.1	0.4	-	-
m/z: 67, 57, 82, 41, 39, 83	-	-	4.0	2.6	1.9	1.0	2.0	-	-	-	-	-	-	-	-	0.9	0.0	1.6	-	-
m/z: 67, 57, 82, 41, 39, 83	-	-	0.1	0.1	0.2	0.1	0.2	-	-	-	-	-	-	-	-	2.0	0.2	1.3	-	-
m/z: 67, 55, 83, 82, 39, 41	-	-	0.1	0.1	0.2	0.0	0.1	-	-	-	-	-	-	-	-	0.0	0.1	0.6	-	-
Methyl decanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aromatics																				
Benzaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.8	7.5	-	-
Benzyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-	66.7	41.9	38.9	-	-	-	-	-
Methyl benzoate[a]	-	-	-	-	-	-	-	-	-	-	-	-	26.7	44.7	27.0	-	-	-	-	-
Methyl salicylate[a]	-	-	-	-	5.0	3.6	1.7	-	-	-	-	-	-	-	-	2.1	1.1	1.6	-	-
p-Anisaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	6.7	13.4	34.2	-	-	-	-	-
Terpenoids																				
Camphene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-β-Ocimene[a]	-	-	-	-	0.1	0.2	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Terpinene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-β-Ocimene[a]	-	-	1.3	3.1	0.8	0.7	0.9	-	-	-	-	-	-	-	-	1.4	0.3	1.3	3.0	6.5
γ-Terpinen[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-Linalool-oxide [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpinolene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-4,8-Dimethyl-1,3,7-nonatriene[a]	4.9	7.6	6.1	5.5	2.6	2.6	3.8	100.0	26.2	36.2	49.7	16.5	-	-	-	3.5	0.8	14.9	30.2	41.8
1,3,8-p-Menthatriene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Camphor[b]	-	8.2	-	-	-	-	-	-	-	-	6.7	3.4	-	-	-	-	-	-	-	-
Borneol[a]	0.1	-	-	-	-	-	-	-	2.6	-	8.0	3.0	-	-	-	-	-	-	-	-
Verbenone[a]	0.4	1.8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl geranate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Lne 4	Lne 5	Lvu 1	Lvu 2	Lvu 3	Lvu 4	Lvu 5	Lco 1	Lco 2	Lco 3	Lco 4	Lco 5	Lat 1	Lat 2	Lat 3	Lcl 1	Lcl 2	Lcl 3	Lde 1	Lde 2
Amount of trapped scent ng / leaf * 12 minutes	0.0	0.0	0.3	0.5	0.2	0.2	0.2	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.5	7.3	2.5	0.0	0.0
Bornyl acetate[b]	0.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 119, 161, 91, 81,120	-	-	0.3	0.2	0.3	0.2	0.2	-	-	-	-	-	-	-	-	0.1	0.2	0.8	-	-
α-Ylangene[b]	-	-	-	-	0.3	0.2	0.1	-	-	-	-	0.9	-	-	-	0.2	2.8	1.6	-	-
α-Copaene[a]	4.9	-	1.9	1.4	2.9	1.4	1.6	-	60.8	26.6	8.9	11.2	-	-	-	1.5	1.2	12.9	-	-
m/z: 105, 91, 106, 204, 92, 107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 81, 79, 80, 123, 77, 39, 161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	-	-
m/z: 105, 161, 91, 133, 204, 119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.8	-	-
β-Bourbonene[b]	-	-	1.4	0.6	1.4	0.6	0.3	-	-	-	-	2.0	-	-	-	0.1	1.0	1.6	-	-
m/z: 91, 105, 133, 77, 93, 76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.1	0.3	1.3	1.3
m/z: 161, 105, 91, 120, 119, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.3	0.3	-	-
m/z: 91, 79, 119, 77, 93, 105	-	-	1.2	0.9	2.2	0.6	1.1	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Caryophyllene[a]	41.3	-	1.1	1.1	0.9	1.2	1.3	-	5.2	31.9	3.8	43.3	-	-	-	0.2	4.1	11.3	21.5	22.1
m/z: 161, 105, 91, 119, 133, 79	-	-	0.4	0.2	0.4	0.1	0.2	-	-	-	-	-	-	-	-	0.0	0.4	0.8	-	-
(Z)-β-Farnesene[b]	-	-	0.2	0.3	0.2	0.1	0.1	-	-	-	-	-	-	-	-	0.3	0.3	3.2	0.6	-
m/z: 105, 91, 161, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.0	-	-
m/z: 105, 91, 119, 79, 78, 81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.2	-	-
α-Caryophyllene[a]	2.1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.3	0.8	1.0	0.4
m/z: 161, 105, 91, 119, 204, 133	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.1	0.2	-
(E)-α-Bergamotene[b]	-	-	0.3	0.2	0.3	0.1	0.2	-	-	-	-	-	-	-	-	-	-	-	11.7	9.0
m/z: 161, 105, 91, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	0.4	1.6	-	-
Germacrene D[a]	-	-	0.3	0.2	0.4	0.1	0.2	-	-	-	-	-	-	-	-	-	-	-	-	-
(E,E)-α-Farnesene[a]	1.3	-	6.2	4.8	9.2	3.9	5.5	-	-	-	-	-	-	-	-	0.4	-	0.8	29.7	14.9
m/z: 121, 105, 161, 136, 91, 204	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.5	0.5	3.2	-	-
m/z: 105, 161, 91, 119, 79, 204	-	-	-	-	-	-	-	-	5.2	5.2	2.9	4.7	-	-	-	-	-	-	1.0	4.2
m/z: 93, 91, 119, 77, 105, 107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 79, 133	-	-	0.0	0.1	0.0	0.0	0.0	-	-	-	-	-	-	-	-	0.1	0.1	1.0	-	-
m/z: 119, 161, 105, 204, 91, 134	1.0	-	0.4	0.3	0.5	0.1	0.3	-	-	-	-	-	-	-	-	0.1	0.3	1.6	-	-
N-containing compounds																				
Methyl nicotinate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknowns																				
m/z: 43, 123, 95, 69, 101, 139	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 153, 138, 128, 53, 81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 179, 69, 107, 39, 95, 40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 43, 125, 107, 108, 126, 135	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 159, 131, 129, 128, 41, 144	0.2	-	-	0.2	-	0.0	0.0	-	-	-	-	-	-	-	-	0.0	0.2	0.6	-	-

	Lde 3	Lde 4	Lde 5	Lep 1	Lep 2	Lep 3	Lep 4	Lep 5	Lfo 1	Lfo 2	Lfo 3	Lfo 4	Lli	Lmau 1	Lmau 2	Lmau 3	Lmau 4	Lmau 5	Lmi 1
Amount of trapped scent ng / leaf * 12 minutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Aliphatics																			
(Z)-3-Hexenol[a]	-	-	-	-	-	-	96.9	100.0	2.9	9.6	-	-	-	-	-	-	88.6	-	-
(Z)-3-Hexenyl acetate[a]	-	-	-	-	-	-	-	-	47.3	28.3	14.8	51.8	-	-	-	-	-	-	80.0
Methyl octanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 41, 42, 40, 82, 55	-	-	-	-	-	-	-	-	0.3	0.5	0.5	0.4	-	-	-	-	-	-	-
m/z: 74, 43, 101, 69, 55, 141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.2
m/z: 67, 41, 42, 40, 82, 55	-	-	-	-	-	-	-	-	0.1	0.8	0.1	0.7	-	-	-	-	-	-	-
m/z: 67, 57, 82, 41, 39, 83	-	-	-	-	-	-	-	-	-	1.0	0.1	0.2	-	-	-	-	-	-	-
m/z: 67, 57, 82, 41, 39, 83	-	-	-	-	-	100.0	-	-	2.6	5.6	0.5	4.6	-	-	-	-	-	-	-
m/z: 67, 55, 83, 82, 39, 41	-	-	-	-	-	-	-	-	0.3	1.0	0.1	0.4	-	-	-	-	-	-	-
Methyl decanoate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Aromatics																			
Benzaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Benzyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	95.6	93.7	73.3	-	97.6	-
Methyl benzoate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl salicylate[a]	-	-	-	-	80.6	-	-	-	-	-	-	-	-	-	-	-	-	-	-
p-Anisaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids																			
Camphene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-β-Ocimene[a]	-	-	-	-	-	-	-	-	-	-	-	-	6.3	-	-	-	-	-	-
α-Terpinene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
(E)-β-Ocimene[a]	3.0	-	-	-	-	-	-	-	8.2	4.8	19.7	7.3	43.2	-	-	-	-	-	-
γ-Terpinen[b]	-	-	-	-	-	-	-	-	8.8	1.0	2.1	0.7	-	-	-	-	-	-	-
(Z)-Linalool-oxide [a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terpinolene[b]	-	-	-	-	-	-	-	-	-	-	-	-	1.9	-	-	-	-	-	-
(E)-4,8-Dimethyl-1,3,7-nonatriene[a]	32.3	97.4	99.8	100.0	18.6	-	3.1	-	8.5	18.8	29.9	16.9	7.6	4.3	4.9	9.5	11.4	2.4	-
1,3,8-p-Menthatriene[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4-Oxoisophorone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	0.0	1.4	-	-	-	1.1
Camphor[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Borneol[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Verbenone[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Methyl geranate[a]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

	Lde 3	Lde 4	Lde 5	Lep 1	Lep 2	Lep 3	Lep 4	Lep 5	Lfo 1	Lfo 2	Lfo 3	Lfo 4	Lli	Lmau 1	Lmau 2	Lmau 3	Lmau 4	Lmau 5	Lmi 1
Amount of trapped scent ng / leaf * 12 minutes	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.1
Bornyl acetate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 119, 161, 91, 81,120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Ylangene[b]	-	-	-	-	-	-	-	-	2.1	3.6	3.9	-	2.0	-	-	-	-	-	-
α-Copaene[a]	-	-	-	-	-	-	-	-	4.5	2.6	3.6	-	5.2	-	-	-	-	-	-
m/z: 105, 91, 106, 204, 92, 107	-	-	-	-	-	-	-	-	1.5	2.8	1.1	1.9	-	-	-	-	-	-	-
m/z: 81, 79, 80, 123, 77, 39, 161	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 161, 91, 133, 204, 119	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Bourbonene[b]	-	-	-	-	-	-	-	-	0.1	0.3	3.9	0.4	0.6	-	-	-	-	-	-
m/z: 91, 105, 133, 77, 93, 76	-	1.4	-	-	-	-	-	-	-	-	-	-	0.9	-	-	-	-	-	-
m/z: 161, 105, 91, 120, 119, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 79, 119, 77, 93, 105	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-Caryophyllene[a]	31.9	1.3	-	-	0.8	-	-	-	6.4	12.3	10.7	6.9	12.5	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 133, 79	-	-	-	-	-	-	-	-	-	0.1	1.9	-	1.7	-	-	-	-	-	-
(Z)-β-Farnesene[b]	-	-	-	-	-	-	-	-	-	-	-	-	2.3	-	-	-	-	-	-
m/z: 105, 91, 161, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 91, 119, 79, 78, 81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Caryophyllene[a]	2.2	-	-	-	-	-	-	-	0.3	3.3	2.3	1.9	1.2	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 204, 133	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	-	-	-	-	-
(E)-α-Bergamotene[b]	9.2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	1.5	-	-	-	-	-	-	-	-
Germacrene D[a]	-	-	-	-	-	-	-	-	0.9	0.2	-	-	-	-	-	-	-	-	-
(E,E)-α-Farnesene[a]	19.5	-	-	-	-	-	-	-	2.7	1.9	0.9	3.0	0.9	-	-	-	-	-	-
m/z: 121, 105, 161, 136, 91, 204	-	-	-	-	-	-	-	-	2.2	1.4	0.9	1.5	-	-	-	-	-	-	-
m/z: 105, 161, 91, 119, 79, 204	0.6	1.3	0.2	-	-	-	-	-	-	-	-	-	1.2	-	-	-	-	-	-
m/z: 93, 91, 119, 77, 105, 107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 79, 133	-	-	-	-	-	-	-	-	-	-	-	-	1.2	-	-	2.7	-	-	-
m/z: 119, 161, 105, 204, 91, 134	-	-	-	-	-	-	-	-	0.1	0.2	1.5	1.5	7.5	-	-	14.5	-	-	-
N-containing compounds																			
Methyl nicotinate[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknowns																			
m/z: 43, 123, 95, 69, 101, 139	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 67, 153, 138, 128, 53, 81	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 179, 69, 107, 39, 95, 40	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10.9
m/z: 43, 125, 107, 108, 126, 135	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.7
m/z: 159, 131, 129, 128, 41, 144	-	-	-	-	-	-	-	-	0.1	0.1	0.1	0.0	2.7	-	-	-	-	-	-

	Lmi 2	Lmi 3	Lmi 4	Lmi 5	Lth 1	Lth 2	Lth 3	Lth 4	Lth 5	Lar 1	Lar 2	Lmar
Amount of trapped scent ng / leaf * 12 minutes	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0	-	-	-
Aliphatics												
(Z)-3-Hexenol[a]	-	-	-	-	-	-	-	-	-	2.0	0.9	27.7
(Z)-3-Hexenyl acetate[a]	81.1	72.2	67.6	60.5	62.4	55.0	76.9	86.9	91.4	17.8	12.7	57.9
Methyl octanoate[b]	-	-	-	-	19.7	14.9	12.6	4.6	0.0	-	-	-
m/z: 67, 41, 42, 40, 82, 55	-	-	-	-	-	-	-	-	-	3.7	0.6	-
m/z: 74, 43, 101, 69, 55, 141	1.3	0.3	3.2	5.1	-	-	-	-	-	-	-	-
m/z: 67, 41, 42, 40, 82, 55	-	-	-	-	-	-	-	-	-	1.5	0.5	0.6
m/z: 67, 57, 82, 41, 39, 83	-	-	-	-	-	-	-	3.2	-	27.0	5.2	-
m/z: 67, 57, 82, 41, 39, 83	-	-	-	-	0.1	0.1	-	5.4	8.6	35.6	4.4	-
m/z: 67, 55, 83, 82, 39, 41	-	-	-	-	-	-	-	-	-	-	-	-
Methyl decanoate[b]	-	-	-	-	11.6	19.0	9.4	-	0.0	-	-	-
Aromatics												
Benzaldehyde[a]	-	-	-	-	-	-	-	-	-	0.9	2.3	6.9
Benzyl alcohol[a]	-	-	-	-	-	-	-	-	-	-	-	-
Methyl benzoate[a]	-	-	-	-	-	-	-	-	-	-	-	-
Methyl salicylate[a]	-	-	-	-	-	-	-	-	-	-	-	-
p-Anisaldehyde[a]	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids												
Camphene[b]	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-β-Ocimene[a]	-	-	-	-	-	-	-	-	-	0.7	9.9	-
α-Terpinene[b]	-	-	-	-	-	-	-	-	-	-	-	0.5
(E)-β-Ocimene[a]	-	-	-	-	-	-	-	-	-	6.1	54.8	2.4
γ-Terpinen[b]	-	-	-	-	-	-	-	-	-	-	-	2.6
(Z)-Linalool-oxide [a]	-	-	-	-	-	-	-	-	-	-	-	0.2
Terpinolene[b]	-	-	-	-	-	-	-	-	-	-	-	-
(E)-4,8-Dimethyl-1,3,7-nonatriene[a]	-	-	-	-	-	-	-	-	-	0.9	2.2	0.9
1,3,8-p-Menthatriene[b]	-	-	-	-	-	-	-	-	-	-	-	0.1
4-Oxoisophorone[a]	0.0	0.0	1.2	-	-	-	-	-	-	-	-	0.3
Camphor[b]	-	-	-	-	-	-	-	-	-	2.9	4.4	-
Borneol[a]	-	-	-	-	-	-	-	-	-	-	-	-
Verbenone[a]	-	-	-	-	-	-	-	-	-	0.2	0.2	-
Methyl geranate[a]	-	-	-	-	-	-	-	-	-	-	-	-

	Lmi 2	Lmi 3	Lmi 4	Lmi 5	Lth 1	Lth 2	Lth 3	Lth 4	Lth 5	Lar 1	Lar 2	Lmar
Amount of trapped scent ng / leaf * 12 minutes	0.1	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0	-	-	-
Bornyl acetate[b]	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 119, 161, 91, 81,120	-	-	-	-	-	-	-	-	-	-	-	-
α-Ylangene[b]	-	-	-	-	-	-	-	-	-	-	-	-
α-Copaene[a]	-	-	-	-	-	-	-	-	-	0.0	0.0	-
m/z: 105, 91, 106, 204, 92, 107	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 81, 79, 80, 123, 77, 39, 161	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 161, 91, 133, 204, 119	-	-	-	-	-	-	-	-	-	-	-	-
β-Bourbonene[b]	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 105, 133, 77, 93, 76	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 120, 119, 79	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 91, 79, 119, 77, 93, 105	-	-	-	-	-	-	-	-	-	-	-	-
β-Caryophyllene[a]	-	-	-	-	-	-	-	-	-	0.5	1.5	-
m/z: 161, 105, 91, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	-	-
(Z)-β-Farnesene[b]	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 91, 161, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 91, 119, 79, 78, 81	-	-	-	-	-	-	-	-	-	-	-	-
α-Caryophyllene[a]	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 204, 133	-	-	-	-	-	-	-	-	-	-	-	-
(E)-α-Bergamotene[b]	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 133, 79	-	-	-	-	-	-	-	-	-	-	-	-
Germacrene D[a]	-	-	-	-	-	-	-	-	-	-	-	-
(E,E)-α-Farnesene[a]	-	-	-	-	-	3.0	-	-	-	0.1	0.3	-
m/z: 121, 105, 161, 136, 91, 204	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 105, 161, 91, 119, 79, 204	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 93, 91, 119, 77, 105, 107	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 161, 105, 91, 119, 79, 133	-	-	-	-	-	-	-	-	-	-	-	-
m/z: 119, 161, 105, 204, 91, 134	-	-	-	-	-	-	-	-	-	-	-	-
N-containing compounds												
Methyl nicotinate[b]	-	-	-	-	6.2	8.0	1.1	-	-	-	-	-
Unknowns												
m/z: 43, 123, 95, 69, 101, 139	1.3	2.9	3.6	4.9	-	-	-	-	-	-	-	-
m/z: 67, 153, 138, 128, 53, 81	-	2.1	0.9	2.9	-	-	-	-	-	-	-	-
m/z: 179, 69, 107, 39, 95, 40	10.1	15.7	15.1	16.1	-	-	-	-	-	-	-	-
m/z: 43, 125, 107, 108, 126, 135	6.3	6.8	8.3	10.4	-	-	-	-	-	-	-	-
m/z: 159, 131, 129, 128, 41, 144	-	-	-	-	-	-	-	-	-	-	-	-

Publication 4

Honest signaling by a private communication channel in a specialized pollination system

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Honest signaling by a private communication channel in a specialized pollination system

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Abstract

Flower scent is an important mediator between angiosperms and their pollinators, however, little is known about the single compounds/compound mixtures responsible for attraction of specific pollinator species. This is true for generalized as well as specialized systems. The interaction between oil flowers and their oil bee pollinators is highly specialized, and here we determined floral volatiles mediating this interaction.

By using a multifaceted approach, we identified floral volatiles in oil, and for comparative purposes in non-oil secreting species (chemical analyses), and determined physiologically (electroantennography) and behaviourally active scent compounds in *Macropis* oil bees (bioassays). We also tested whether oil bees have, compared to non-oil bees, olfactory adaptations towards a widespread compound in oil secreting plants (electroantennography).

The most widespread compound among oil secreting plants was diacetin, and this acetylated glyceride was electrophysiologically and behaviourally active in *Macropis*. Diacetin was the key compound in attracting *Macropis*, whereas few other electrophysiologically active compounds increased its attractiveness. Because only oil but not non-oil bees responded to this compound in electroantennographic analyses, it seems to serve as a private communication channel between oil secreting plants and their oil collecting bee pollinators. Diacetin is structurally similar to the floral oils and likely produced by similar metabolic pathways as the non-volatile fatty oils. Therefore it represents a reliable and honest cue for bees in search for oils.

Introduction

Most angiosperms are pollinated by animals (1), and several flowering plants are involved in specialized pollination systems, i.e. they are pollinated by only a single/few species or functional group of animals (2-4). In a high number of specialized pollination systems floral scent is the most important floral signal for pollinator attraction (5, 6). Specificity in pollinator attraction has been suggested to be achieved either by sensory 'private channels', i.e. unusual compounds which are well detected by intended but not by non-intended receivers, or by specific ratios of ubiquitous compounds (5, 7). Indeed potential private channels as well as specific ratios of widespread compounds were identified in several case studies as mediators of specialized pollination systems. For example, sexually deceptive orchids which mimic female sex pheromones of Hymenoptera, emit either uncommon compounds, such as chiloglottone (8) or 9-hydroxydecanoic acid (9), or specific ratios of widespread hydrocarbons (alkenes, alkanes) (10) for attracting single male pollinator species. The above mentioned uncommon compounds are good candidates for private channels, however, the second assumption for a private communication channel that these compounds are well detected by the pollinators but hardly by other species of the available flower-visiting fauna has not been tested explicitly in any deceptive and also not in any reward-based pollination system where private channels were assumed (5). Further it is unknown, whether pollinators have adaptations in their olfactory circuit, e.g. specific receptors, binding proteins, or neurons, which do not occur in non-pollinators for detecting these compounds.

In sexually deceptive systems mediated by uncommon compounds, the plants exploit already available olfactory capabilities/preferences of the specific pollinators, whereas in reward-based pollination systems, the olfactory capability in detecting the uncommon compound(s) may be the result of a specific adaptation in the olfactory circuit of the specific pollinator towards these compounds. Though such adaptations towards specific compounds of host plants are not demonstrated in any pollinator yet, it is known that some insects detect specific compounds differently from their habitat demonstrating that evolutionary shifts in the periphery of the olfactory circuit are possible among insects, even if they are closely related (11, 12).

A highly specialized pollination system evolved between oil secreting plants and oil collecting bees (13-16). Plant species in more than ten families produce and secrete fatty oils, mostly instead of nectar, in their flowers are involved. Such plants occur in the Neotropical, the Palaeotropical, the Cape, and the Holarctic floristic regions (17). This oil is only collected by the few specialized oil bees that are members of the Apidae and Melittidae. The oil is used by these bees as material for lining the cells within the nest (18, 19) and as larval food provisions (e. g. 19). In bees, oil foraging evolved minimally seven times, and in plants, oil as a floral reward developed at least 28 times independently

(17). As in other specialized pollination systems, floral scent is important for the interaction between oil plants and their bee pollinators. Behavioral experiments with European *Macropis fulvipes* revealed that olfactory cues of *Lysimachia punctata* L. host plants are most important for host location in this bee species. This use of olfactory cues for locating hosts seems to have a genetically basis, as not only bees with but also bees without previous foraging experience (naive) on *Lysimachia* flowers responded strongly (20, 21). Compounds responsible for attracting *M. fulvipes* are present in solvent extracts of complete flowers and floral fatty oils only (20, Schäffler unpublished). Both complete flowers and floral oils release a wide variety of compounds (20), however, the specific compound(s) eliciting the behavioral response in bees is/are not known yet. An uncommon compound, 1-hydroxy-1-phenyl-2-propanone, which occurred in both flower as well as oil samples, and which was suggested to play a role in attracting *Macropis* bees (5, 20), did not attract bees in behavioral tests (22). The finding that solvent extracts of oil are capable in attracting *Macropis* bees allows us to speculate that the floral oils or compounds involved in the biosynthesis of these oils are involved in pollinator attraction. Such compounds would be an ideal signal for *Macropis* in search for oils because it would directly point towards oils. Interestingly, oil offering plants around the world produce quite similar oils. They consist typically of mono, di,- or triacetylated glycosides or free fatty acids, whereas a common trait of the fatty acids (typical chain length: C16, C18) is an acetyl group on the beta carbon (14, 16, 23-25). Due to this similarity, oil flowers around the world might advertise their oil rewards by a similar signal. Generally, in most pollination systems, pollinators use volatile signals derived from biosynthetic pathways (e.g. terpenoids, aromatics, fatty acid derivatives), which do not have a direct link to its reward like sugar and protein (26) for finding rewarding flowers. Pollination systems, in which the signal is the reward itself or compounds biosynthetically very similar to the rewards are very rare, but can be found in systems involving male, perfume collecting euglossine bees (27) and male tephritid flies (28). Euglossine males use these compounds during courtship behavior (29), whereas male flies collect and either convert these compounds into male sex pheromones (30), or directly use collected compounds for mate attraction (31).

Based on first the similarity in chemical structure of floral oils offered by the different plants around the world, second the finding that floral scent are attractive for oil collecting bees, and third the fact that oil flowers are mainly/exclusively pollinated by oil bees, we address the hypothesis that the oil flower oil bee pollination system is mediated by a volatile private communication channel which is derived from the floral oil.

Materials and Methods

Bees

The oil collecting bee *Macropis fulvipes* (Fab.) (Melittidae, Melittinae) is distributed in Europe and is, as all species in this genus, specialized on *Lysimachia* (Primulaceae) oil offering flowers (15, 16, 32). Fatty floral oils and pollen of these plants are the only food collected for the offspring. Adult males and female also feed on pollen of *Lysimachia* and females use the oil additionally to line the brood cells (16, 19). Individuals used for behavioral tests were from a flight cage population (see below), those used for electrophysiological measurements (see below) from a natural population in the Ecological Botanical Garden of the University of Bayreuth (EBG).

Rediviva (Melittidae, Melittinae) oil bees are closely related to *Macropis*, occur in Southern Africa, and also collect floral oils as food for the offspring (33). *Rediviva neliana* Cock. is widespread in the summer rainfall area. Specimens for electrophysiological measurements (see below) were collected in the Drakensberg area close to Wietsieshoek when visiting oil or nectar/pollen plants.

Melitta bees which occur in the Holarctic and in Africa, are from the same subfamily as *Macropis* and *Rediviva*, i.e. Melittinae, but species do not collect floral oils. *Melitta haemorrhoidalis* (Fab.) is distributed in Europe and specialized on pollen of *Campanula* species . Specimens for electrophysiological measurements were collected from natural populations in the EBG.

The non-oil collecting honey bee, *Apis mellifera* L., occurs throughout the world and is, in contrast to the other bee species used, an Apidae. Individuals used for electroantennographic measurements were collected in the EBG from established hives.

Plant material and volatile collection

Floral scent was collected from 58 plant species (50 oil and 8 non-oil) for chemical analyses by elution. Samples of four oil offering species thereof were additionally used for electrophysiological analyses, and samples of *L. punctata* were used for bioassays as well. Samples were either collected from plants growing in the natural habitat or from material collected in different green houses (supplementary data Tab S1). In total, 11 700 flowers (in the mean 80 (SE: 12) flowers per sample in oil species, and 277 (SE: 75) flowers per sample in non-oil species) were removed from the plants using clean forceps and extracted for one minute in 2-3 ml pentane (p.a., 99%, Grüssing, Germany). Obtained samples were subsequently filtered with silanized glass wool (Supelco) to remove particles and concentrated by evaporation to a volume of 0.5 ml. The solvent without flowers was used as negative control.

Flight cage

A *Macropis fulvipes* population was established in a flight cage (7.2 x 3.6 x 2.2 m; wood-framed mesh gauze; see also 20), which was placed in a greenhouse in the EBG, the same as described

by Dötterl and Schäffler (20). Each bee was individually marked with a plastic disc commonly used for marking honey bee queens (Opalith number plates, 1-99, in five colors) after emerging. We did not offer *Lysimachia* host plants to the bees or other floral oil secreting plants so bees used for behavioral experiments (see below) were Lysimachia-naïve with respect to oil and pollen foraging. However, we offered the bees flowering *Geranium sanguineum* L. and *Origanum vulgare* L. as nectar sources and they were additionally provided with a sugar solution (30%, a 1:1 v : v mixture of glucose and fructose), that was added to the *Geranium* flowers or to an artificial feeder (see also 21).

Gas Chromatography with Electroantennographic Detection (GC-EAD)

Electrophysiological experiments were performed with flower samples on a gas chromatograph (HP 5890 series 2) equipped with a flame ionization detector (FID) and an EAD setup (temperature controller TC-02, two-channel universal serial bus acquisition controller IDAC-2 and stimulus controller CS-01) provided by Syntech (Hilversum, Netherlands), the same as described by Dötterl et al. (34). The gas chromatograph was equipped with a ChromatoProbe kit (CPAV6890, Aviv Analytical LTD, Hod Hasharon, Israel) allowing to analyze "dirty" samples (35), and a ZB-5 column (length 30 m, inner diameter 0.32 mm, film thickness 0.25µm, Phenomenex). The ChromatoProbe kit was needed for analysis of pentane floral extracts, which not only contained volatiles but also also "non-volatile" floral oils. One micro liter of a flower sample in a small vial was placed into the injector port by means of the ChromatoProbe (injector temperature: 260°). Compounds not vaporized remained in the sample vial which was discarded after use. The samples were injected split less at an oven temperature of 40°C, followed by opening the split vent after 1 min and heating the oven at a rate of 10°C/min to 260°C. The end temperature was held for 5 min. The column was split at the end by the four arm flow splitter GRAPHPACK 3D/2 (Gerstel, Mühlheim, Germany) into two pieces of deactivated capillary (length 50 cm, ID 0.32 mm) leading to the FID and EAD-setup, respectively. Makeup gas (He, 16ml/min) was introduced through the fourth arm of the splitter. For measurements the insect antenna was cut at the basis and the top, and mounted between glass micropipette electrodes filled with insect ringer (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l CaCl$_2$). The electrodes were connected to silver wires.

Both sexes of *M. fulvipes* were used because in previous analyses we did not find differences in antennal responses between sexes (Dötterl and Vereecken 2010). Antennae were tested on scent samples of four different oil secreting species from three different plant orders and two different continents. By using this approach, compounds widespread among oil secreting plants (independent of their relatedness) and potentially important in oil flower oil bee pollination systems can be identified. Five *Lysimachia punctata* flower extracts were tested on antennae of 7 male and 6 female bees (one antenna per bee), and EAD-active compounds were determined when elicited antennal response in at least three antennae. Additionally, one flower extract of *L. congestiflora* and one of *Diascia interregima* were tested on two different males' antennae each (compound treated as EAD-active

when response in one of the runs each), and the flower extract of *Corycium dracomontanum* was tested on one male antenna.

Electroantennography (EAG)

For the EAG tests (see e. g. 36) we treated five antennae of female *M. fulvipes*, six antennae from *Rediviva neliana* (five males, one female), five antennae from female *Melitta haemorrhoidalis*, and nine antennae from honey bee workers as described above, and used these antennae to measure dose-response curves for diacetin (diluted in acetone; $10^{-2} – 10^{-5}$). Antennae of *Melitta haemorrhoidalis* were only tested on the two most concentrated dilutions.

As positive control we used linalool (10^{-2} in acetone), a compound widespread among plants pollinated by bees (37), and acetone was used as negative control. Antennae were stimulated at 2 min intervals using following sequence: acetone, linalool, different diacetin dilutions (starting with the lowest), linalool, acetone.

For every stimulus 2 ml of the test solution was applied on filter paper (0.3 x 5cm). The solvent was allowed to evaporate before the strip was placed in a glass pasteur pipette (15 cm in length). Stimuli were released into a continuous flow of humidified air passing over the antenna with a pulse duration of 0.5 sec, and a flow of 10 ml/sec regulated by the Syntech CS-01 Stimulus Controller (Bayreuth lab, Germany; antennae of *Macropis*, *Melitta*, and *Apis*) or the Syntech CS-55 Stimulus Controller (Pietermaritzburg lab, South Africa; antennae of *Rediviva neliana*). Data were analysed using the software EAGPro 1.0 provided by Syntech. To counterbalance for the loss of antennal sensitivity during the measurements, the antennal responses to linalool were used to normalize the antennal responses towards acetone and diacetin ("normalize data" option in EAGPro). Thereafter, the normalized linalool response was set as 100%, and the responses to the different diacetin dilutions as well as acetone are given in relation to the normalized response of linalool.

To test whether different bee species respond differently to the dilution series of diacetin, data were analysed using a repeated measurement ANOVA (38) with the different *dilutions* and *species* as categorical factor. Tukey was used as post hoc test. Responses of *Melitta* were excluded from these analyses as only two of the four diacetin dilutions were tested in this species. Instead, we tested for a *dilution* effect in *Melitta* using a t-test for dependent samples (38).

A t-test for dependent samples was also used to test, in each species, for differences in responses to acetone and the 10^{-2} dilution of diacetin.

Chemical Analyses

To identify the EAD-active compounds in the four species used for GC-EAD measurements, 1 µl of the flower samples was analyzed on a Varian Saturn 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector (GC-MS). Additionally, the

occurrence of each of the EAD-active compounds was studied among all the 50 oil and eight non-oil species available in present work.

The sample was inserted in a quartz vial and in the injector by using the ChromatoProbe kit of Varian (39, 40). The injector split vent was opened and the injector heated to 40°C to flush any air from the system. After 2 min, the split was closed and the injector heated with a rate of 200°C/min to 260°C, then held this temperature for 2 min, after which the split vent opened and the injector cooled down. A ZB-5 column (5% phenyl polysiloxane) was used for the separation (60 m long, inner diameter 0.25 mm, film thickness 0.25 μm, Phenomenex). Helium carrier gas flow was 1.0 ml/min. GC oven temperature was held for 4.5 min at 40°C, then increased by 6°C/min to 300°C and held at this end temperature for 15 min. The MS-interface temperature was 290°C and the ion trap worked at 175°C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan/s from m/z 30 to 650. The GC-MS data were processed using the Saturn Software package 5.2.1.

Component identification was carried out using the NIST 08 mass spectral database or MassFinder 3, and confirmed by comparison with retention times of authentic standards. Retention time and mass spectra of diacetin isomers were compared with data provided by Nebel (41). In all samples analysed, diacetin occurred as 1,2- and 1,3-isomer, and in *L. vulgaris*, we also detected both enantiomers of 1,2-diacetin (Schäffler and Dötterl, unpublished data). In present study we do not discriminate between the different isomers.

To quantify the absolute amount of the emitted EAD-active compounds, we added 1 μg of 3-chloro-4-methoxytoluene (used as an internal standard) in five *L. punctata* flower extract samples.

Preparation of synthetic flower extracts

For testing the attractiveness of EAD-active substances on *Macropis* bees we prepared dilutions of the synthetic substances in acetone (99.9%, AnalaR NORMAPUR, VWR): diacetin (after purification, see below), geranic acid (98%, ABCR), heptanoic acid (99%, Aldrich), (*E*)-2-dodecenal (93%, Aldrich) and 2-tridecanone (98%, ABCR).

Though EAD-active, we didn't include 1-hydroxy-1-phenyl-2-propanone in our behavioral experiments because it failed to attract bees in previous tests (22) and was not available in present work.

The absolute amount of synthetic compounds offered the bees per bioassay in 10 μl solvent was 12.3 μg, which is equivalent to the quantity of compounds found in extracts of 100 flowers (2 μg heptanoic acid, 4 μg geranic acid, 2 μg (*E*)-2-dodecenal, 4 μg 2-tridecanone, and 0.3 μg diacetin).

Purification of diacetin

Technical diacetin (technical grade 50%, ABCR) is contaminated with glycerin, mono- and triacetin. To separate diacetin from the other substances, the mixture was chromatographed on a silica gel column (Merck, Silica gel 60, 63 – 200 μm) and eluted with EtOAc/*n*-hexane (4:1, v/v). Fractions

containing diacetin (2 g) were evaporated to dryness and redissolved in 3 mL CH$_3$CN. Further purification was obtained by preparative HPLC (Merck- L-7150 LaChrom pump, Hitachi L-7400 UV Detector, Degasser, Hitachi D-2500 Chromato Integrator). Isocratic chromatography was carried out with CH$_3$CN/H$_2$O (9:1, v/v) on a YMC-Pack column (ODS-A, 150x20 mm, 5 µm particle size) at a flow rate of 10 mL min^{-1}. 1,2- and 1,3-Diacetin eluted between 5 and 8 minutes. Purification was monitored by ESI-MS (Applied Biosystems API-150EX) and ^{1}H- NMR-Spectroscopy (Varian NMRS 600).

Bioassays

Two-choice bioassays were performed for testing the importance of EAD-active volatiles in *L. punctata* for host plant location in *M. fulvipes* female bees. Diacetin was tested against an acetone negative control and against a natural flower extract of *L. punctata* (positive control).

We further tested a natural against the complete synthetic (5 EAD-active compounds) extract as well as the complete synthetic extract against reduced synthetic mixtures that missed one of the compounds. To obtain a mixture without diacetin, we additionally needed to eliminate geranic acid as we found by GC-MS analyses trace amounts of diacetin (0.24 ng in 4 µg geranic acid) as contamination in synthetic geranic acid.

All experiments were conducted in June 2010 on sunny days during 9 a.m. and 5 p.m., when flight activity of bees was high. Test substances were offered on a glass surface (bottom of a reversed beaker, Schott DURAN; cleaned with pentane and ethanol and heated for 2 hours at 250°C).

The bioassays were stopped when no bee approached any more (max. 5 minutes), otherwise tests lasted 20 minutes. In a specific two-choice assay that lasted 20 minutes, the positions of the glasses were exchanged at half-time. This was, however, not possible when testing synthetic samples, as bees typically responded very quickly and only at the beginning of the experiments (1-3 min). For getting valuable data to test for attractiveness of samples, we repeated two of the tests once (diacetin against negative control; natural extract against synthetic extract). Bees responding in both replicates were counted only the first time. The position of the treatments was randomized.

Bees approaching to at least within 5 cm of a glass were caught with an insect net and stored on ice until the test was finished. Exact binomial tests were used to prove the hypothesis that the samples tested against each other attracted the same number of bees. The spreadsheet provided by http://udel.edu/~mcdonald/statexactbin.html (accessed 2011, August 8; see also McDonald 2009) was used for calculations.

Results

Electrophysiology and chemical analyses:

Detection of EAD-active compounds in oil flowers

In the GC-EAD analyses with antennae of *M. fulvipes* and scent samples collected from four different oil plants, we found one compound, diacetin, which occurred in all the plant species and additionally elicited a signal in bee´s antennae. Other EAD-active compounds occurred only in one ((*E*)-2-octenal, 1-hydroxy-1-phenyl-2-propanone, triacetin, (*E*)-2-dodecenal, 3,5-dimethoxytoluene, 4-hydroxy-3-methoxystyrene, UNK RI: 1264) or three of the tested plant species (heptanoic acid, 2-tridecanone), respectively.

1: (*E*)-2-Octenal
2: Heptanoic acid
3: 1,3-Diacetin + 1,2-Diacetin
4: 1-Hydroxy-1-phenyl-2-propanone
5: Geranic acid

6: (*E*)-2-Dodecenal
7: 2-Tridecanone
8: 3,5-Dimethoxytoluene
9: 4-Hydroxy-3-methoxystyrene
10: UNK RI: 1264

* : artefact
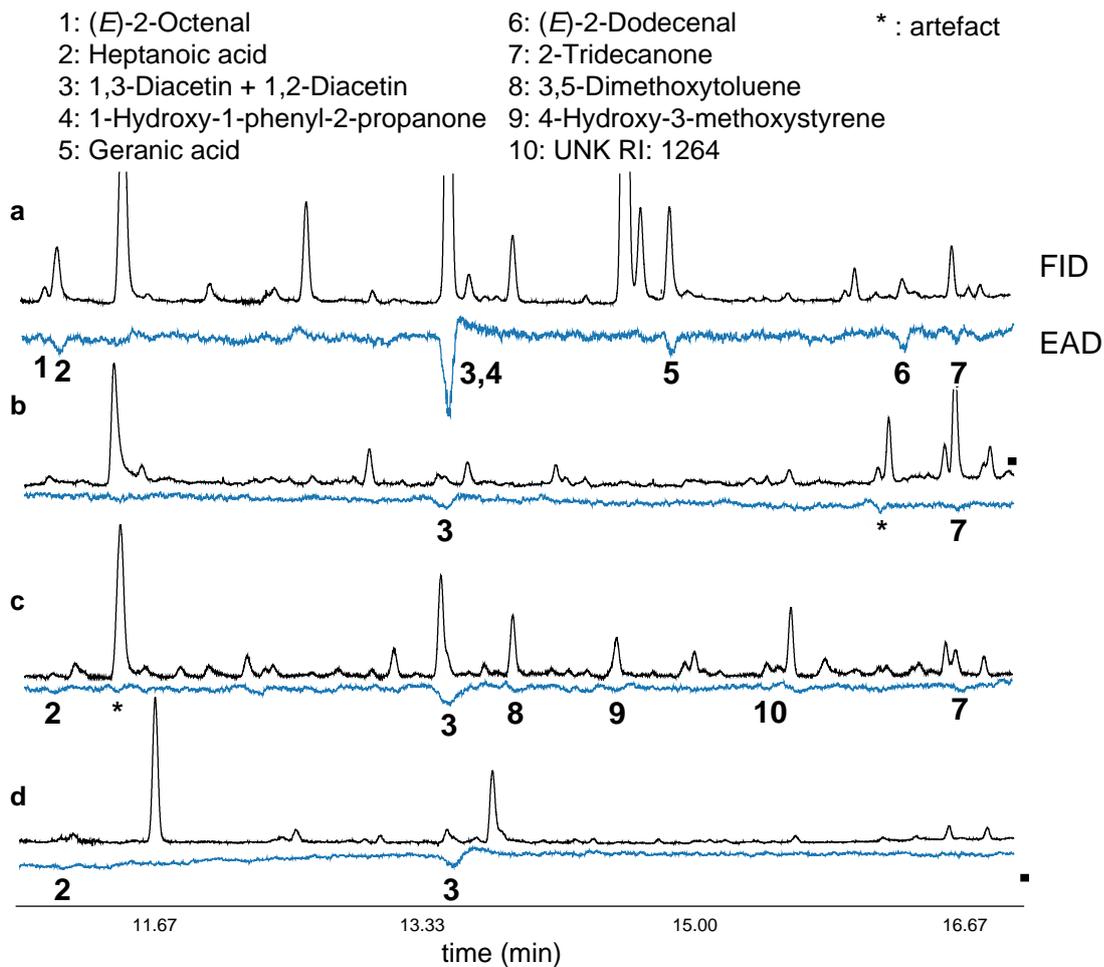
Figure 1: Examples of responses of *M. fulvipes* antennae to flower extracts of *L. punctata* (a), *L. congestiflora* (b), *Diascia interregima* (c), and *Corycium dracomontanum* (d); (UNK RI 1264: unknown compound (Retention index 1264 m/z: 122, 78, 106, 51, 50)

100

GC-MS analyses:

Occurrence of EAD-active compounds

We analyzed as well oil-offering and few non-oil offering plants of different floral regions and different systematic position by GC-MS for the presence of the EAD-active compounds. The most widespread compound was diacetin, which occurred in 41 of the 50 (82%) studied oil species and in one of the eight studied non-oil species. It was present in plants from three of the four studied floristic regions and overall in all of the Holarctic (seven) and South African (18) oil species, as well as in 16 (73 %) of the Neotropical oil species (Tab. 1, for complete list see Table S1). Nearly as widespread as diacetin was 2-tridecanone, which was found in 34 (68%) oil species and in one non-oil species. Heptanoic acid was detected in 20 (40%) and 4-hydroxy-3-methoxystyrene in 18 (36%) oil species, whereas the remaining EAD-active compounds occurred in less than 10 (20%) oil species.

Tab. 1. Occurrence of EAD-active substances in oil and non-oil secreting plants of different families/genera and floristic regions. (For detailed information see Table S1).

number of taxa studied in floristic regions

	Holactic	Cape	Neotropis	Palaeotropis
families	1	3	6	1
genera	1	7	13	1
species oil/non-oil	7/7	18/0	22/0	3/1

percentage of oil/non-oil species with a specific EAD-active compound

diacetin	100/14	100	73	0/0
2-tridecanone	100/0	72	55	100/100
heptanoic acid	71/0	55	23	0/0
4-hydroxy-3-methoxystyrene	29/0	50	18	100/0
(*E*)-2-octenal	29/0	17	5	0/0
unkown (RI 1264 m/z: 122,78, 106, 51, 50)	0/0	17	0	66/0
geranic acid	29/0	0	2	0/0
(*E*)-2-dodecenal	43/0	0	0	0/0
1-hydroxy-1-phenyl-2-propanone	29/0	0	0	0/0
3,5-dimethoxytoluene	0/0	11	0	0/0

EAG - antennal responses to diacetin in oil and non-oil bees

An overall analysis revealed a significant *species* (RM-ANOVA: $F_{2,17} = 15.97$, $p < 0.001$), *dilution* (RM-ANOVA: $F_{3,51} = 60.21$, $p < 0.001$), and interaction (*species* x *dilution:* RM-ANOVA: $F_{6,51} = 17.94$, $p < 0.001$) effect indicating differences in responses to diacetin among species. Only in *M.*

fulvipes and *Rediviva neliana* oil bees but not in the honey bee did the antennal responses increase with increasing concentration of diacetin (Fig. 2). Similar to the honey bee, there was no *dilution* effect of diacetin in *M. haemorrhoidalis* (t-test: t = 1.00, df = 4, p = 0.37).

Responses to the highest concentration of diacetin were stronger than to acetone in antennae of *M. fulvipes* (t-test: t = -6.32, df = 4, p < 0.01) and *R. neliana* (t-test: t = -9.44, df = 5, p < 0.001), but not in *M. haemorrhoidalis* (t-test: t = 1.00, df = 4, p = 0.37) and *A. mellifera* (t-test: t = 1.13, df = 8, p = 0.29).
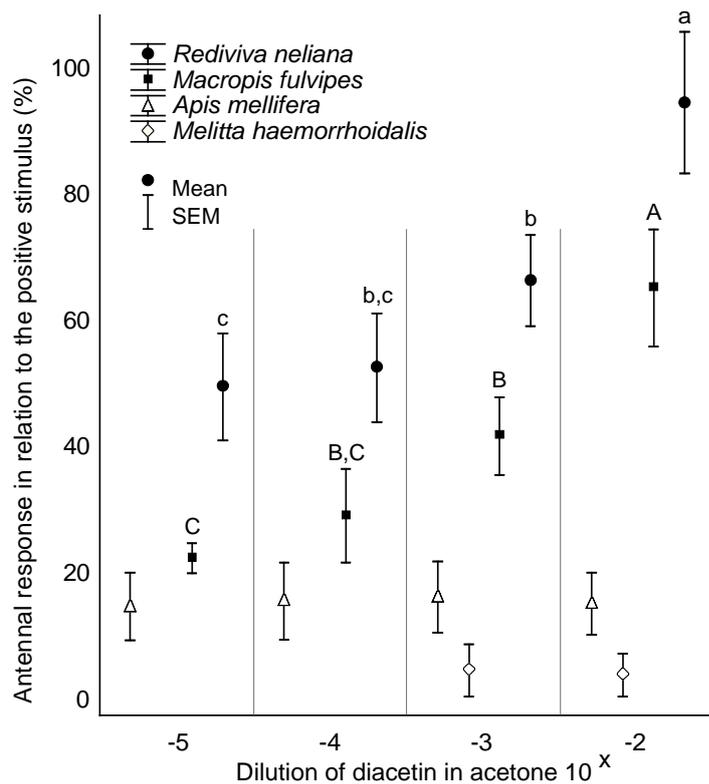
Fig. 2. Electroantennographic responses (EAG) of *M. fulvipes* / *R. neliana* oil bees, and *Melitta haemorrhoidalis* / *Apis mellifera* non-oil bees to different dilutions of diacetin. SEM = standard error of the mean

Behavioral experiments:

In two-choice experiments, conducted in the flight cage, diacetin attracted significantly more bees than a negative control but significantly less bees compared to a natural flower extract (Fig. 3). The complete mixture of EAD-active compounds, which included diacetin and four other compounds, had the same attractiveness as a natural flower extract. To test the importance of the single compounds, we performed subtractive experiments. Reduced mixtures missing geranic acid and 2-tridecanone, respectively, had the same attractiveness compared to the complete mixture, blends without heptanoic

acid and (*E*)-2-dodecenal, however, were less attractive than the complete mixture. A blend missing diacetin (together with geranic acid, see material and methods) did not attract any bee; instead, all bees preferred the complete mixture.
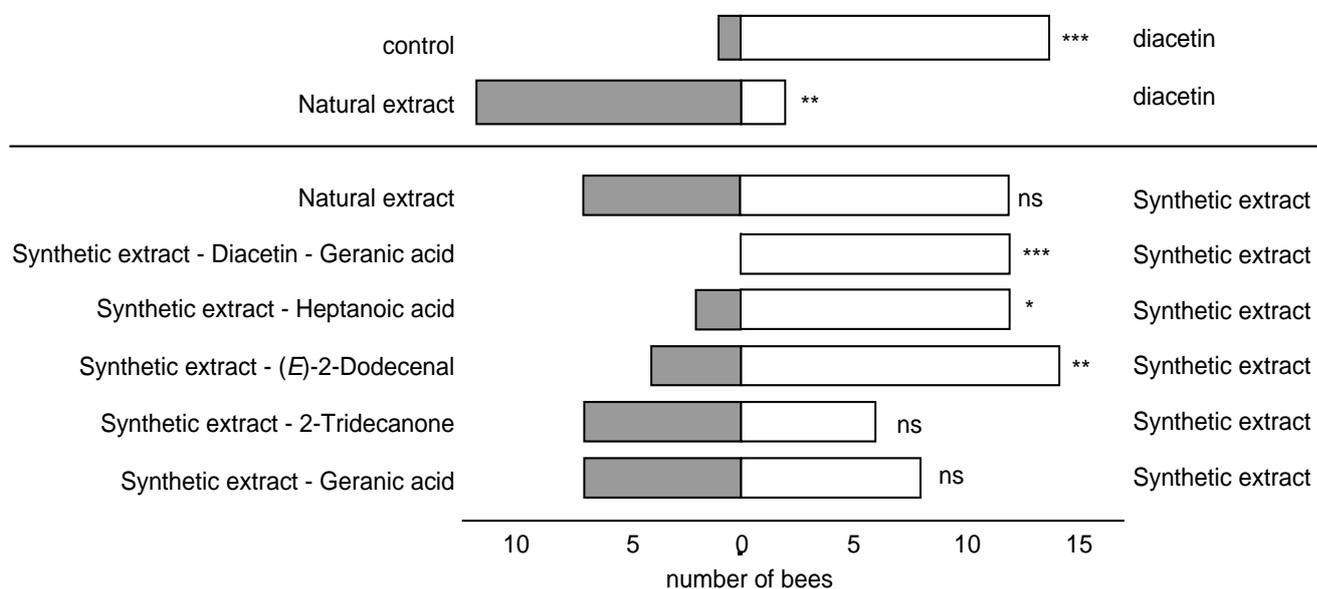
Figure 3. Approaches of naive *Macropis fulvipes* females to diacetin and natural as well as synthetic complete and reduced blends of EAD-active compounds identified in *L. punctata* flower extracts*.* Exact binomial test: ns: $p > 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Discussion

Floral oil secreting plants around the world are pollinated by highly specialized bees, which collect the fatty oils as food for the larvae and to line their brood cells. Only rarely oil flowers are visited by insects others than oil collecting bees. As oil plants and oil bees are dependent on each other, an effective communication system can be expected to have (co)evolved in this pollination system. Our data demonstrate that flowers of most of studied oil species around the world emit the fatty acid derivative diacetin. This compound elicits strong antennal responses in bees of different floral regions and continents, whereas it does not elicit antennal responses in non-oil bees indicating an olfactory adaptation in oil bees towards this uncommon compound. Diacetin is a main signal in the *Lysimachia-Macropis* pollination system, in which other components increase attractiveness of this key component. Overall, our data suggest that diacetin is a private communication channel and honest signal in the oil flower oil bee pollination system, as it is structurally similar to the floral oils and may be a reliable marker for presence of floral oils (Fig. 4).

Diacetin, an honest signal for oil bees?

Diacetin, for the first time described as floral compound just recently (42), and only found in a few extracts of leaves (43, 44), essential oils (45), and propolis (46), occurs as a floral scent compound

in most (82%) of the studied Holarctic, Neotropic, and South African oil secreting plant species from quite different lineages (Asparagales; Malpighiales, Ericales, Lamiales). It can strongly be assumed, that the production of diacetin in oil flowers has evolved independently several times, in accordance with the independent evolution of oil secretion in these flowers (17, 47, 48).

In contrast to the widespread occurrence of diacetin in oil secreting plants, we did not find diacetin in non-oil species with the exception of one. In the non-oil secreting *Lysimachia thyrsiflora*, this compound was detected in flower extracts and we recently also detected it in headspace samples (49). The presence of diacetin in this non-oil species may have to do with its close relatedness to the oil secreting and diacetin emitting *L. vulgaris*, and in addition to this we can not absolutely exclude, that flowers of this species produce and secrete some oils, which might explain the emission of diacetin.
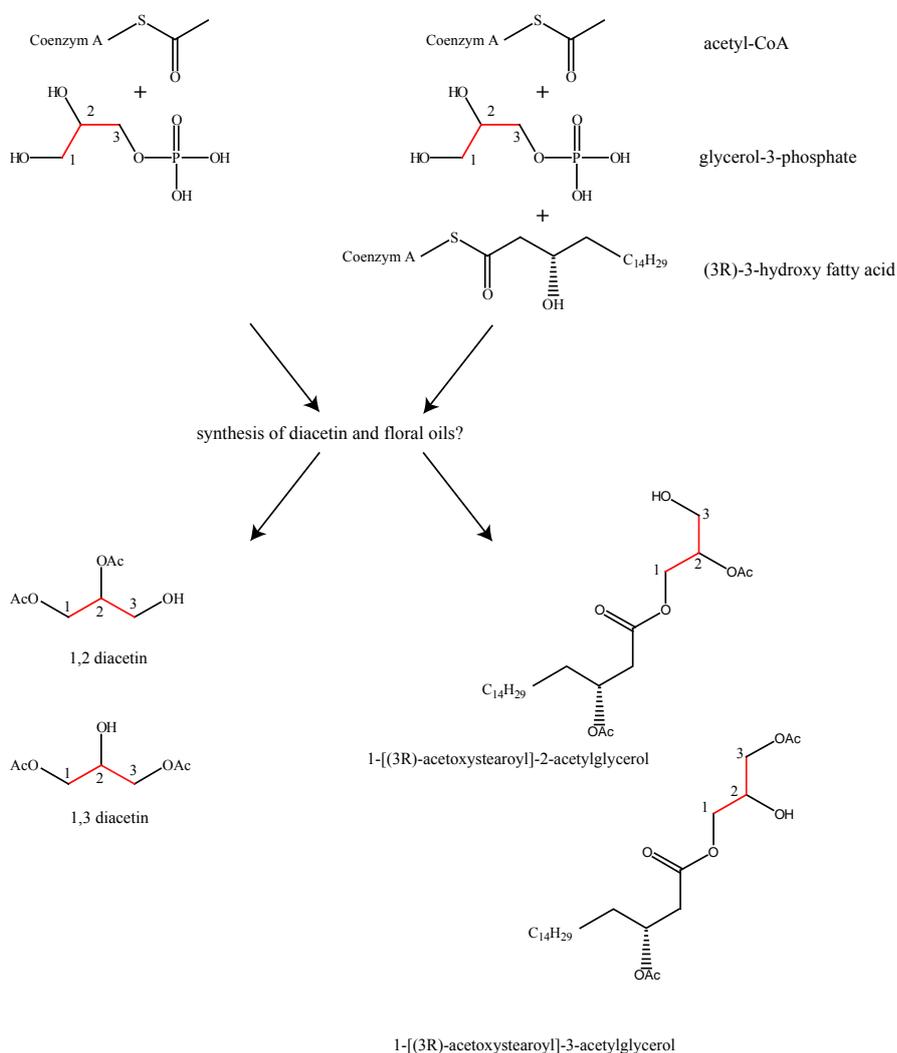
Fig. 4. Schematic overview of proposed biosynthesis of diacetin and abundant floral oil components found in *Lysimachia punctata*

We did not find diacetin in several oil secreting species based on dynamic headspace collections (20, 50), though we identified diacetin in the same species based on flower solvent extracts used in this work, suggesting that diacetin is released in the headspace in only small and not detectable amounts

from the flowers (see also below). Interestingly, diacetin was not identified so far in any of the studies focusing on the chemistry of the floral oils itself, most probably because it was not a target compound (smaller and higher volatility compared to the non-volatile oils) in that studies (e. g. 16, 25) and treatment of samples did not allow detecting it. The amount of diacetin available in the samples was often quite small compared to the oils and this small amount may have got lost in the process of evaporating the "oil samples" to dryness.

The basic structure of floral oils (i.e. acylglycerols) is similar in oil species around the world, and it resembles the volatile compound diacetin as well as lipids in plant tissues. It can be assumed that metabolic pathways proceeds similarly in lipids, in floral oils, as well as in diacetin production (Fig. 4), while enzymes must be involved in esterification of the fatty acids (51) (in floral oils: 3-hydroxy/3-acetoxy fatty acids or acetic acid) with the 1-3 hydroxy groups of glycerol.

As exemplified for *L. punctata*, main compounds in the floral oil are 1-[(3R)-acetoxystearoyl]-2-acetylglycerol and 1-[(3R)-acetoxystearoyl]-3-acetylglycerol, in both compounds of which one of the hydroxyl groups of glycerol is esterified with acetic acid, one other with an acetylated fatty acid (Fig. 4). Structural and biosynthetic similarities of these two compounds with 1,2- and 1,3-diacetin, in which glycerol is esterified with two molecules of acetic acid, are evident.
Based on these similarities between "non-volatile" floral oils and diacetin, we expect that diacetin is present in all oils that consist of a glycerol backbone and additionally have one or more acetyl group(s) whereas it may not be present in oils made up of other classes of compounds (e.g. free fatty acids). Indeed, we found diacetin in all plants having oils congruent with these criteria with the exception of *Momordica* and *Bunchosia* species, whereas we did not find diacetin in *Nierembergia*, the oils of which do not consist of acetylated glycerols (Table S2) (25, 52). The common occurrence of diacetin and "acetylated" floral oils supports the idea that diacetin and the floral oils derive from the same metabolic pathway, and makes diacetin an ideal and honest volatile signal for bees looking for floral oils.

Olfactory adaptation in oil bees to diacetin

Our data not only show that diacetin is widespread among oil secreting species and a good candidate used by oil bees around the world as honest signal for oil rewards, but also indicate that diacetin represents a private communication channel between oil flowers and oil bees. In our electrophysiological measurements diacetin elicited antennal responses in an European (*Macropis fulvipes*) and South African (*Rediviva neliana*) melittid oil bee (and also in *M. europaea* Warncke 1973, *R. brunnea* Whitehead & Steiner, and *R. pallidula* Whitehead & Steiner (Dötterl and Steiner, unpublished data)), but did not elicited significant antennal responses in a close related melittid non-

oil bee (*Melitta haemorrhoidalis*) and the honey bee (*Apis mellifera*, Apidae). This difference in antennal response to diacetin between oil and non-oil bees demonstrates that the oil bees have specific olfactory adaptations (e.g. on the level of olfactory receptors or olfactory binding proteins (11, 53, 54) in the periphery of the olfactory circuit to detect diacetin. Such adaptations have not been described for any other pollinators before this study and in a next step perception of diacetin by Apidae oil bees need to be analyzed to test if Apidae oil bees respond similarly to diacetin as melittid oil bees do.

Our bioassays with *M. fulvipes* and EAD-active scent compounds of its host plant *L. punctata* point towards a key function of diacetin in host location. Diacetin alone was capable in attracting *Macropis* bees and a mixture containing diacetin and four other EAD-active compounds had the same attractiveness as a natural flower extract. Subtractive experiments revealed that two of the added compounds (2-tridecanone, geranic acid) are not involved in bee attraction, but two other compounds (heptanoic acid, (*E*)-2-dodecenal) are. However, a mixture without diacetin (and geranic acid, see below) did not attract any bee compared to a synthetic mixture with all the compounds.

We needed to exclude geranic acid additionally to diacetin as we found trace amounts of diacetin as contamination in the synthetic geranic acid. These trace amounts seems to be enough to elicit behavioral responses in *Macropis*, because a synthetic flower extract without diacetin but with geranic acid attracted *Macropis* bees (Schäffler, unpublished data). When removing only geranic acid from the complete synthetic mixture the bees did not discriminate between this reduced mixture and the complete mixture demonstrating not only that geranic acid has no influence on bee behavior, but also that the lacking of trace amounts of diacetin (contamination of geranic acid) did not influence the choice of bees. Overall, we conclude that diacetin and not geranic acid was responsible for the loss of attractiveness compared to the complete scent mixture when excluding both substances from the complete mixture, which points towards diacetin as the key compound in attracting *Macropis*.

In addition to diacetin, heptanoic acid and (*E*)-2-dodecenal are used by *Macropis* bees for finding oil flowers. Heptanoic acid was detected in about 20 oil secreting species in three floristic regions, and recently in a few oil and non-oil species (50, 55), whereas only once it was reported as a kairomone in a host-parasitic communication systems (56). Even rarer is the floral scent compound (*E*)-2-dodecenal, that we found in only three *Lysimachia* oil species. Until now this compound was found only in a few South African oil orchids (50) and in two non-oil species (57, 58), and only once reported towards a insect deterrent produced by a millipede (59). In contrast the EAD-active compound 2-tridecanone, not influencing the attractiveness of the synthetic mixture, is very widespread among our studied oil species as well as in a higher number of oil orchids of South Africa (50), and also known as floral scent compound in several non-oil species (55, 57, 58, 60). As we could show that 2-tridecanone is not involved in pollinator attraction, this compound, known as a repellent for insects (61) including generalized bee pollinators (62), could act as a floral filter (see also 50, 63)

at least in the *Macropis-Lysimachia* pollination system to reduce visitation rates from inappropriate visitors avoiding pollen loss.

Interestingly, while diacetin is very widespread among oil secreting plants, the plants additionally emit other compounds, several of which are not that widespread and occur only in one or a few of the species (49, 50, 58). There is high overall variation in floral scent among oil plants, which is true for species within floristic regions and even for species pollinated by the same oil bee (Holarctic: (49), South Africa: (50)) as well as among floristic regions. These findings led us to speculate that diacetin is a reliable volatile marker for 'non-volatile' fatty oils around the world, whereas the emission of other compounds, like (*E*)-2-octenol, geranic acid, 3,5-dimethoxytoluene, or (*E*)-2-dodecenal, may be important for allowing bees to discriminate among co-blooming species. Scents distinguishable among plant species are known to promote effective pollen transfer within species and species integrity through flower constancy of pollinators (64). However, we did not find diacetin in all of the oil producing species suggesting that they emit diacetin in amounts too low for detection or that diacetin is not produced by these plants. If the latter is true, other compounds than diacetin may be important as pollinator attractants.

Conclusion:

In summary, we could show that diacetin occurs in several floral oil secreting plants around the world and that it is important for host plant finding in a Holarctic *Macropis* oil bee. Suggesting that diacetin and floral oils share the same metabolic pathways the production of diacetin is likely linked with the production of oil and therefore represents an honest signal for oil collecting bees. Our data also point towards diacetin as a private communication channel, since oil bees (melittids) can detect it, whereas there is no indication that non-oil bees have the olfactory capability for perceiving this compound.

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Supplementary Information:

Table S1: Oil and non-oil secreting species of the different floral regions used for chemical analyses. The number of samples collected per species and those with diacetin is also given. Samples were either collected in Germany (BT: Bayreuth, Heckl. Hecklingen, M: Munich) or in South Africa (SAf).

Floristic region *Plant family* Plant genus	*Plant species*	Oil (O) Non-oil (N)	Nr. of samples collected/Nr. of samples with diacetin	Collected in
Holarctis				
Primulaceae				
Lysimachia L.				
	L. ciliata L.	O	3/3	BT
	L. christinae Hance	O	3/3	BT
	L. congestiflora Hemsl.	O	3/3	BT
	L. nummularia L.	O	3/3	BT
	L. punctata L.	O	3/3	BT
	L. vulgaris L.	O	5/5	BT
	L.patungensis Hand.-Mazz.	O	2/2	BT
	L. arvensis L.	N	1/0	BT
	*L. atropurpurea*L.	N	1/0	BT
	*L. clethroides*Duby	N	3/0	BT
	L. ephemerum L.	N	2/0	BT
	L. nemorum L.	N	3/0	BT
	*L. maritima*Galasso, Banfi & Soldano	N	1/0	Heckl.
	L. minoricensis J.J.Rodr.	N	1/0	BT
	L. thyrsiflora L.	N	5/1	BT
Capensis				
Scrophulariaceae				
Diascia Link&Otto				
	D. capensis Britten	O	1/1	SAf
	D. cordata N.E.Br.	O	1/1	SAf
	D. barbarae Hook. f.	O	4/4	BT
	D. interregima E.Mey. ex Benth.	O	1/1	SAf
	D. purpurea N.E.Br.	O	3/3	SAf
	D. vigilis Hilliard & B.L.Burtt	O	4/4	SAf
Hemimeris L.				
	H. racemosa (Houtt.) Merr.	O	1/1	SAf
Orchidaceae				
Corycium Sw.				
	C. dracomontanum Parkman & Schelpe	O	2/2	SAf
	C. orobanchoides Sw.	O	1/1	SAf
	C. nigrescens Sond.	O	1/1	SAf
Pterygodium Sw.				
	P. catholicum Sw.	O	1/1	SAf
	P. magnum Rchb. f.	O	1/1	SAf
Disperis Sw.				
	D. fannineae Harv.	O	1/1	SAf
	D. oxyglossa Bolus	O	1/1	SAf
	D. villosa Sw.	O	1/1	SAf
Huttonaea Harv.				
	H. grandiflora Rolfe	O	1/1	SAf
	H. pulchra Harv.	O	1/1	SAf

Floristic region / Plant family / Plant genus / Plant species	Oil (O) Non-oil (N)	Nr. of samples collected/Nr. of samples with diacetin	Collected in
Stilbaceae			
<u>*Bowkeria* Harv.</u>			
B. sp.	O	1/1	SAf
Neotropis			
Orchidaceae			
<u>*Oncidium* Sw.</u>			
O. sotoanum R. Jiménez & Hágsater	O	2/2	M/BT
<u>*Sigmatostalix* Rchb.f.</u>			
*S. cuculigera (*Schltr.) Garay	O	1/1	M
S. guatemalensis Schltr.	O	1/1	M
S. radicans Rchb.f.	O	1/1	BT
<u>*Dipteranthus* Barb. Rodr.</u>			
D. obliquus (Schnee) Garay & Dunst.	O	1/1	M
Iridaceae			
<u>*Trimezia* Salisb. ex Herb.</u>			
T. sincorana Ravenna	O	1/1	M
<u>*Ennealophus* N.E.Br.</u>			
E. euryandrus Ravenna	O	1/1	M
Calceolariaceae			
<u>*Calceolaria* L.</u>			
C. elatior Griseb.	O	1/1	M
C. integrifolia L.	O	1/1	M
C. sp	O	1/1	BT
Malpighiaceae			
<u>*Bunchosia* Rich. ex Juss.</u>			
B. spec.	O	2/0	M
<u>*Gaudichaudia* Kunth</u>			
G. mucronata A. Juss	O	1/1	M
<u>*Heteropteris* Kunth</u>			
H. chrysophylla (Lam.) Kunth	O	1/1	M
<u>*Malpighia* L.</u>			
M. coccigera L.	O	1/1	BT
M. fucata Ker Gawl.	O	1/0	M
M. glabra var. undulata Nied.	O	1/0	M
M. urens L.	O	2/2	M
<u>*Stigmaphyllon* A. Juss.</u>			
S. ciliatum A. Juss.	O	2/2	M
S. sinuatum A. Juss.	O	1/0	M
Solanaceae			
<u>*Nierembergia* Ruiz & Pav.</u>			
N. scoparia Sendtn.	O	1/0	M
N. hippomanica Miers	O	4/0	BT
Plantaginaceae			
<u>*Angelonia* Humb. & Bonpl.</u>			
A. angustifolia Benth.	O	1/1	BT
Palaeotropis			
Cucurbitaceae			
<u>*Momordica* L.</u>			
M. cissioides Planch.ex Benth male	O	1/0	M
M. foetida Schumach. male	O	1/0	M
M. boivinii Blume female	O	2/0	BT
M. charanthia L. male flowers	N	1/0	BT
M. charanthia L. female flowers	N	1/0	BT

Table S2. Overall floral oil chemistry and presence of diacetin in plant genera from which species were included in our analyses. Diacetin was expected to occur in plants that have oils with a glycerol moiety and an acetyl group, and to not occur in plants having other oils. Unexpected results are printed in bold.

floristic region	genus[references]	oil chemistry		diacetin expected in the samples	detected diacetin in our samples
		presence of			
		glycerol moiety	acetyl group		
Holarctis	*Lysimachia* [1, 2, 3, 5]	yes	yes	yes	yes
Capensis	*Diascia* [1, 4, 5]	yes	yes	yes	yes
	Trimezia [1]	yes	yes	yes	yes
	Corycium [5]	yes	yes	yes	yes
	Pterygodium [5]	yes	yes	yes	yes
Neotropis	*Angelonia* [1, 5]	yes	yes	yes	yes
	Nierembergia [1, 6]	no	yes	no	no
	Malpighia [1, 5]	yes	yes	yes	yes
	Stigmaphyllon [5]	yes	yes	yes	yes
	Bunchosia [5]	yes	yes	**yes**	**no**
	Sigmatostalix [1]	yes	yes	yes	yes
	Calceolaria [7]	yes	yes	yes	yes
	Oncidium [8, 5]	yes	yes	yes	yes
Palaeotropis	*Momordica* [5, 9]	yes [9]	yes [5]	**yes**	**no**

References: [1] (1); [2] (2); [3] (3); [4] (4); [5] (5); [6] (6); [7] (7); [8] (8); [9] (9)

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Hiermit erkläre ich, dass ich die Arbeit selbständig verfasst und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe.

Ferner erkläre ich, dass ich anderweitig mit oder ohne Erfolg nicht versucht habe, diese Dissertation einzureichen. Ich habe keine gleichartige Doktorprüfung an einer anderen Hochschule endgültig nicht bestanden.

Bayreuth