Pollinator attraction in
Wasp-flowers

Dissertation
zur Erlangung des Doktorgrades Dr. rer. nat.
der Fakultät für Naturwissenschaften der Universität Ulm
vorgelegt von

Jennifer Brodmann
aus
Tettnang, Deutschland

Ulm 2010
Titelfoto:
Vespula germanica auf Epipactis helleborine
Vespula vulgaris auf Steveniella satyrioides
Vespa bicolor an Dendrobium sinense
Vespula germanica an Scrophularia umbrosa

© Jennifer Brodmann
<table>
<thead>
<tr>
<th>Rolle</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amtierender Dekan</td>
<td>Prof. Dr. Axel Groß</td>
</tr>
<tr>
<td>Erstgutachter</td>
<td>Prof. Dr. Manfred Ayasse</td>
</tr>
<tr>
<td>Zweitgutachter</td>
<td>Prof. Dr. Harald Wolf</td>
</tr>
<tr>
<td>Tag der Promotion</td>
<td>1. Oktober 2010</td>
</tr>
</tbody>
</table>
Table of contents

Summary ................................................................................................................................. 1
Introduction ............................................................................................................................ 1
Results .................................................................................................................................. 5
Discussion ............................................................................................................................. 11
Conclusion ............................................................................................................................ 15
References ............................................................................................................................. 16

Zusammenfassung .................................................................................................................. 21
Einleitung ............................................................................................................................... 21
Ergebnisse & Diskussion ....................................................................................................... 26
Schlussfolgerung ................................................................................................................... 30

Chapters ................................................................................................................................. 31

1. Orchid mimics green leaf volatiles to attract prey-hunting wasps for pollination ............ 31
2. Orchid mimics honeybee alarm pheromone in order to attract hornets for pollination .... 42
3. Orchid mimics alarm pheromone of the pollinator to attract alerted wasps for pollination . 54
4. Pollinator attraction of the wasp-flower Scrophularia umbrosa ....................................... 66
5. Multifaceted ways of attracting various pollinators in the orchid genus Epipactis ......... 80
6. The role of innate and learnt floral odour preferences in the pollinator attraction of the orchid Epipactis helleborine ................................................................. 97

Publications from this thesis ................................................................................................. 108
Contributions of co-authors ................................................................................................. 109
Danksagung .......................................................................................................................... 110
Curriculum vitae ................................................................................................................... 111
Eidesstattliche Erklärung ....................................................................................................... 115
Summary

Pollinator attraction in wasp-flowers

Introduction

The huge diversity of pollination systems in orchids has fascinated evolutionary biologist since the very beginning (Darwin 1862). One main factor behind this fascination was the fact that one third of the approximately 30,000 orchid species described have evolved a deceptive pollination mechanism (Nilsson 1992; Pijl and Dodson 1966). Deceptive flowers do not offer a reward (e.g. nectar or pollen) to the pollinators but attract them by visual and/or olfactory mimicry. The deceptive mechanisms include e.g. food deception, brood site mimicry, sexual deception, or prey mimicry (Brodmann et al. 2009; Dafni 1984; Jersáková et al. 2006). In deceptive orchids in contrast to rewarding ones there is often a higher pollinator specificity, resulting in more efficient pollen transfer from orchid flowers in a single pollinator visit (Dafni 1984).

The discovery by Sprengel (1793) that many orchid flowers do not contain nectar, and thus deceive their insect pollinators, was greeted with incredulity and disbelief. More than one-third of orchid species do not provide their pollinators with either pollen or nectar rewards (Ackerman 1986; Dressler 1981; Pijl and Dodson 1966). The evolution of such a rewardless strategy is difficult to explain, because many rewardless species appear to have lowered reproductive success compared with their rewarding relatives (Neiland and Wilcock 1998). Deceptive orchids have been intensively studied since Darwin (1838), but the evolution of deceptive flowers still presents a major puzzle for evolutionary biology. The explanations why deception is overrepresented in the orchid family include an efficient removal and deposition of pollinaria from orchid flowers in a single pollinator visit, and thus an efficient transport of orchid pollen (Dafni 1984). There are two general hypotheses as to how deception could increase fitness in plants. The first is that deception allows reallocation of resources from rewards to fruit production. The second is that deception results in pollinators visiting fewer flowers on a plant, thereby promoting outcrossing (Jersáková et al. 2006; Nilsson 1992). Arguments that deception evolves because rewards are costly are consequently problematic because that small amounts of nectar are unlikely to have a significant effect on the energy budget of orchids (Jersáková et al. 2006).

Plants attract their pollinators with the help of various floral cues to their flowers. One of these floral cue is odour, which is known as an important cue for many plants to attract their pollinators (Knudsen et al. 2006). In sexually deceptive orchids the odour is even the most important cue to attract pollinators. The flowers mimic female sex pheromones and attract only male insects, which pollinate the flowers in an attempted copulation routine (Ayasse 2006; Schiestl 2005; Schiestl et al. 2003). But also the optical floral cues are involved in
pollinator attraction. It was shown that honeybees are able to discriminate between dummy flowers on the basis of colour pattern (Hempel de Ibarra et al. 2002), or that bumblebees are directed by floral guides to their flowers (Lunau et al. 2006). Besides olfactory and optical cues of the flower, nectar plays a crucial role in plant reproduction by rewarding the pollinators (Simpson and Neff 1983). The main ingredient of the nectar in the majority of plants is sugar: glucose, fructose and sucrose (Baker and Baker 1983). Apart from it, nectar contains amino acids, lipids, organic acids, as well as various vitamins, enzymes, antioxidants, mineral ions and secondary metabolites (Baker 1975; Galetto et al. 1998). It is known that diverse pollinator species prefer different sugar compositions of the nectar (Baker and Baker 1983). For instance hummingbirds prefer relatively high sucrose concentrations, whereas birds exploit only simple hexose sugars, such as glucose or fructose (del Rio 1990). The coevolution of plants and their pollinators formed the phrase of “pollination syndromes”. Pollination syndromes are groups of floral traits such as colour, morphology, scent and nectar characteristics, which are thought to be associated with certain groups of pollinators (Fenster et al. 2004). Therefore pollinators can be organized into functional groups. Robertson (1928) made a classification of the visitors into nine functional groups: long-tongued bees, short-tongued bees, other Hymenoptera, Diptera, Coleoptera, Lepidoptera, Hemiptera, Neuroptera, and birds. To give an example, bird-attracting flowers are typically large, showy and robust, having red, orange or bright yellow colours, no scent and copious amount of dilute nectar (Faegri and Van der Pijl 1979; Proctor et al. 1996).

Although 5 % of the known orchids are pollinated by wasps (Pijl and Dodson 1966), the “syndrome of wasp-pollination”, which belongs to the group of “other Hymenoptera”, is nearly overlooked. Social wasps (Vespidae), as well as bees and ants belong to the genus of Hymenoptera (Wilson 1971). In contrast to bees, wasps do not feed their brood with pollen collected from flowers; instead they feed their larvae with meat nutriments, like small arthropods (Schremmer 1962). This is the reason, why in literature wasps are not mention as typical flower visitors.

A distinction is drawn between the pollination by social wasps, living in colonies, and solitary wasps. Amongst the solitary wasps many studies have been performed to investigate the coevolutionary association between figs and their pollinating fig-wasps (Anstett et al. 1997). Furthermore certain species of sexually deceptive orchids are pollinated by solitary wasps (Ayasse et al. 2003; Schiestl 2005). In contrast the pollination by social wasps was nearly overlooked. Some examples of flowers pollinated by social wasps are listed in literature, under them some species of the orchid genus *Epipactis*, some species of *Scrophularia*, as well as *Helix hedera*, and *Cotoneaster vulgaris* (Schremmer 1962). Because little was known about the floral signals responsible for the highly specific attraction of social wasps, Faegri and Van der Pijl (1979) believed that it would be “hardly possible to establish a syndrome of wasp blossoms”. Meanwhile it has been shown that wasp-flowers exhibit physiological and morphological adaptations for the pollination by social wasps (Müller 1873). For instance the head of the wasp fits optimally in the flower shape, which benefits an optimal pollination. In general, flowers visited by social wasps tend to have a dull coloration and exposed nectar (Müller 1881; Schremmer 1962; Werth 1956). In case of the rewarding wasp-flower *Epipactis helleborine* the pollinating social wasps are thought to be attracted by
visual cues, namely dull brown, dirty-red to washed yellow-green colours, only (Wiefelspütz 1970). Further behavioural observations of Keppert (2001) leads to the suggestion, that floral scent is also involved in wasp attraction. Furthermore energetic quality of the nectar, certain nectar constituents, or taste, may be important factors in the foraging choice of flower visitors (Baker and Baker 1983). Baker & Baker (1983) found, that the nectar of wasp-pollinated flowers is, in contrast to flowers of other pollination syndromes, sucrose dominated. To answer the question, why wasp-flowers are nearly overlooked by other flower visitors, they expected that there could be a sugar with a repellent function, like cellobiose and gentiobiose, in the nectar (Baker and Baker 1983), or nectar may even contain toxic alkaloids, amino acids or other unpleasant substances that repel nectar thieves (Baker 1975; Baker and Baker 1977; Baker et al. 1978). The chemical composition of the wasp-flower *E. helleborine* nectar was studied and a number of compounds, amongst them ethanol, with potential narcotic properties, were identified (Jakubska et al. 2005). The authors assumed that these substances would make the insects, which drink the nectar to become “sluggish”. As a consequence, the time, which they spent on the inflorescence would be prolonged and therefore the chance of pollinating larger number of flowers would be increased.

**Thesis topic**

Because little was known about the pollinator attracting floral signals that are used by flowers pollinated by social wasps, in my thesis I investigated in a comparative approach the floral cues, which are responsible for the specific attraction of social wasps to the flowers. I wanted to answer the question, if there is a wasp specific attractant that can be found in all wasp-flowers or if various wasp-flowers use different strategies to attract wasps. Furthermore I explored if there is a difference between orchids and non-orchids and between rewarding and deceptive wasp-flowers concerning the attractants used for the specific wasp attraction.

The following questions were addressed:

1. *Which floral cues are responsible for the wasp attraction in wasp-flowers?*
2. *Do orchids and non-orchids use the same floral cues to attract wasps for pollination?*
3. *Do rewarding and deceptive species use the same floral cues to attract wasp for pollination?*

**Investigated species**

In my studies I investigated several species of the orchid genus *Epipactis* (Orchidaceae, subfamily: Epidendroideae) in Germany, *Dendrobium sinense* (Orchidaceae, subfamily: Epidendroideae) in China, and *Steveniella satyrioides* (Orchidaceae, subfamily: Orchidoiceae) in Turkey. As an example of a non-orchid wasp-flower I involved plants of the genus *Scrophularia* (Scrophulariaceae) from Germany in my study.
The orchid genus *Epipactis* is represented by about 18 species and 20 subspecies in Europe (Baumann 2005), which are known to be pollinated by various insects. While some species have a broad pollinator spectrum and are, amongst others, visited by bees (Vöth 1999) and solitary wasps (Nilsson 1978), others are very specialised. *Epipactis helleborine* (L.) Crantz is the most common and widely distributed species of the genus (Wiefelspütz 1970), and is a prime example for a wasp-flower, as well as *E. purpurata*, because both are mainly pollinated by social wasps (Hymenoptera: Vespidae), like *Vespula vulgaris* and *V. germanica* (Müller 1873). Their flowers exhibit physiological, morphological and ecological adaptations for the attraction of pollinating social wasps (Müller 1873). As noted by Darwin (1888), this species are almost entirely overlooked by other potential pollinators, despite a large nectar reward. The flowering period of this orchid ranges from June to July.

The epiphytic orchid *Dendrobium sinense*, is an endemic species of the Chinese island Hainan (Tang and Chen 1977). The flowers of *D. sinense* are white with a red strip in the centre, and the flowering period ranges from September to October in the raining season. This orchid offers no nectar reward to its sole pollinator, the hornet *Vespa bicolor*.

The orchid *Steveniella satyrioides* (Stev.) Schltr. is currently the only species described in the genus. The natural habitat covers Anatolia, Northern Iran, Caucasus and Crimea (Baumann and Künkele 1988; Nevski 1935). The orchid, with small reddish-brown flowers, is nectarless and as pollinators so far two species of wasps, *Vespula vulgaris* and *Dolichovespula sylvestris*, are described (Nazarov 1995). Dependent on the location the flowering period ranges from April to June.

The genus *Scrophularia* (Scrophulariaceae) is represented by approximately 268 species and is mainly distributed in the holoarctic, with its primary diversification centre in Asia (Ortega Olivencia and Devesa Alcaraz 1992; Stiefelhagen 1910). The flowers offer pollen and nectar as a reward to pollinators (Ortega Olivencia and Devesa Alcaraz 1992). Although *Scrophularia* species are visited from a broad range of pollinators, certain species like *S. nodosa* (Sprengel 1793) and *S. umbrosa* (Müller 1881) are often described as wasp-flowers, because they are mostly visited by social wasps. But in contrast to the other investigated wasp-flowers in my thesis this species are not so specialized in pollination by social wasps, and several other pollinators, like honeybees, solitary bees, syrphid flies, and solitary wasps can be observed on the flowers (Emer 2005; Faegri and Van der Pijl 1979; Schremmer 1958).

**Methods used**

To investigate the attractiveness of the floral cues responsible for the wasp attraction of the investigated wasp-flowers I used behavioural experiments combined with chemical (GC: gas chromatography, GC-MS: gas chromatography coupled with mass spectrometry, HPLC: high pressure liquid chromatography) and electrophysiological investigations (GC-EAD: gas chromatography coupled with an electroantennographic detector). Electrophysiological techniques can determine which volatiles present in a floral bouquet are “active”, implicating that the insect can detect this compound. Once active volatiles have been identified, their
function in modifying insect behaviour (e.g. as attractants or deterrents) can be explored using behavioural experiments.

In behavioural experiments I investigated the attractiveness of optical and olfactory floral cues in order to discover, which floral cues play the key role in the wasp attraction. To explore the attractiveness of optical versus olfactory floral cues I performed experiments with a modifiable quartz glass cylinder, so that visual and olfactory floral cues could be offered to wasps singly or in combination. To test the olfactory cues in more details and identify key compounds, I conducted olfactometer experiments using a Y tube and performed tests in the wind tunnel under semi natural conditions. Furthermore I investigated various synthetic odour mixtures in field experiments, in a flight cage, or in a wind tunnel.

Using a combination of chemical (GC, GC-MS) and electrophysiological (GC-EAD) investigations I identified the floral compounds which can be detected by the wasps, and thus could be responsible for the wasp attraction. Afterwards the identified compounds were tested in behavioural experiments.

Furthermore I performed nectar analysis using a HPLC to compare wasp-flowers and non wasp-flowers of the genus Epipactis concerning their nectar sugar composition. Optical measurements using an Ocean Optics S2000 spectrophotometer were used to investigate the optical cues of various Epipactis flowers, which are pollinated by various pollinators.

Summary Chapter 1

Orchid mimics green leaf volatiles to attract prey-hunting wasps for pollination

In this study we investigated the wasp-flowers Epipactis helleborine and E. purpurata to identify pollinator attracting floral cues and to answer the question why this orchid is exclusively visited and pollinated by social wasps.

The orchid Epipactis helleborine (L.) Crantz is a prime example of a wasp-flower; it is mainly pollinated by social wasps (Hymenoptera: Vespidae) like Vespula vulgaris and V. germanica (Müller 1873). Social wasps feed their larvae with insects like caterpillars (Schremmer 1962), amongst them Pieris rapae. In order to locate their prey they use a combination of visual and olfactory cues (Cornelius 1993).

In parasitic wasps it is known, that they use volatiles (e.g. green leaf volatiles) emitted by green plant tissue, in response to herbivory, to locate insect prey (Paré and Tumlinson 1999; Turlings et al. 1990). we hypnotized that social wasps may do likewise. “Green leaf volatiles” (GLVs), mostly six-carbon aldehydes, alcohols and acetates and other volatile organic compounds (VOCs), are emitted by many plants infested by herbivores, e.g. caterpillars (Whitman and Eller 1990). we suspected that E. helleborine flowers may produce GLVs in order to attract prey-hunting social wasps for pollination.

Within the genus Epipactis certain species are pollinated by social wasps, whilst others attract, amongst others, bees for pollination (Baumann 2005). With electrophysiological investigations (GC-EAD) and chemical analyses (GC, GC-MS) we demonstrated for the first
time that the wasp-flowers *E. helleborine* and *E. purpurata* emit GLVs, which are attractive to
foragers of the social wasps *Vespula germanica* and *V. vulgaris*. we also detect these GLVs,
found in the orchid flowers, in cabbage infested with *Pieris rapae* larvae. Furthermore we
could show that *E. atrorubens*, a species that is visited by a broad spectrum of pollinators,
mainly bumblebees (Baumann 2005), do emit GLVs only in trace amounts. This results
clearly show that the two wasp-pollinated species *E. helleborine* and *E. purpurata* emit
significantly higher amounts of GLVs than *E. atrorubens*, and using behavioural experiments
we could show that these GLVs definitely have a key function in the wasp attraction
(Brodmann et al. 2008). This was the first example in which GLVs have been implicated in
chemical mimicry for the attraction of pollinating insects.

**Summary Chapter 2**

*Orchid mimics honeybee alarm pheromone in order to attract hornets for pollination*

The aim of this study was to determine the floral scent involved in pollinator attraction of the
rewardless orchid *Dendrobium sinense*, an endemic species of the Chinese island Hainan,
which is pollinated by the hornet *Vespa bicolor* (Brodmann et al. 2009).

The flowers of *D. sinense* are white with a red strip in the centre. The pollinator of the orchid
was unknown prior to our studies of eight populations of *D. sinense* in the Bawang National
Reserve in Hainan, China. Via observations we identified the hornet *Vespa bicolor*
(Hymenoptera: Vespoidea) as the sole pollinator of the orchid. Rather than landing and
pausing on the flowers as would be typical for most pollinators, the hornets instead pounced
on the red centre of the flower, much like their behaviour when attacking prey. Contact with
the flower during these pounces was typically less than one second.

Hornets belong to the group of social wasps that feed their brood with meat nutriments,
mainly insects (Schremmer 1962). Foraging hornets are known to often capture honeybees,
either in the surroundings of a colony or while they forage for pollen and nectar on flowers
(Ono et al. 1987; Schremmer 1962). Behavioural experiments have shown that prey-
searching wasps use a combination of visual and olfactory cues to locate their victims (Reid
et al. 1995).

Using a combination of chemical analyses (GC, GC-MS) and electrophysiological
methods (GC-EAD), we demonstrated that the flowers of *D. sinense* produce Z-11-eicosen-
1-ol amongst other volatiles and that the pollinator *V. bicolor* can smell this compound. Z-11-
eicosen-1-ol is a major compound in the alarm pheromones of the Asian (*Apis cerana*) and
European honeybees (*Apis mellifera*) (Pickett et al. 1982; Schmidt et al. 1997), and is also
exploited by the European beeewolf (*Philanthus triangulum*) to locate its prey, honeybees
(Herzner et al. 2005). In comparative chemical analysis we found three compounds, under
them Z-11-eicosen-1-ol, in common in the orchids scent an in the surface extracts of the
Asian honeybee *A. cerana*. This was the first time that Z-11-eicosen-1-ol has been identified
as a floral volatile. In behavioural experiments, we demonstrated that the floral scent of the
orchid, as well as the synthetic Z-11-eicosen-1-ol are attractive to the foraging females of the hornet *V. bicolor* (Brodmann et al. 2009). Since hornets frequently capture honeybees to feed to their larvae, we suggested that the flowers of *D. sinense* mimic the alarm pheromone of honeybees in order to attract prey-hunting hornets for pollination.

**Summary Chapter 3**

*Orchid mimics alarm pheromone of the pollinator to attract alerted wasps for pollination*

In this study we investigated the orchid *Steveniella satyrioides*, which is exclusively pollinated by social wasps. In my investigation we wanted to answer the question, how the nectarless orchid lures the wasps to their flowers for pollination.

The wasp-pollinated orchid *Steveniella satyrioides* (Stev.) Schltr. occurs in Anatolia, Northern Iran, Caucasus and Crimea (Baumann and Künkele 1988; Nevski 1935). It is nectarless, and entirely purple-brown in colour. It is pollinated by social wasps (Hymenoptera: Vespidae), like *Vespula vulgaris* and *Dolichovespula sylvestris* (Nazarov 1995). Because wasps feed their larvae with meat, Nazarov (1995) hypothesized that the orchid would attract the wasps by mimicking a piece of meat. He described reddish-brown papillae on the base of the lip, at the spur entrance as a visual signal and suggested that the wasps may take them as food. Therefore he entitled the pollination mechanism as “false-prey”-syndrome.

In behavioural experiments, we proved that not the optical cue, but the odour of the orchid has a superior function to attract wasps from a distance and elicits, after landing on a flower, stinging and biting in the wasps. Using a combination of electrophysiological investigations (GC-EAD) and chemical analyses (GC, GC-MS) we revealed that the flowers of *S. satyrioides* produce, amongst other compounds, acetophenone and 2-ethylhexan-1-ol, volatiles that the pollinator can smell and which are also produced in the venom gland of several species of social wasps with a function as an alarm pheromone (Fortunato et al. 2004; Ono 2005). In social insects, including wasps, alarm pheromones have a function for colony defence, release aggressive behaviour in females (Maschwitz 1964; Veith et al. 1984), are used to kill the victims (Olson 2000) and mark prey items. In behavioural experiments we could show that the orchid mimics the alarm pheromone compounds in order to attract alerted wasps for pollination. We showed for the first time that a deceptive plant mimics the alarm pheromone of its pollinator species and thereby exploits the social communication system of the pollinator. This is a new evidence, that prey mimicry is a common syndrome of wasp-pollinated orchids (Brodmann et al. 2008; Brodmann et al. 2009).
Summary Chapter 4

Pollinator attraction of the wasp-flower Scrophularia umbrosa

In this study we investigated Scrophularia umbrosa, which was mostly pollinated by social wasps. The background idea was to compare the floral signals responsible for wasp attraction from the non-orchid S. umbrosa with the orchid Epipactis helleborine. In contrast to the other wasp-flowers included in my study, this is the only non-orchid.

The genus Scrophularia (Scrophulariaceae) is represented by approximately 268 species, mainly distributed in the holoarctic, with its primary diversification centre in Asia (Ortega Olivencia and Devesa Alcaraz 1992; Stiefelhagen 1910). The flowers offer pollen and nectar, as a reward to their pollinators (Ortega Olivencia and Devesa Alcaraz 1992). Scrophularia nodosa (Sprengel 1793) and S. umbrosa (Müller 1881) are thought to be wasp-flowers and show small, bulbous rust-brown or greenly-brown coloured flowers (Haeupler and Muer 2000; Sebald et al. 1996), with exposed nectar. However in contrast to other wasp-flowers, like E. helleborine, S. umbrosa exhibit a broader visitor spectrum. Expect social wasps, also other flower visitors could be found, amongst them especially honeybees (Faegri and Van der Pijl 1979), Syrphidae (Schremmer 1958), and solitary wasps (Emer 2005).

Using a combination of chemical (GC, GC-MS) and electrophysiological analyses (GC-EAD) we could identify the compounds in the complex floral odour bouquet that are detectable by the wasps. As well as in the wasp-flower E. helleborine we found so called “green leaf volatiles” (GLVs) in the floral odour (Brodmann et al. 2008). GLVs, mostly six-carbon aldehydes, alcohols and acetates and other volatile organic compounds (VOCs), are emitted by many plants infested by herbivores, e.g. caterpillars (Whitman and Eller 1990). Behavioural experiments demonstrated that in contrast to the other investigated wasp-flowers (Brodmann et al. 2008; Brodmann et al. 2009) the floral odour alone is not responsible for the wasp attraction. Behavioural experiments with synthetic mixtures in a Y tube showed that the identified GLVs alone are highly attractive to wasps, but in combination with the other biological active compounds of the flower odour the synthetic mixture lose its attractiveness for wasps. One possible explanation for this result could be that one or several floral compounds mask the attractiveness of the wasp attractive GLVs. Furthermore we performed sugar analysis of S. umbrosa flowers, which proved the statement that the nectar of wasp-flower is sucrose dominated (Baker and Baker 1983).

The results of the study showed that the non-orchid S. umbrosa use the same floral cues than the orchid E. helleborine to attract social wasps for pollination to their flowers, which leads us to the assumption that there is a convergent evolution of these pollinator attractive substances.
Summary Chapter 5

Multifaceted ways of attracting various pollinators in the orchid genus Epipactis

The intention of this study was to compare pollinator attracting floral cues in various species of *Epipactis* with different pollinators. Therefore we analyzed the floral odour, optical cues and the nectar sugar composition of *E. helleborine*, *E. purpurata*, *E. atrorubens*, *E. palustris*, and *E. muellerie*.

The orchid genus *Epipactis* is represented by about 18 species and 20 subspecies in Europe (Baumann 2005), which are known to be pollinated by various insects. While some have a broad visitor spectrum, others are rather specialised. *Epipactis helleborine* (L.) Crantz is the most common and widely distributed species of the genus (Wiefelspütz 1970), and is a prime example for wasp-flowers, as well as *E. purpurata*; both are mainly pollinated by social wasps (Hymenoptera: Vespidae) (Müller 1873). Darwin (1888) already noticed that *E. helleborine* is almost exclusively ignored by bees and bumblebees, a mystery that was recently disclosed (Brodmann et al. 2008). Besides the wasp-pollinated *Epipactis* species there are other species, like *E. atrorubens*, that is mainly pollinated by bumblebees, or *E. palustris*, which exhibits a broad spectrum of pollinators, amongst them honeybees (Vöth 1999) and solitary wasps (Nilsson 1978). *E. muellerie* is an autogamous species (Baumann 2005). Because of the choice of different pollinators within the genus *Epipactis*, we assumed that the species occupied different niches to avoid competition. Furthermore we hypothesized that as an adaptation to different pollinator groups different species of *Epipactis* should use diverse floral cues. Because floral size, shape, colour and fragrance are all means by which plants advertise to foraging pollinators (Heinrich and Raven 1972; Waser 1983), we compared various *Epipactis* species concerning their fragrance, optical cues, and nectar sugar composition. We wanted to answer the questions, which floral cues are responsible for pollinator attraction of the various *Epipactis* species, and if there is a difference in scent production of allogamous and autogamous species.

By using a combination of chemical (GC, GC-MS) and electrophysiological (GC-EAD) analyses, we found differences in the floral scent of the investigated species. We could show that all wasp-pollinated *Epipactis* species exploit prey mimicry and produce specific odour compounds, so called “green leaf volatiles” (GLVs). Behavioural experiments demonstrated that the floral odour plays the key role in pollinator attraction, while the cryptic coloration is assumed to reduce the attraction of other potential flower visitors. Whereas in the mainly bumblebee-pollinated *E. atrorubens* colour in combination with an intensive vanilla scent (Delforge 1994) are responsible for pollinator attraction (Appel 2008). In contrast to the typical odour of the wasp-pollinated species the compounds of the scent of *E. atrorubens* are very common and well known in flowering plants (Knudsen et al. 2006). Therefore the visitor spectrum is broader than in the wasp-pollinated species. The nearly scentless *E. palustris* lures their pollinator with the help of UV trades on the flowers and in a recent investigation it was shown that it co-occurs with other species which emit high amounts of floral volatiles that do have a function as magnet species and attract pollinators of *E. palustris* from a distance (Schuffenhauer 2009). Because it is not necessary to allure pollinators using flower
cues, the autogamous specie *E. muellerie* neither have a strong scent production, nor a flashy colouration, so the orchid reduce cost. But besides this factors, the choice of the habitat surely also plays a role in pollinator attraction. The two wasp-pollinated species grow in shadow habitats in the edge of forests, where the occurrence of bees is rare. Whereas the bee and bumblebee-pollinated species prefer open, sunny habitats. The autogamous species *E. muellerie* grow in the middle of forests, where not so many insects appear. This is a fascinating example, how orchids species of the same genus evolved different strategies, like production of specific odour compounds, optical cues, or nectar sugar composition, to ensure their reproductive success.

**Summary Chapter 6**

*The role of innate and learnt floral odour preferences in the pollinator attraction of the orchid Epipactis helleborine*

Since Darwin made the first published observations of learning in honeybees, they became a model organism for studies on the neural substrates of learning and memory (Menzel and Mueller 1996). Although plant-insect interaction is from high interest, and floral volatiles play a major role in such a communication, comparatively few is known about which compounds of a complex flower odour bouquet are recognized by innate pollinators and which ones are learned while visiting a flower.

In the wasp-flower *E. helleborine* (Orchidaceae) it is known, that the floral odour has the primacy function in the wasp attraction (Brodmann et al. 2008). The floral odour compounds that are responsible for this attraction are so called “green leaf volatiles” (GLVs). GLVs are released by green plant tissue infested with herbivores, and parasitic (Whitman and Eller 1990), as well as social wasps (Brodmann et al. 2008) are highly attracted to this compounds and exploit them to locate their prey. Because GLVs are compounds that are very common and widespread in nature, we hypnotized that, besides the GLVs, the orchid should also produce more specific odour compounds that could be learned while the first flower visit, to ensure that the wasp would visit other flowers of the same species.

Among the floral volatiles that were found to be electrophysiologically active in wasp pollinators of the orchid *E. helleborine*, there were unspecific GLVs in headspace samples (Brodmann et al. 2008), and more specific less volatile compounds that were found in solvent extracts of flowers (Hölzler 2003). The later ones were identified as heptanal, pentadecanal, hexadecanal, hexacecan-1-ol, and vanillin. Using a dual-choice wind tunnel test we studied the preferences for synthetic mixtures of these compounds in naive and experienced wasps after conditioning for 48 h.

The results showed that naive wasps are attracted by GLVs. In parasitic wasps it could already be shown, that naive wasps oriented to certain GLVs the first time they encountered the odours (Whitman and Eller 1990). These results imply that wasps have a highly refined, innate, and genetically based proclivity to respond to these substances, and suggests a long evolutionary association between wasps and GLVs. This and my results showed that wasps
are extremely sensitive to GLVs. In contrast to the GLVs, the more specific floral compounds found in the solvent extract became more attractive after learning the compounds with a sugar reward. These compounds are probably learned while drinking nectar at the first flower contact on the orchid *E. helleborine*. Learning flower odours while visiting a flower is a very common phenomenon. In honeybees is known that associative learning becomes an essential component of the foraging behaviour (Gould 1993; Menzel 1985).

When a wasp smells the GLVs of an *E. helleborine* flower, it assumes to find prey for its brood. But instead of an insects prey the wasp finds nectar that it also need as a carbon hydrate source for themselves. While drinking the first time nectar, it may learn the more specific compounds of the flowers and associate them with a nectar reward. The relatively high amount of amino acids found in nectar of wasp flowers (Baker and Baker 1986) may further increase the number of visiting, experienced wasps. The combination of the more unspecific GLVs and more specific compounds learned by experience, guarantees a successful reproduction in *E. helleborine* flowers, because the wasps surely will visit other flowers of the orchid and thereby ensure a successful reproduction.

**Discussion**

**Pollinator attracting floral signals in orchid wasp-flowers**

Already the famous naturalist Charles Darwin (1888) noticed that the flowers of certain plant species are exclusively visited by social wasps, and are almost entirely overlooked by other potential pollinators, despite a large nectar reward. But until now the mechanism for the attraction of pollinating social wasps was something of a mystery. In my thesis I wanted to clarify the question, how wasp-flowers exclusively attract social wasps to their flowers. My investigations on different wasp-flowers exposed the primacy of floral scent in wasp attraction (Brodmann et al. 2008; Brodmann et al. 2009) and is confirmed by a study of a Chinese research group working on the orchid *Coelogyne fimbriata*, which is exclusively pollinated by female *Vespula* wasps (Cheng et al. 2009).

In the wasp-flowers *E. helleborine* and *E. purpurata* we identified “green leaf volatiles” (GLVs), which are emitted by the flowers, responsible for the specific wasp attraction. GLVs, mostly six-carbon aldehydes, alcohols and acetates and other volatile organic compounds (VOCs), are emitted by many plants infested by herbivores, e.g. caterpillars (Whitman and Eller 1990). We suspected that the wasp-flowers produce GLVs in order to attract prey-hunting social wasps for pollination (Brodmann et al. 2008). This was the first example in which GLVs have been implicated in chemical mimicry for the attraction of pollinating insects.

In the deceptive orchid *D. sinense* we could show that the alcohol Z-11-eicosen-1-ol, a very rare chemical compound, never identified in plants before, is responsible for the pollinator attraction (Brodmann et al. 2009). This compound is known as a compound of the alarm pheromone of honeybees (Pickett et al. 1982; Schmidt et al. 1997), a common prey item of the pollinator. We could show that the orchid produces this compound to attract prey-hunting wasps for pollination (Brodmann et al. 2009).
In the deceptive orchid *S. satyrioides* we revealed that the flowers of the orchid produce, amongst other compounds, acetophenone and 2-ethylhexan-1-ol, volatiles that the pollinator can smell and which are also produced in the venom gland of several species of social wasps with a function as an alarm pheromone (Fortunato et al. 2004; Ono 2005). In behavioural experiments we could show that the orchid mimics the alarm pheromone compounds in order to attract alerted wasps for pollination.

In summary in all investigated wasp-flowers we found chemical mimicry responsible for the wasp attraction. In all cases the flowers mimic prey of the wasps, and thereby exploit the unique selling point of wasps - the fact that they feed their larvae with meat, instead of pollen. But besides the olfactory cues, the optical floral cues also may play an important role in pollinator attraction. The flowers of *D. sinense*, with their red stripes on the white background, may mimic optically a honeybee sitting on a flower. Due to the fact that wasps are not able to see the colour red (Beier and Menzel 1972), in their eyes the red strip appears black, and so formed a strong contrast with the white back. The presence of contrast on flowers is known to be very important for pollinator attraction (Lunau 1992). Behavioural experiments on *D. sinense* revealed, that in contrast to natural flowers, modified flowers with covered strips are not attractive for the wasps (Song Xi-qiang, unpublished data), which leads to the suggestion that besides the olfactory cues also the optical cues are important for close range attraction to the wasps. Although our experiments clearly showed that in the orchid *S. satyrioides* optical cues alone are not attractive to the wasps, the influence of the optical cues may play a role to increase searching behaviour in the wasps and optical cues may also play a role in close range attraction. Already Nazarov (1995) hypothesized that the orchid would attract the wasps by mimicking optically a piece of meat. He described reddish-brown papillae on the base of the lip, at the spur entrance as a visual signal and suggested that the wasps may take them as food. Therefore he entitled the pollination mechanism as “false-prey” - syndrome. Despite in all investigated orchid wasp-flowers behavioural experiments revealed that the optical cues alone, without the scent, are not attractive for wasps, it is quite possible that the optical cues may enforce the attractiveness of the flowers.

**Pollinator attracting floral signals in non-orchid wasp-flowers**

To compare the attracting floral cues used in orchids and non-orchids we investigated the non-orchid *Scrophularia umbrosa*. Like in the wasp-pollinated *Epipactis* species we found GLVs in the floral scent of *S. umbrosa*. Behavioural experiments with synthetic mixtures showed that the floral GLVs identified in *S. umbrosa* tested alone are highly attractive to wasps, but in combination with the other biological active compounds of the flower odour the synthetic mixture lose its attractiveness to the wasps. Therefore we assumed that *S. umbrosa* emits one or several floral odour compounds which could mask the highly attractive GLVs in the floral scent of *S. umbrosa*, and thereby the floral odour of *S. umbrosa* lose its attractiveness to wasps. We hypothesized that *S. umbrosa* assumes a trade-off, to attract, besides wasps, also other pollinator species, and thus ensure its reproductive success. But in contrast to the orchid *E. helleborine* (Brodmann et al. 2008) behavioural experiments demonstrated that besides olfactory cues also optical cues are important for the wasp
attraction and consequently pollinator attraction. But the fact that the non-orchid *S. umbrosa* uses the same compounds, namely GLVs, than the orchid wasp-flowers of the *Epipactis* species leads us to the conclusion that there is a convergent evolution of these pollinator attractive substances.

In contrast to the investigated orchids, *S. umbrosa* is more generalized in pollinator attraction (Emer 2005), and hence *S. umbrosa* is a less specialized wasp-flower in comparison to *E. helleborine*. With their small bulbous red flowers and the highly accessible nectar both exhibit the typical characteristics of wasp-flowers (Müller 1881; Sebald et al. 1996). But in contrast to *E. helleborine*, *S. umbrosa* possesses some elementary differences in flower penology and habitat choice. While the flowering time of *E. helleborine* is exactly overlapping with the peak of the wasp occurrence in July, *S. umbrosa* is flowering earlier in the year, when the abundance of social wasps is still low. During this time of the year, other flower visitors, like bees and flies are active and do have a function as pollinators (Emer 2005).

Furthermore the choice of the habitat could play a role in flower visitor attraction. While *S. umbrosa* mostly grows on sunny exposed places, *E. helleborine* often prefers dark forest understory, where other flower visitors are rare. In contrast to the orchid *E. helleborine*, *S. umbrosa* exhibits UV reflection (Kugler 1963; Rosen and Barthlott 1991), and in contrast to the cryptic coloured *E. helleborine* uses optical cues in addition to floral scent to attract pollinators. It is known that UV marks on the flowers are important for luring honeybees to the flowers (Vöth 1999). This could be a reason why *S. umbrosa* also lures other flower visitors and thus is not so specialized in wasp attraction. Furthermore it is assumed that nectar contents of *E. helleborine*, deter other flower visitors (Jakubska et al. 2005). Our results present further evidence that orchids are more specialized in pollinator attraction than non-orchids.

**The advantage to be a pollinator-specialized flower**

Because wasps do not feed their larvae with pollen, they just visit flowers for their own sugar requirements. For this reason wasps are not mention as an effective pollinator in literature. But the pollination of wasps possesses one essential advantage: wasps visit not so many plant species, because their morphology is not suitable for so many plants, e.g. because of their short tongues, they can only exploit exposed nectaries. For orchids this is an essential benefit.

A characteristic for orchids is that they store all their pollen in pollen packets, so called “pollinaria” (Dafni 1984; Jersáková et al. 2006) that can be removed by pollinators only once. For this reason it is very important for orchids to have a specific pollinator, who carries the pollen to another plant of the same species. Therefore for orchids a specialization of a specific pollinator is reasonable and very common in orchids. In case of rewarding flowers usually many different pollinators can be expected and loss of pollen is usually higher than in deceptive species. In sexually deceptive orchids there is often a one to one Ophrys - pollinator species relationship and sympatric orchid species are reproductively isolated via the exploitation of different pollinators (Stökl et al. 2005; Stökl et al. 2008). While mimicking
the specific sex pheromones of the own pollinator species, the orchid attracts only one pollinator species (Schiestl 2005).

**Why are wasp-flowers not visited by other pollinators?**

Despite the large nectar contents in many wasp-flowers, they are often rather specific and do not attract other insect visitors, which would exploit the nectar without pollinating. The wasp-specific odour of wasp-flowers is surely the main reason of this phenomenon. We found compounds in the flower odour responsible for the wasps attraction, which are very rare and some have never been found in flower fragrance before. For example the alcohol Z-11-eicosenol, found in the orchid *D. sinense* (Brodmann et al. 2009), was so far not described in any plant species. The GLVs found in *E. helleborine* and *E. purpurata* (Brodmann et al. 2008), and *S. umbrosa* are usually produced in green plant tissue and not by flowers. Because all this odour compounds play a specific role in the life of wasps, by mimicking their prey, the flowers exclusively attract wasps for pollination. Hence wasp-flowers reduce the attractiveness for other flower visitors (deterring nectar thieves) and avoid thus a loss of pollen. Furthermore, it was shown that wasps-flowers have a dull coloration (Johnson 2005) and use the offered nectar as a visitor filter, while offering distasteful nectar (Adler 2000; Johnson et al. 2006; Shuttleworth and Johnson 2009; Shuttleworth and Johnson 2006; Stephenson 1981; Stephenson 1982), or exhibit a specific sugar concentration, not acceptable for other flower visitors (Baker 1975; Waller 1972). The orchid wasps-flowers investigated in my study, even offer no nectar reward, like *D. sinense* and *S. satyrioides*, or in the case of *E. helleborine* and *E. purpurata* exhibit a dull coloration to reduce the attractiveness for other insects, which would exploit the offered nectar. Baker & Baker (1983) found wasp-pollinated species to be rather rich in sucrose, whereas many flowers pollinated by bees, butterflies, and other insects produce higher amounts of glucose. My investigations showed that the nectar of the wasp-pollinated *Epipactis* species is sucrose dominated.

In the wasp-pollinated *Epipactis* species also the habitat specificity may play a role in reducing other visitors than wasps. The wasp-pollinated *Epipactis* species, *E. helleborine* and *E. purpurata*, often grow in dark forest understory where other insect pollinators like honeybees, solitary bees, butterflies, etc. are rare or absent (Van der Cingel 1995). In *Epipactis* species pollinated by social wasps probably several floral traits, like the production of GLVs in combination with the dull coloration and in addition habitat selection result in the highly specific attraction of social wasps.

**Different strategies in rewarding and deceptive species to attract wasps for pollination**

In all my investigated wasp-flowers the plants exploit the fact that wasps feed their larvae with meat, and thereby have to search for prey items. We found that the deceptive wasp-flowers use a more specific kind of prey mimicry, by producing compounds, which all play an important role in the life of wasps, than the ones which offer nectar to the wasps. The rewarding *E. helleborine* and *S. umbrosa* emit GLVs, which mimics prey of the wasps. But the identified GLVs are very common in green plant tissue, and are therefore not an indicator for a specific prey species. My investigations also could show that these GLVs release searching behaviour in naive wasps. After reaching the flower the wasps get a nectar reward
and will visit other flowers of the same species, and thereby ensures the reproductive success of the orchid.

In contrast, the deceptive orchids *D. sinense* and *S. satyrioides*, emit more specific floral compounds and mimic semiochemicals that may release innate behaviour in the pollinators. *Dendrobium sinense* emit specific prey substances, the scent of their common prey, honeybees. *Steveniella satyrioides* mimics compounds of the alarm pheromone of the pollinator itself, to attract alerted wasps for pollination. With the mimicry of these specific compounds, which play all an essential role in the life of a wasps, the orchids are able exclusively luring wasps to there flowers. With this wasp specific attractant wasp-flowers get very specialized pollinators, which guarantee an optimal pollination.

In the rewarding *E. helleborine* it could be shown that learning of floral odour is involved in pollinator attraction. The orchid exploits the naive reaction of the wasps to GLVs to attract them to the flowers. During this first visit of a flower the wasps are capable to learn other, more specific floral cues, in association with the offered nectar. This enforced the urge of the wasps to visit more flowers of the orchid, and thereby ensure a successful reproduction. The deceptive species do not apply the act of learning in the wasps. With their specific odour compounds they release an instinctual behavioural pattern, like a specific prey, or alarming behaviour in the wasps, which allows them to disclaim an reward, and anyway ensure an successful reproduction. Because the fouled wasps are frequently confronted with honeybees and aggressive behaviour, they will visit the orchid flowers over and over again.

**Conclusion**

The results of my thesis represent fascinating examples of chemical mimicry in the pollination system of orchids. Our results clearly show that prey mimicry is a characteristic of wasp-flowers to attract their pollinator. In all of the investigated orchids the floral scent plays the key role in pollinator attraction. All of the involved scent compounds play an important role in the life of wasps, helping them in the location of prey. Furthermore I revealed that the investigated wasp-flowers use specific odour compounds, like GLVs, compounds emitted by infested green plants, Z-11-eicosen-ol, a compound of the alarm pheromone of honeybees, or the compounds acetophenone and 2-ethylhexan-1-ol, also identified in the alarm pheromone of several social wasp species, to attract the wasps to their flowers. All this compounds are seldom or even never found in flower fragrance before. With these results I can declare that prey mimicry is a characteristic of wasp-flowers to attract their pollinator, and therewith we get a big step to discover the undisclosed syndrome of wasp-pollinated orchids. Furthermore I revealed that there is a convergent evolution of the pollinator attracting GLVs in orchids and non-orchids.

An exception is the non-orchid *S. umbrosa*, where floral scent alone is less attractive to wasps and visual cues contribute more to pollinator attraction. In contrast to the other investigated species *S. umbrosa* is less specialized in wasp attraction, because the flowers are also visited and pollinated by other insects (Emer 2005). In all of the investigated species the floral scent involved plays an important role in the life of wasp, and is essential in the location and finding of prey.
A difference between nectar rewarding and non-rewarding orchids is in the compounds involved in pollinator attraction. While rewarding species use common occurring GLVs in combination with a nectar reward, deceptive species use more specific cues that are involved in the communication system of wasps in the case of S. satyrioides or in prey location like in D. sinense. Nectar reward is not necessary in the deceptive systems since there is no problem of habituation. Females of Vespa bicolor will be more often rewarded while interacting with prey items (honeybees) than they will visit cheating orchids and there is also no risk of habituation in the wasps that pollinate Steveniella, because S. satyrioides is a rare occurring species and foraging wasps are probably regularly reinforced to react on their alarm pheromone compounds if a colony is attacked by enemies or while wasps mark larger prey items, and this compounds are likely to release an innate behaviour in wasps.

Apart from the fundamental research these wasp-specific substances could found appliance in developing an environmentally responsible system for trapping pest wasps. In the time of my thesis we already tested the floral odour compounds found in E. helleborine in specific wasp traps in field experiments in cooperation with Sterling International, USA. The aim of this traps is to reduce wasp abundance in places where many people occur, for example in a nursery.

References

Baker HG (1975) Sugar concentrations in nectars from hummingbird flowers. Biotropica 7:37-41
Baumann H (2005) Die Orchideen Deutschlands. Arbeitskreise Heimische Orchideen (Bad Hersfeld, Germany: Hoehl-Druck)
Darwin CR (1862) The various contrivances by which orchids are fertilised by insects. (London: John Murray)
Darwin CR (1888) The various contrivances by which orchids are fertilised by insects, 2nd edition (London: John Murray)
del Rio C (1990) Sugar preferences in hummingbirds: the influence of subtle chemical differences on food choice. Condor 92:1022-1030


Robertson C (1928) Flowers and insects. Lists of visitors to four hundred and fifty-three flowers. C. Robertson, Carlinville, IL-221


Sprengel CK (1793) Das entdeckte Geheimnis der Natur im Bau und in der Befruchtung der Blumen. Berlin


Stephenson AG (1982) Iridoid glycosides in the nectar of Catalpa speciosa are unpalatable to nectar thieves. Journal of Chemical Ecology 8:1025-1034

Stiefelhagen H (1910) Systematische und pflanzengeographische Studien zur Kenntnis der Gattung Scrophularia. Botanische Jahrbücher 44: 406-496


Veith HJ, Koeniger N, Maschwitz U (1984) 2-Methyl-3-buten-2-ol, a major component of the alarm pheromone of the hornet Vespa crabro. Naturwissenschaften 71


Werth E (1956) Bau und Leben der Blumen. Ferdinand Enke Verlag, Stuttgart


Zusammenfassung

Bestäuberanlockung bei Wespenblumen

Einleitung


Um ihren Bestäuber an die Blüten zu locken, setzen Pflanzen diverse Blütensignale, wie Blütenfarbe und Form (Vogel 1993), sowie Blütenduft (Knudsen et al. 2006) ein. Es konnte beispielsweise gezeigt werden, dass Honigbienen zwischen Blütenattrappen unterschiedlicher Farbe unterscheiden können (Hempel de Ibarra et al. 2002), oder dass sich Hummeln bei der Landung auf einer Blüte an farbigen Saftmalen auf der Blüte orientieren.
Zusammenfassung


**Thema der Doktorarbeit**

Zusammenfassung

unsuchen, ob sich Unterschiede in den Blütenmerkmalen zu wespenbestäubter Orchideen finden lassen. Da Orchideen durch ihre Pollenpakete all ihren Pollen an einen einzigen Bestäuber verlieren, ging ich davon aus, dass Orchideen spezialisierter in ihrer Bestäuberwahl sein sollten als nicht-Orchideen.

Folgende Fragen sollten beantwortet werden:

1. Welche Blütensignale sind für die Wespenattraktivität von Wespenblumen verantwortlich?
2. Benutzen Orchideen und nicht-Orchideen dieselben Blütensignale, um Wespen anzulocken?
3. Benutzen belohnende und täuschende Arten dieselben Blütensignale, um Wespen anzulocken?

Untersuchte Arten

Für meine Untersuchungen arbeitete ich mit den Orchideen der Gattung Epipactis (Orchidaceae, Unterfamilie: Epidendroideae) in Deutschland, Dendrobium sinense (Orchidaceae, Unterfamilie: Epidendroideae) in China und Steveniella satyrioides (Orchidaceae, Unterfamilie: Orchidoiceae) in der Türkei. Als nicht-Orchidee wurden Pflanzen der Gattung Scrophularia (Scrophulariaceae) in Deutschland in die Untersuchungen miteinbezogen.


Die Orchidee Steveniella satyrioides (Stev.) ist derzeit die einzig beschriebene Art dieser Gattung, und das natürliche Vorkommen erstreckt sich über Anatolien, Nordiran, Kaukasus


**Verwendete Methoden**

Um die Attraktivität der Blütensignale von Wespenblumen auf soziale Wespen zu testen, wurden Verhaltensexperimente kombiniert mit chemischen (GC: Gaschromatographie, GC-MS: Gaschromatographie gekoppelt mit Massenspektrometrie, HPLC: Hochleistungsflüssigkeitschromatographie) und elektrophysiologischen (GC-EAD: Gaschromatographie gekoppelt mit Elektroantennographie) Methoden verwendet.

In Verhaltensexperimenten wurde die Attraktivität von optischen und olfaktorischen (Duft) Blütensignalen untersucht, welche Blütensignale eine Schlüsselrolle bei der Wespenaktivität spielen. Um die Attraktivität von optischen und olfaktorischen Blütensignalen gegeneinander zu testen, wurden Experimente mit einem modifizierbarem Quarzglassylinder durchgeführt. Dies ermöglicht es, die optischen und olfaktorischen Blütensignale jeweils alleine, oder in Kombination zu testen. Um die olfaktorischen Reize weiter zu testen, wurden Olfaktometertests in einem Y-Rohr, sowie Experimente im Windtunnel und Flugzelt durchgeführt.

Eine Kombination aus chemischen (GC, GC-MS) und elektrophysiologischen (GC-EAD) Analysemethoden ermöglichte es die Komponenten im Blütenduft zu identifizieren, die von den Wespen wahrgenommen werden können. Die Attraktanz dieser Substanzen auf die Wespen wurde im anschließenden Verhaltensexperiment mit synthetischen Substanzen getestet.

Ergebnisse & Diskussion

Bestäuberanlockende Blütensignale von Wespenblumen - Orchideen


Bei der täuschen Orchidee S. satyrioides produzieren die Blüten der Orchidee unter anderem die Substanzen Acetophenon und 2-Ethylhexan-1-ol, beides Substanzen, die die bestäubenden Wespen riechen können. Diese Substanzen wurden ebenfalls in der Giftdrüse mehrerer Wespenarten identifiziert, mit der Funktion eines Alarmpheroms (Fortunato et al. 2004; Ono 2005). In Verhaltenstests konnte gezeigt werden, dass die Orchidee diese Alarmpheromonkomponenten produziert, um alarmierte Wespen zur Bestäubung anzulocken, und damit das soziale Kommunikationssystem seines Bestäubers ausnutzt, um die Bestäubung zu sichern.

Zusammenfassend kann man sagen, dass bei allen von uns untersuchten Wespenblumen chemische Mimikry in die Wespenanlockung involviert ist. In allen Fällen ahmen die Blüten Beute der Wespen nach, und nutzen dadurch das Alleinstellungsmerkmal der Wespen - die Tatsache, dass diese ihre Brut mit fleischlicher Nahrung füttern, anstatt mit Pollen - aus.

**Bestäuberanlockende Blütensignale von Wespenblumen der Gattung Scrophularia**

Um die bestäuberanlockenden Blütensignale von Orchideen und nicht-Orchideen zu vergleichen haben wir die nicht-Orchidee *Scrophularia umbrosa* in unsere Untersuchungen miteinbezogen. Wie in den wespenbestäubten *Epicatis* Arten fanden wir neben anderen Substanzen GLVs im Blütenduft von *S. umbrosa*. Die im Blütenduft identifizierten GLVs waren im Biotest für die Wespen hoch attraktiv, verloren aber in Kombination mit den anderen elektrophysiologisch aktiven Verbindungen des Blütenduftes ihre Attraktivität für die Wespen. Ich vermute daher, dass eine oder mehrere Substanzen im Blütenduft die Attraktivität der GLVs maskieren könnten, wodurch die Attraktivität des Blütendufts für die Wespen abnimmt. Da die Blühzeit von *S. umbrosa* nicht mit dem Wespenpeak korreliert, ist *S. umbrosa* neben Wespen auch auf andere Insektenarten als Bestäuber angewiesen. Dies könnte erklären, dass *S. umbrosa* neben den für Wespen hoch attraktiven GLVs ebenfalls andere Blütenduftstoffe produziert, die jedoch die Attraktivität der GLVs herabsetzen könnte. Man könnte vermuten, dass *S. umbrosa* ein Trade-off eingeht, um neben Wespen auch andere Insekten zur Bestäubung anzulocken, und dadurch ihre Reproduktion zu sichern. Im Gegensatz zu der Orchidee *E. helleborine* zeigten Verhaltensexperimente, dass neben dem Blütenduft auch die optischen Merkmale der Blüte eine Rolle zur Bestäuberanlockung spielen. Die Tatsache, dass die nicht-Orchidee *S. umbrosa* dieselben Komponenten, nämlich GLVs, wie die wespenbestäubten Arten der Orchideengattung *Epipactis* verwendet, lässt
vermuten dass der Gebrauch dieser Substanzen für die Wespenanlockung konvergent entstanden sein muss.


**Die Vorteile einer Wespenblume**

Zusammenfassung

Warum Wespenblumen von anderen potenziellen Blütenbesuchern ignoriert werden


Unterschiedliche Strategien bei belohnenden und täuschenden Arten zur Wespenanlockung

weitere Blüten derselben Art zu besuchen, und sichert somit den Fortpflanzungserfolg der Orchidee.

Im Gegensatz dazu produzieren die täuschenden Orchideen *D. sinense* und *S. satyrioides* sehr spezifische Blütenduftstoffe. *Dendrobium sinense* produziert Alarmpheromonkomponenten von Honigbienen, der Beute ihres Bestäubers, nämlich Hornissen. *S. satyrioides* produziert Komponenten im Alarmpheromon von Wespen, um alarmierte Wespen zur Bestäubung anzulocken. Die Mimikry all dieser spezifischer Substanzen, die alle eine wichtige Rolle im Leben von Wespen spielen, ermöglicht es der Pflanze lediglich Wespen, ihre optimalen Bestäuber, an ihre Blüten zu locken. Dieser wespenspezifische Blütenduft ermöglicht der Pflanze eine sehr spezifische Bestäuberwahl, was eine optimale Bestäubung garantiert.

Im Vergleich zwischen belohnenden und täuschenden Arten konnte gezeigt werden, dass täuschende Arten eine noch spezifischere chemische Mimikry betreiben als belohnende Arten. Die täuschenden Arten haben keine Notwendigkeit auf Lernen zu setzen, weil ihre spezifischen Beutesignale ein instinktives Verhalten in der Wespe auslösen. Dadurch, dass die Wespe viel häufiger mit dem Modell Honigbiene oder dem Alarmpheromon der eigenen Arte ausgesetzt ist als dem Mimet, der Blüte, wird sie trotz einer Enttäuschung an der Blüte immer weitere Blüten besuchen.

**Schlussfolgerung**


Chemical mimicry in wasp-pollinated orchids

1.

Orchid mimics green leaf volatiles to attract prey-hunting wasps for pollination

Jennifer Brodmann¹, Robert Twele², Wittko Francke², Gerald Hölzler³, Qing-He Zhang⁴, Manfred Ayasse¹

¹Institute of Experimental Ecology, University of Ulm, 89069 Ulm, Germany
²Institute of Organic Chemistry, University of Hamburg, 20146 Hamburg, Germany
³Department of Evolutionary Biology, University of Vienna, 1090 Vienna, Austria
⁴Sterling International, Spokane, WA 99216-1616, USA

Published in Current Biology 18, 1-5. 2008.
Summary

An outstanding feature of orchids is the diversity of their pollination systems [1]. Most remarkable are those species that employ chemical deceit for the attraction of pollinators [2]. The orchid Epipactis helleborine is a typical “wasp-flower”, exhibiting physiological and morphological adaptations for the attraction of pollinating social wasps [3]. As noted by Darwin [1], this species is almost entirely overlooked by other potential pollinators, despite a large nectar reward. Therefore, the mechanism for the attraction of pollinating social wasps was something of a mystery. Using a combination of behavioral experiments, electrophysiological investigations, and chemical analyses we demonstrate for the first time that the flowers of E. helleborine and E. purpurata emit “green leaf volatiles” (GLVs), which are attractive to foragers of the social wasps Vespula germanica and V. vulgaris. GLVs, emitted by damaged plant tissues, are known to guide parasitic wasps to their hosts [4]. Several E. helleborine GLVs, that induced response in the antennae of wasps, were also emitted by cabbage leaves infested with caterpillars (Pieris brassicae), which are common prey items for wasps [5]. This is the first example in which GLVs have been implicated in chemical mimicry for the attraction of pollinating insects.

Results and Discussion

Visual versus olfactory cues for wasp attraction

The orchid Epipactis helleborine (L.) Crantz is a prime example of a ‘wasp-flower’; it is mainly pollinated by social wasps (Hymenoptera: Vespidae) like Vespula vulgaris and V. germanica [3]. Wasp-flowers exhibit physiological and morphological adaptations for the attraction of pollinating social wasps. Although wasp-pollinated flowers have been the subject of a number of studies [3, 6-9] little is known about the floral signals that are responsible for the highly specific attraction of wasps.

Social wasps feed their larvae on insects like caterpillars [5], amongst them Pieris rapae [10]. In order to locate their prey they use a combination of visual and olfactory cues [11]. Parasitic wasps use volatiles emitted by plants to locate insect prey [4, 12]. Social wasps may do likewise. Indeed, the ability of plants to induce resistance in response to herbivory has been reported for many species [13], and plants may even produce carnivore-attracting volatiles [4].

To investigate the relative importance of floral signals to foraging wasps, we compared the attractiveness of whole inflorescences, inflorescences covered with a quartz glass cylinder (visual cues), and natural scent of E. helleborine (olfactory cues) which were offered to workers of V. germanica. The results of our field bioassays showed that olfactory cues were significantly more attractive to wasps than visual cues (Mann-Whitney U test, U = 5.5, p < 0.001) and released the same number of approaches in the wasps as the whole inflorescences (Mann-Whitney U test, U = 65.5, p = 0.7, whole inflorescence: mean ± SD:
4.83 ± 1.69, n = 12, scent: 5.41 ± 2.02, n = 12, visual cues: 1.8 ± 1.13, n = 10). *E. helleborine* grows in shaded areas, often in dark coniferous forests with a shortage of pollinators [14, 15]. Foraging wasps are obviously attracted from a distance by the flower's fragrance. This is evident by the optomotor anemotaxis mediated searching behavior of *V. germanica* workers who approach the flowers in a characteristic zigzag flight. The results of our behavioral experiments support the primacy of olfactory cues in the long distance attraction of wasps. The floral scent offered without visual cues clearly attracted the wasps to visit flowers.

**Do *Epipactis* flowers release green leaf volatiles?**

“Green leaf volatiles” (GLVs), mostly six-carbon aldehydes, alcohols and acetates, are emitted by many plants infested by herbivores, e.g. caterpillars [16]. GLVs may attract predators or parasitoids of herbivorous insects [4, 17-19], and we suspected that *E. helleborine* flowers may produce GLVs in order to attract prey-hunting social wasps for pollination.

Gas chromatography coupled with an electroantennographic detector (GC-EAD) was used to identify those compounds in the complex flower scent perceived by the antennae of worker wasps, a technique we have found to be an effective method to identify volatile pollinator attractants in *Ophrys* flowers [2, 20]. In headspace samples collected from *E. helleborine* flowers we found seven compounds inducing an electrophysiological response in antennae of workers of *V. germanica* and of *V. vulgaris* (Figure 1). Using gas chromatography coupled with mass spectrometry (GC-MS) we identified the aldehydes octanal, nonanal and decanal, as well as benzaldehyde and the GLVs hexyl acetate, Z-3-hexenyl acetate and Z-3-hexen-1-ol in inflorescences.

In parallel investigations we found the same compounds to be present when caterpillars of *Pieris brassicae* infest cabbage (*Brassica oleracea gemifera*), a plant that is known to release GLVs to herbivore attack [21-23]. The total amount of emitted volatiles was significantly higher in infested cabbage (mean 1.7 µg ± 0.17 SE / cabbage) than in *E. helleborine* (mean 0.4 µg ± 0.08 SE / inflorescence) (Mann-Whitney U test, U = 5.0, p = 0.013, n = 14), and the relative proportions of volatiles differed too (Figure 1). However, several studies have shown that plant species emit different patterns of volatiles when attacked by herbivorous insects, and different species of *Pieris* caterpillars induce varying amounts of emitted volatiles [21-23]. Therefore, differences in the scents of cabbage infested with *P. brassicae* caterpillars and of *E. helleborine* flowers were not surprising. Since prey hunting wasps search for insects that feed on many different plant species that produce different bouquets of volatiles, we expected the wasps to react instantly to certain key compounds, even if quantitative volatile compositions were not identical. Our finding that octanal, hexyl acetate, Z-3-hexenyl acetate, and Z-3-hexen-1-ol were produced in higher amounts in cabbage plants damaged by *Pieris* caterpillars [21] is consistent with our hypothesis that *E. helleborine* flowers produce GLVs in order to attract prey-hunting social wasps for pollination.

In behavioral experiments we tested the attractiveness of various odors to *V. vulgaris* and *V. germanica* workers. In a Y-tube olfactometer the wasps significantly preferred the odor of *Pieris* infested cabbage compared to the empty control (Sign test, p < 0.001, n = 24) and
compared to uninfested cabbage (Sign test, p < 0.001, n = 43) (Figure 2), indicating that insect hunting wasps find their prey using GLVs. In further tests we could show that floral volatiles emitted by *E. helleborine* flowers (Sign test, p < 0.001, n = 34), as well as a synthetic mixture of all EAD active compounds identified in *E. helleborine* (Sign test, p < 0.01, n = 24), and a mixture consisting of the three GLVs hexyl acetate, Z-3-hexenyl acetate, and Z-3-hexen-1-ol (Sign test, p < 0.02, n = 28), which are produced by *E. helleborine* flowers, were significantly more attractive than the empty control. In addition, the attractiveness of a synthetic mixture consisting of all of the electrophysiologically active *E. helleborine*-compounds that were found to co-occur in damaged cabbage was confirmed as attractive by a choice experiment in a flight cage in the field (Sign test, p = 0.01, n = 170). Over 60% of the foraging wasps selected the flowers impregnated with a synthetic blend of *Epipactis* volatiles.

In former investigations it was shown that hunting wasps can use several different kinds of cues to find their prey, including frass odors [24]. Our results show for the first time that prey hunting foragers of social wasps use GLVs to find herbivorous insects. Until now this was only known in parasitic wasps [4]. We do not exclude the possibility that visual cues have an additional function, e.g. in close-range orientation. Like other wasp-flowers, *E. helleborine* is characterized by a dull coloration which may play an additional role in the attraction of wasps and which may even prevent visual attraction of insects that are morphologically unsuitable as vectors for the pollinaria. Some of the compounds that are particularly attractive to foraging wasps may even repel other potential visitors [9]. It is likely that learning of the odor and association with visual cues of prey may optimize foraging activities of the wasps [25, 26].

**Figure 1.** Electrophysiologically active compounds.
Simultaneous recordings of GC (FID) and EAD signals obtained with headspace samples of *E. helleborine* flowers (above) and *Pieris* infested cabbage (below) by using the antenna of a *V. vulgaris* worker. The GC analyses were performed on a polar DB-Wax capillary column. Hexyl acetate and octanal could not be separated with the GC parameters used. We found seven EAD-active compounds common to both plants that are present in quantitatively different compositions.
The importance of GLVs for wasp attraction

Within the genus *Epipactis* certain species are pollinated by social wasps while others attract bees [27]. We expected the GLVs found in *E. helleborine* to also occur in other wasp-pollinated species of *Epipactis*. Therefore, we looked for the presence of the GLVs hexyl acetate, Z-3-hexenyl acetate and Z-3-hexen-1-ol in two wasp-pollinated species of *Epipactis*: *E. helleborine* and *E. purpurata*, and in *E. atrorubens*, a species that is visited by a broad spectrum of pollinators, mainly bumblebees [27]. Our results clearly show that both wasp-pollinated species produce significantly higher amounts of GLVs than *E. atrorubens* (Figure 3). In a comparative olfactometer test the wasp-flower *E. helleborine* was significantly more attractive to wasps than the bumblebee pollinated species *E. atrorubens* (Sign test, p = 0.05, n = 28) (Figure 2). The two wasp-pollinated species *E. helleborine* and *E. purpurata* emit significantly higher amounts of GLVs than *E. atrorubens*, and these GLVs definitely have a key function in wasp attraction.

The fact that other insect species rarely visit *E. helleborine* and *E. purpurata* may primarily be a consequence of the habitat specificity. These wasp-pollinated *Epipactis* species, *E. helleborine* and *E. purpurata*, grow in dark forest understory, where other insect pollinators like honey bees, solitary bees, butterflies, etc. are rare or absent [14]. In addition, quality and quantity of nectar of *E. helleborine* and other wasp-pollinated species could be different from that of flowers of species that are visited by other insect pollinators. Baker & Baker [28] found wasp pollinated species seem to be rather rich in sucrose, whereas many flowers pollinated by bees, butterflies, and other insects produce higher amounts of glucose. Whether nectar collected by *Vespula* females is inappropriate for honey bees or bumble bees or whether non-sugar components are present that repel other insects is unknown so far.

To test our prediction that the scent of *E. helleborine* flowers does not attract other potential pollinators like honeybees, we also performed electrophysiological investigations and behavioral experiments with workers of the honeybee *Apis mellifera*. We found that antennae of *A. mellifera* workers respond to the same compounds as *V. germanica* and *V. vulgaris* in the electrophysiological investigations but were neither attracted by the synthetic mixture of *E. helleborine* flowers nor by the mixture of the GLVs in the Y-tube experiment (Sign test, p > 0.5, n = 20).
Figure 2. Attraction of wasps to various odor samples.
Comparison of the attractiveness of the odor from *Pieris* infested cabbage, *E. helleborine* and *E. atrorubens* flowers, a synthetic mixture of GC-EAD active substances of *E. helleborine* and a synthetic mixture of GLVs to social wasps in a Y-tube olfactometer (Sign test * * ≤ 0.05, ** p ≤ 0.01).

Figure 3. Comparison of absolute amounts (mean ± 2 SE) of GLVs in *E. helleborine*, and *E. purpurata*, both wasp-pollinated, as well as in *E. atrorubens*, a species that is mainly visited by various bee species. Different letters indicate significant differences (Mann-Whitney U test + Benjamini-Hochberg correction [31], p < 0.05).

### Conclusion

The refinement of adaptations for insect pollination has led to a high morphological diversity within the Orchidaceae. There are approximately 10,000 pollinator-deception species, amongst them food deceptive orchids that mimic the floral structures of food providing species and that represent the most numerous group of cheaters [29]. The pollination system that we found in the GLV-producing *E. helleborine* has not been described so far and represents a new form of chemical mimicry. By constitutively emitting volatiles that are usually emitted transiently by wounded plants infested by herbivores and, thus, deceptively indicating the presence of prey, the flowers are capable of attracting their pollinators. After reaching a flower, wasps most likely associate the odor of the orchid with its nectar reward and visit further flowers of the same species, ensuring a highly specific and effective pollination system. This is the first time that GLVs have been found to be involved in chemical mimicry for the attraction of pollinating insects. We are presently investigating other wasp-pollinated species in order to see whether there are common chemical principles responsible for wasp attraction by plant volatiles.
Experimental Procedures

Volatile collection
Floral scent emitted from *E. helleborine* flowers and Brussels sprouts (*Brassica oleracea gemifera* cv. Titurel) infested by *Pieris brassicae* (Lepidoptera: Pieridae) caterpillars was collected using dynamic headspace adsorption techniques. Intact inflorescences and infested cabbage plants were carefully enclosed in polyester oven bags (Toppits®, Germany), and volatiles were trapped in an adsorbent tube containing a thin layer of activated charcoal (CLSA, 1.5 mg, Gränicher & Quartero) or 5 mg Super Q (Waters Devision of Millipore) by using a membrane pump, adjusted to a flow rate of 500 ml / min for ca. 9 h. The inflowing air stream was cleaned of atmospheric pollutants by a charcoal filter (activated charcoal, Superco, Orbo 32 large). The trapped volatiles in the adsorbent tube were eluted with 40 µl dichloromethane (Sigma-Aldrich, HPLC grade). After each sampling session, the sorbent tubes were cleaned three times, using ethanol, dichloromethane, and pentane.

Chemical analyses
Headspace samples were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a polar DB-Wax capillary column (J&W, 30 m x 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml / min constant flow) was used as carrier gas. One µl of the sample was injected splitless at 40°C. Subsequently, the splitter was opened and the oven temperature increased at a rate of 5°C / min to 240°C.

Coupled gas chromatography-mass spectrometry (GC-MS) was performed with a double focusing VG70/250 SE mass spectrometer (Vacuum Generators, Manchester, England) linked to a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, US) that was equipped with a fused silica column (FFAP, 50 m x 0.25 mm, operated at an initial temperature of 60°C and programmed to 220°C at a rate of 5°C / min). Structural assignments were based on comparison of analytical data obtained with natural products, data reported in the literature [30] and those of synthetic reference compounds. Structures of candidate active compounds were verified by co-injection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as internal standard.

Electrophysiology
Electrophysiological analyses of headspace samples from *E. helleborine* flowers and cabbage infested by *P. brassicae* caterpillars were performed on a HP 6890 gas chromatograph (Agilent Technologies) equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae from workers of *V. germanica* and *V. vulgaris*, caught from two nests in the surrounding of the campus of the University of Ulm, were tested. *Apis mellifera* workers were captured in the field and used for GC-EAD analyses with headspace samples from *E. helleborine* flowers. For each EAD the tip of an excised antenna was cut off and the antenna mounted between two glass-capillary electrodes filled with insect Ringer solution. The electrode at the antenna’s base was grounded via an Ag-AgCl wire, and the
recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 10°C / min. The effluent was split and 30 ml / min of make-up gas (nitrogen) was added (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD=1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the female wasp's antenna. Natural samples (collected scent) and synthetic compounds (identified upon GC/MS-analyses) were run under the same conditions.

Behavioral experiments

Bioassays were performed in July and August 2001 at the Institute of Zoology (Vienna) and in July and August 2007 at the Institute of Experimental Ecology in Ulm.

In the first bioassay the importance of visual versus olfactory cues of *E. helleborine* flowers was examined in a field experiment. All tests were made under sunny conditions and temperatures of about 26-29°C at the terrace of the Institute of Zoology where the abundance of *V. germanica* workers was high. A plant covered with a UV permeable quartz glass cylinder with two holes for incoming and outgoing air (enriched with scent of the flowers) allowed testing of the importance of optical versus olfactory cues of *E. helleborine* flowers. With this setup we performed three test series: 1) to test the combination of visual and olfactory cues, the whole plant was presented in the cylinder. 2) To test the olfactory cues only, the cylinder was covered with an additional cardboard cylinder so that the wasps could not see but could smell the flowers. In these tests, the inflowing air stream (200 ml / min) was cleaned from atmospheric pollutants by a charcoal filter (activated charcoal, Supelco, Orbo 32 large), passed the flowers, and left through the second hole. 3) To test the importance of visual cues alone, the holes of the cylinder were closed. Each test lasted 20 min and was performed at least 10 times in the field.

The olfactometer experiment involved a Y-tube olfactometer (length 22 cm, diameter 0.8 cm), horizontally fixed in a polystyrene box (18 x 18 x 16 cm). To avoid visual disturbance, the only light resource was a cold light lamp (Schott KL 1500 LCD, 2950K) placed above the centre of the Y-tube. The test plants were put into glass cylinders (length 25 cm, diameter 15 cm), which were connected with Teflon or silicon tubing to the Y-tube. Both glass cylinders were connected by equally long Teflon tubes to a motor pump (Volcraft, Laboratory Power Supply, PS-302A). Air forced into each glass chamber (50 ml / min) through a single inlet was filtered and cleaned from atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (Orbo-32, Supelco). After having passed the glass chamber containing the test plants or a blank control, the air streams were directed into the shanks of the Y-tube. To test synthetic volatiles, 10 µl (representing 5 plant equivalents, PE) of the test-mixtures (the composition is given below) or of the pure solvent was applied on a piece of filter paper (3 x 0.5 cm) and placed at each end of the shorter Y-tube arms. In all tests, an insect (wasp or honeybee) was released into the long arm of the Y–tube, and its choice was registered. A site was counted as “chosen” if the insect touched the filter paper baring it at the end of the tube. For each test, a new wasp (honeybee), a new Y-tube, and
new filter papers were used. To avoid preference of the insects for one side of the Y-tube, the positions of shanks for treatment and blank control were shifted after every run.

The following samples were used in the Y-tube tests: 1) infested and non-infested cabbage plants, 2) five flowers of either *E. helleborine* or *E. atrorubens* put in each shank of the Y-tube (for direct comparison), 3) synthetic test mixture of EAD active compounds of *E. helleborine* consisting of 0.03 µg hexyl acetate, 0.09 µg octanal, 0.04 µg Z-3-hexenyl acetate, 0.01 µg Z-3-hexenol, 1.28 µg nonanal, 0.22 µg decanal and 0.37 µg benzaldehyde dissolved in pentane. 4) Synthetic mixture of the GLVs consisting of 0.03 µg hexyl acetate, 0.04 µg Z-3-hexenyl acetate and 0.01 µg cis-3-hexenol dissolved in pentane. The qualitative and quantitative composition of the synthetic mixtures was the same as natural samples, as verified by GC analyses. Synthetic compounds were obtained from Sigma-Aldrich; purity ranged from 95-99%.

In addition to the Y-tube experiments we performed a further *choice experiment under semi-field conditions in a flight cage* (3 x 4 x 3 m). A table was placed in the center of the flight cage that was used as a foraging area for the wasps. Four dishes (diameter 3 cm) containing a 50% sugar solution of API-Invert® (72.7% glucose; Südzucker AG, Germany; 1 g citric acid and 3 g potassium sorbate were added per liter API-Invert solution for preservation) and each with an artificial paper flower (radially symmetric flower shape, yellow, diameter 4.5 cm) were placed on top of the table. Two of the artificial flowers were impregnated with 10 µl (5 PE) of the synthetic mixtures, the other two with solvent only (control). Every 5 min the artificial flowers were replaced with a new impregnated artificial paper flower. During a test period of 60 min, numbers of visiting wasps for each of the four dishes were counted.

**Data analysis**

We compared the total number of approaches in the field experiment by a Mann-Whitney U-test. For the statistical analysis of the Y-tube experiments and the choice experiments in the flight cage we used the “Sign test”. In the flight cage test the registered numbers of behavioral events for the two flowers impregnated with the same samples (solvent or test mixtures) were pooled. Comparison of the total amount of GLVs released by *E. helleborine*, *E. purpurata* and *E. atrorubens* and infested cabbage was done with the Mann-Whitney U-Test with a Benjamini-Hochberg correction [30].

**Acknowledgments**

We thank Werner Hiller, Manfred Kalteisen and Hannes Paulus who helped us to collect plant material. We thank Rob Hodgkison, Stefan Jarau and Robert Paxton for critical reading of the manuscript and linguistic advice and Johannes Stökl for methodological advice. We also like to thank Rod Schneidmiller (Sterling International, Inc.) for financial support. W.F. wishes to thank the Fonds der Chemischen Industrie for financial support.
References


2.

Orchid mimics honeybee alarm pheromone in order to attract hornets for pollination

Jennifer Brodmann¹, Robert Twele², Wittko Francke², Luo Yi-bo³, Song Xi-qiang⁴, Manfred Ayasse¹

¹Institute of Experimental Ecology, University of Ulm, Germany
²Institute of Organic Chemistry, University of Hamburg, Germany
³State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Science, China
⁴Key Laboratory of Tropical Horticultural Plant Resources and Genetic Improvement, Hainan University, China

Published in Current Biology 18, 740-744. 2009.
Summary

Approximately one third of the world’s estimated 30,000 orchid species are deceptive and do not reward their pollinators with nectar or pollen [1]. Most of these deceptive orchids imitate the scent of rewarding flowers or potential mates [2, 3]. In this study, we investigated the floral scent involved in pollinator attraction to the rewardless orchid *Dendrobium sinense*, an endemic species of the Chinese island Hainan, pollinated by the hornet *Vespa bicolor*. Using a combination of chemical analyses and electrophysiological methods, we demonstrate that the flowers of *D. sinense* produce Z-11-eicosen-1-ol and that the pollinator can smell this compound. This is a major compound in the alarm pheromones of both Asian (*Apis cerana*) and European honey bees (*Apis mellifera*) [4, 5] and is also exploited by the European beewolf (*Philanthus triangulum*) to locate its prey [6]. This is the first time that Z-11-eicosen-1-ol has been identified as a floral volatile. In behavioral experiments, we demonstrate that the floral scent of *D. sinense* and synthetic Z-11-eicosen-1-ol are both attractive to foraging hornets (*V. bicolor*). Since hornets frequently capture honeybees to feed to their larvae, we suggest that the flowers of *D. sinense* mimic the alarm pheromone of honeybees in order to attract prey-hunting hornets for pollination. This is an exciting new example of prey mimicry, which adds yet another dimension to the already diverse array of pollination strategies in orchids.

Results and Discussion

The orchid and its pollinator

In this study, we investigated the floral scent involved in pollinator attraction of the rewardless orchid *Dendrobium sinense*, an endemic species of the Chinese island Hainan [7]. The flowers of *D. sinense* are white with a red center (Figure 1). The pollinator of the orchid was unknown prior to our studies of eight populations of *D. sinense* in the Bawang National Reserve in Hainan. During an observation time of 121 h, we counted 35 visiting insects, 30 of them being identified as the hornet *Vespa bicolor* (Hymenoptera: Vespoidea). The other visitors were bees, wasps, and a butterfly. Rather than landing and pausing on the flowers as would be typical for most pollinators, the hornets instead pounced on the red center of the flower, much like their behavior when attacking prey. Contact with the flower during these pounces was typically less than one second. Of the visiting insects, only hornets were observed to effect pollination, with both pollinia deposition and pollinia removal on the pronotum of the insects observed in the field. Removal or deposition of polinia by *V. bicolor* was observed in 5 of 30 visits. Furthermore, during a time period of 30 min we registered 277 nest entering or nest leaving wasps from three colonies from that 30 females carried pollinia.

During the flowering time of *D. sinense*, there are two other sympatrically occurring orchids in bloom: *Epigeneium fargesii* and *Coelogyne fimbriata*. The hornets are not interested in the flowers of *C. fimbriata*, but occasionally visit the flowers of *E. fargesii*. However, we never observed them to remove pollinia (Song Xi-qiang, own observation).
Therefore, the pollinia that the hornets carry are definitely from *D. sinense*. This result supports that *V. bicolor* is the pollinator of *D. sinense*. Our observations led us to hypothesize that *V. bicolor* is the sole pollinator of the orchid *D. sinense*. This hypothesis is supported by a comparison of the pollinator and orchid flower size (Figure 1). The orchid flowers are assumed to have morphological adaptation to the visits of and pollination by the hornet *V. bicolor*. The mean height of the thorax of *V. bicolor* is 0.554 ± 0.022 cm SD (n = 16) and the mean width is 0.538 ± 0.025 cm SD (n = 16) and allowing the pollinator to fit optimally within the flower passage with a height of 0.567 ± 0.061 cm SD and a width of 0.544 ± 0.101 cm SD (n = 118). These morphological adaptations of the flowers maximize the chance that pollinia are removed by the hornets and ensure secure transfer of the pollinia to another flower. We assessed the fruit set of *D. sinense* in three different locations and found about 13% of the flowers (n = 703) to be pollinated, as can be expected for a nectarless orchid [8].

Hornets belong to the group of social wasps that feed their brood with meat nutrients, mainly insects [9]. Foraging hornets are known to capture honeybees often, either in the surroundings of a colony or while they forage for pollen and nectar on flowers [9, 10]. Behavioral experiments have shown that searching wasps use a combination of visual and olfactory cues to locate their prey [11]. However, so far, the chemical structures of corresponding volatile signals remained unknown.

In a recent investigation, flowers of *Epipactis helleborine*, another wasp-pollinated orchid, have been shown to emit “green leaf volatiles” (GLVs), which are attractive to foragers of the social wasps *Vespuia germanica* and *V. vulgaris* [12]. GLVs are emitted by plant tissues upon damage by herbivores insects, for example, by cabbage leaves infested with caterpillars (*Pieris brassicae*), which are common prey items for wasps [9]. Therefore, we suggested that the flowers of *D. sinense* are mimicking a signal of their prey in order to attract prey-hunting hornets for pollination.

**Figure 1.** *D. sinense* flower (A) and *V. bicolor* forager (B) with pollinia that stick onto the thorax (Photos: Brodmann, Song Xi-qiang).
Is the floral scent of the orchid attractive to hornets?
To investigate the relative importance of floral signals to foraging hornets, we compared the attractiveness of single flowers and of odorless European honeybee dummies impregnated with floral scent to hornets in a flight cage. Hornets significantly more often approached dummies impregnated with the flower extract and whole flowers in comparison with the control consisting of an odorless honeybee dummy (Figure 2). Pentane extracts of flowers released the same number of approaches in the hornets as intact flowers (Mann-Whitney U test, U = 17.5, p = 0.935, whole flower: mean 7.67 ± 3.983 SE, n = 6, flower extract: mean 6.50 ± 1.378 SE, n = 6). Therefore, we concluded that these extracts contained the most important compounds used by the hornets while searching for food. In interactions with flowers or scent-impregnated dummies, the hornets showed a similar behavior to that observed in interactions with orchid flowers in the field. They pounced on the flowers or the dummies impregnated with floral scent. This implies that the scent of the flowers plays an important role for hornets searching for prey.

Figure 2. Approaches of hornets to various odor samples tested in a flight cage. Comparison of the attractiveness of the scent from an orchid flower, a flower extract, and the solvent pentane on impregnated honeybee dummies in a flight cage (Mann-Whitney U test + Benjamini-Hochberg correction [28], p ≤ 0.05, n = 6). Bars represent median with error bars: 95% confidence interval. Different letters indicate significance differences between the tests.

Do orchid flowers mimic honeybees in scent?
Gas chromatography coupled with an electroantennographic detector (GC-EAD) in combination with gas chromatography coupled with mass spectrometry (GC-MS) was used to identify those compounds in the complex flower scent that are perceived by the antennae of worker hornets, a technique that we previously found to be effective for the identification of volatile pollinator attractants in the wasp-pollinated orchid Epipactis helleborine [12]. In pentane extracts collected from D. sinense flowers, we detected five compounds inducing an electrophysiological response in the antennae of workers of V. bicolor (Figure 3). Using GC-MS, we identified not only benzyl acetate and benzyl alcohol, two compounds that belong to the most common components of floral scent [13], but also octadecan-1-ol, eicosan-1-ol, and Z-11-eicosen-1-ol. The occurrence of octadecan-1-ol, eicosan-1-ol, and especially Z-11-eicosen-1-ol in the floral scent of D. sinense supports our hypothesis that flowers mimic odor.
cues emitted by honeybees. The reason for the lower electrophysiological response we found for Z-11-eicosen-1-ol in comparison to benzyl acetate and benzyl alcohol is probably a result of the lower volatility of the compound. All of these electrophysiological active compounds have previously been described in the stinging apparatus of the Asian honeybee A. cerana [4, 14]. We also identified them in body surface extracts of workers of the species by chemical analyses (Figure 4). The total amounts of octadecan-1-ol and Z-11-eicosen-1-ol were nearly the same (octadecan-1-ol: A. cerana: 1.1 ± 0.4 SE µg / sample, n = 6, D. sinense: 1.22 ± 0.12 SE µg / sample, n = 13; Z-11-eicosen-1-ol: A. cerana: 17.3 ± 5.3 SE µg / sample, n = 6, D. sinense: 10.7 ± 2.2 SE µg / sample, n = 13) in the bees and in the orchids. In contrast, amounts of eicosan-1-ol (A. cerana: 0.2 ± 0.05 SE µg / sample, n = 6, D. sinense: 22.2 ± 3.7 SE µg / sample, n = 13), vary between honeybee and flower surface. Z-11-eicosen-1-ol is known as a major compound in the alarm pheromone of both the Asian (Apis cerana) and the European (Apis mellifera) honeybee [4, 5]. Presently, the biological significance of the compounds is not fully understood. It is highly attractive for honeybee foragers, and in behavioral experiments performed at the hive entrance of honeybees, it elicits aggressive behavior as well as stinging in bees [5]. In A. cerana, floral or other resources have been assumed to be marked with Z-11-eicosen-1-ol to attract other foragers [5]. It has also been described as a major component in the secretion of Dufour’s gland in the neotropical stringless bee Frieseomelitta varia [15] and in the thoracic glands of male carpenter bees Xylocopa micheneri [16]. Males of the solitary wasp, the European beewolf Philanthus triangulum, produce Z-11-eicosen-1-ol in the secretion of a cephalic gland [17, 18] to attract females, which exclusively hunt honeybees, Apis mellifera, as provisions for their larvae. The females of these solitary wasps use olfactory cues to find and identify honeybees on flowers [6]. Although Z-11-eicosen-1-ol is only a minor component among the cuticle volatiles in honeybees, it is used as an essential component for prey recognition in hunting female wasps [6]. However, Z-11-eicosen-1-ol has hitherto not been reported in non-hymenopteran insects and, least of all, in flowers.
**Chemical mimicry in wasp-pollinated orchids**

**Figure 3.** Electrophysiologically active compounds in the flower extract of *D. sinense* (A) and comparison of the electrophysiologically active compounds of a flower extract of *D. sinense* and the body surface extract of an *A. cerana* forager (B). Simultaneous recordings of GC (FID) and EAD signals obtained with flower extracts of *D. sinense* by using the antenna of a *V. bicolor* worker were performed on a polar DB-Wax capillary column. Benzyl acetate, benzyl alcohol, octadecan-1-ol, eicosanol, and Z-11-eicosen-1-ol were electrophysiologically active in the flower extract, octadecan-1-ol, eicosanol, and Z-11-eicosen-1-ol also occurred in the extract of the body surface of *A. cerana*.

**Mimicry of the alarm pheromone**

In order to test our hypothesis that Z-11-eicosen-1-ol is produced by the orchid in order to mimic the alarm pheromone of honeybees and to attract hunting hornets to pollinate flowers, we performed further behavioral experiments. In a Y tube olfactometer experiment, the hornets significantly preferred a synthetic mixture of all EAD active compounds identified in *D. sinense* (Sign test, $p = 0.01$, $n = 20$), and Z-11-eicosen-1-ol alone (Sign test, $p = 0.05$, $n = 20$) (Figure 4) compared with the empty control. *D. sinense* flowers (Sign test, $p = 0.001$, $n = 20$) and solvent extracts of the flowers (Sign test, $p = 0.02$, $n = 20$) were significantly more attractive than an empty control or a solvent control (Figure 4). However, in a Y tube olfactometer experiment that gave the hornets the choice between the flower scent and the synthetic mixture, they showed no preference (Sign test, $p > 0.05$, $n = 20$), which implies that the synthetic mixture contains all essential components for pollinator attraction including Z-11-eicosen-1-ol. The attractiveness of a synthetic mixture consisting of all of the
electrophysiologically active *D. sinense* compounds found to co-occur in the honeybee *A. cerana* was confirmed by a field experiment in a flight cage. The mixture attracted the same number of hornets as natural flowers (Mann-Whitney U test, $U = 3$, $p > 0.05$, $n = 11$).

**Figure 4.** Attraction of hornets to various odor samples.

- * Comparison of the attractiveness of the odor from an orchid flower, a flower extract, the synthetic mixture of the EAD-active compounds of the flower extract (*s. D. sinense*), and the substance Z-11-eicosen-1-ol in a Y tube olfactometer (Sign test, $* p \leq 0.05$, $** p \leq 0.001$, $n = 20$ for each test).

### Conclusion

Orchids show a remarkable variation of floral forms and a high diversity in pollination systems. Non-rewarding flowers are widespread among Orchidaceae [19], and deceptive orchid species are well known for their specific pollination systems in which only one or a few animal species are attracted [20]. Orchid species pollinated by social wasps are rare, and most of them offer edible rewards [12, 21] or are assumed to mimic food [22, 23]. The pollination system that we have found in the orchid *D. sinense* represents another fascinating example of chemical mimicry in deceptive pollination. By emitting volatiles indicating the presence of prey, the flowers are capable of attracting their pollinators, foragers of the social wasp, *V. bicolor*. Moreover, to the best of our knowledge, this is the first time that Z-11-eicosen-1-ol has been identified as a floral volatile. We are presently investigating pollinator attraction in a further wasp-pollinated deceptive orchid, *Steveniella satyrioides*, in order to determine whether the principles that we have found in *D. sinense*, i.e., the mimicking of the scent of prey, are common in deceptive wasp-pollinated orchids.

Various species of *Vespa* are problems to beekeepers, because they plunder the hives. Besides this their ravages of fruit crops make hornets a serious pest to man [24, 25]. Our results may be the first step for developing an environmentally responsible system for trapping pest hornets.
Experimental Procedures

Pollination system
Insects visiting flowers of *D. sinense* were observed for a total 121 hours in the moss forest of Bawang National Reserve on the island of Hainan in South China. The fruit set data were collected from 703 flowers at three locations in 2003 and 2004. For the size measurements of *V. bicolor* foragers, the mean height and the mean width of the thorax was measured (n = 16). Furthermore, in *D. sinense*, we measured the height of the flower passage (the distance between the column as the dorsal part of the flower and the labellum as the ventral part of the flower opening) and the width of the passage (the distance between the two side lobes of the labellum) (n = 118).

In three colonies of *V. bicolor* we registered during a time period of 30 min the number of nest entering or nest leaving wasps that carried pollinia.

Hornets
Workers of the hornet *Vespa bicolor*, which were needed for behavioral experiments and for electrophysiological analyses, were collected in the moss forest of Bawang National Reserve near three nests from 20th August – 11th September 2007 and from 1st – 11th September 2008.

Collection of volatiles
Samples of *D. sinense* were collected in the moss forest of Bawang National Reserve in 2007. For the collection of odor samples, individual, freshly opened, unpollinated flowers were cut off from plants and extracted in 10 ml pentane (99%, for high-pressure liquid chromatography; Chromasolv, Sigma-Aldrich, Munich, Germany) at room temperature for 24 h. The flowers were then removed, and the samples were stored at -20°C.

Surface body extracts from foragers of the honeybee *A. cerana* were collected by Mananya Phiancharoen (Department of Biology, Chulalongkorn University, Bangkok, Thailand) in 2008. Worker honeybees were killed by freezing and extracted for 30 sec in 1 ml of the solvent hexane. The samples were subsequently stored in the freezer until used for chemical analyses.

Chemical analyses
Flower extracts of the orchid *D. sinense* (n = 13) and the surface extracts of honeybees *A. cerana* (n = 6) were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a polar DB-Wax capillary column (J&W, 30 m x 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml / min constant flow) was used as the carrier gas. The sample (1 µl) was injected splitless at 40°C. After 1 min delay, the splitter was opened, and the oven temperature increased at a rate of 5°C / min to 240°C.

Coupled gas chromatography-mass spectrometry (GC-MS) was performed with a double focusing VG70/ 250 SE mass spectrometer (Vacuum Generators, Manchester, UK) linked to an HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, US) equipped with a fused
silica column (FFAP, 50 m x 0.25 mm, operated at an initial temperature of 60°C and programmed to reach 220°C at a rate of 5°C / min). Structural assignments were based on a comparison of the analytical data obtained with natural products, data reported in the literature [26], and those of synthetic reference compounds. The structures of candidate active compounds were verified by co-injection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as an internal standard.

**Electrophysiology**

Electrophysiological analyses of flower extracts of the orchid *D. sinense* were performed on an HP 6890 gas chromatograph (Agilent Technologies) equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae from workers of *V. bicolor* imported from China were tested. For each EAD, the tip of an excised antenna was cut off, and the antenna mounted between two glass-capillary electrodes filled with insect Ringer solution. The electrode at the base of the antenna was grounded via an Ag-AgCl wire, and the recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 5°C / min. The effluent was split, and 30 ml / min of make-up gas (nitrogen) was added (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD = 1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the antenna of the female hornet. We declared a substance to be EAD active when it proved to be active in a minimum of 15 replicates.

**Behavioral experiments**

Bioassays were performed in August and September 2007 and September 2008 in the moss forest of Bawang National Reserve on the island of Hainan in South China.

We performed single choice experiments in a flight cage (60 x 60 x 60 cm). The hornets were tested in the flight cage under natural climatic and humidity conditions in the field with an orchid flower or an odorless honeybee dummy. We used a honeybee as dummy, because they are common prey items for hornets. We extracted honeybee foragers for 24 h in dichloromethane using Soxhlet extraction. The odorless soxhlet extracted, honeybee dummy was impregnated with 10 µl of the flower extract (containing 5 flower equivalents), the synthetic mixture of the EAD-active components of the flower extract, or the solvent pentane as a control. In each case, a single hornet was placed into the flight cage, and the behavior of the hornet was observed for a period of 10 min. We counted approaches (short contact) to the odor source. Each test with one odor sample was repeated with six different hornets.

The olfactometer experiment involved a Y tube olfactometer (length 22 cm, diameter 0.8 cm). To avoid visual disturbance, the Y tube was horizontally fixed in a box covered with a red foil. A glass cylinder (length 10 cm, diameter 2.5 cm) containing an orchid flower and an empty control glass cylinder were connected with silicon tubing to the Y tube. Both glass cylinders were connected by equally long silicon tubes to a motor pump (Volcraft, Laboratory Power Supply, PS-302A). Air forced into each glass chamber (50 ml / min) through a single inlet was filtered and cleaned from atmospheric pollutants by a cylindrical borosilicate glass
cartridge packed with activated charcoal (Orbo-32, Supelco). After having passed the glass chamber containing the orchid flower or a blank control, the air streams were directed into the arms of the Y tube. To test synthetic volatiles, 10 µl (containing 1 flower equivalent) of the test mixtures (the composition is given below) or of the pure solvent was applied to a piece of filter paper (3 x 0.5 cm), which was placed at each end of the shorter Y tube arms. In all tests, a hornet was released into the long arm of the Y tube, and its choice of tube arm was registered. A site was counted as “chosen” when the insect touched the filter paper at the end of the tube. For each test, a new hornet, a new Y tube, and new filter papers were used. To avoid preference of the insects for one side of the Y tube, the positions of the shanks for treatment and blank control were changed after every run. Each test series was repeated 20 times.

The following samples were used in the Y tube tests: 1) a flower of *D. sinense*, 2) a flower extract in pentane (1 flower equivalent), 3) one flower equivalent of a synthetic test mixture of EAD active compounds of *D. sinense* consisting of 2.8 µg benzyl acetate, 9.1 µg benzyl alcohol, 1.2 µg octadecan-1-ol, 22.2 µg eicosan-1-ol, and 10.7 µg Z-11-eicosen-1-ol dissolved in pentane. The qualitative and quantitative composition of the synthetic mixture was the same as those found in the flowers, as verified by GC analyses. Synthetic compounds were obtained from Sigma-Aldrich; purity ranged from 95-99%, except for Z-11-eicosen-1-ol. The latter was synthesized from commercially available methyl Z-11-eicosenoate upon reduction with lithium aluminium tetrahydride according to standard methods [27].

**Data analysis**

Comparison of the total amount of the five identical compounds of *D. sinense* flowers and the honeybee surface was performed with the Mann-Whitney U-Test.

For the dummy experiments in the flight cage, we compared the number of approaches made to the flower of *D. sinense*, the flower extract, and the solvent on a honeybee dummy by a Mann-Whitney U test with a Benjamini-Hochberg correction [28]. For the statistical analysis of the Y tube experiments, we used the Sign test.

**Acknowledgements**

We thank Chengjin, Shijing, and Lee for helping in the field and M. Phiancharoen for collecting the honeybee surface samples. The Robert-Bosch Foundation and Fazit Foundation are gratefully acknowledged for financial support.
References


Orchid mimics alarm pheromone of the pollinator to attract alerted wasps for pollination

Jennifer Brodmann\textsuperscript{1}, Joachim Ruther\textsuperscript{2}, Stephan Franke\textsuperscript{3}, Wittko Francke\textsuperscript{3}, Amots Dafni\textsuperscript{4}, Nejded Bozkurt\textsuperscript{5}, Qing-He Zhang\textsuperscript{6}, Manfred Ayasse\textsuperscript{1}

\textsuperscript{1}Institute of Experimental Ecology, University of Ulm, Germany
\textsuperscript{2}Institute of Zoology, University of Regensburg, Germany
\textsuperscript{3}Institute of Organic Chemistry, University of Hamburg, Germany
\textsuperscript{4}Institute of Evolution and Department of Evolutionary and Environmental Biology, Haifa University, Israel
\textsuperscript{5}Kehribar Sok. MESA Yankı Sitesi No. 17/14, 06700 Gaziosmanpaşa, Ankara, Türkei
\textsuperscript{6}Sterling International, Inc., Spokane, Washington 99216-1630, USA
Summary

Among the known 30,000 orchid species approximately one-third are deceptive and do not offer any reward such as nectar or pollen to their pollinators [1]. The orchid *Steveniella satyrioides* is such a deceptive species, which is exclusively pollinated by social wasps [2]. In our investigation we wanted to answer the question, how the orchid attracts the wasps to their flowers for pollination. In behavioral experiments, we showed that the scent of the orchid plays a decisive role in the attraction of wasps from a distance. In addition, it elicits stinging and biting behavior in the wasps after they landed on the flower. Using a combination of electrophysiological investigations, and analyses of headspace volatiles of *S. satyrioides*, we identified 6 compounds that are perceived by the insects’ antennae: ethanol, methyl methoxyacetate, 1,8-cineol, 2-ethylhexan-1-ol, benzaldehyde and acetophenone. In behavioral experiments we could show that a blend of synthetic compounds mixed in naturally occurring proportions induces the same aggressive behaviour in the wasps as does the natural scent of the orchids. A scenario where the orchid mimics alarm pheromones of wasps in order to attract alerted individuals for pollination is discussed.

We show for the first time that a deceptive plant mimics the alarm pheromone of its pollinator species and thereby exploits the pollinator’s social communication system of the pollinator. This is a new evidence that prey mimicry is a common syndrome of wasp-pollinated orchids [3, 4].

Results and discussion

Visual versus olfactory cues for wasp attraction

The wasp-pollinated orchid *Steveniella satyrioides* (Stev.) Schltr. occurs in Anatolia, Northern Iran, Caucasus and Crimea [5, 6]. It is nectarless, entirely purple-brown in color, and blooms in April and May. It is pollinated by social wasps (Hymenoptera: Vespidae) such as *Vespula vulgaris* and *Dolichovespula sylvestris* [2]. Because wasps feed their larvae with meat nutrient, Nazarov [2] hypothesized that the orchid would attract the wasps by mimicking a piece of meat. He described reddish-brown papillae on the base of the lip, at the spur entrance as a visual signal and suggested that the wasps may take them as food. Therefore, he entitled the pollination mechanism as “false-prey”-syndrome.

To investigate the relative importance of floral signals to wasps, we compared the attractiveness of whole inflorescences, inflorescences covered with a quartz glass cylinder (visual cues), and natural scent of *S. satyrioides* (olfactory cues) which were offered to queens of *V. vulgaris* wasps. The results of our bioassays showed that olfactory cues were significantly more attractive to wasps than visual cues (Mann-Whitney U test, approach: $U = 38, p < 0.001$; landing: $U = 123, p = 0.009$) and elicited the same number of landings by the wasps as the whole inflorescences (Mann-Whitney U test, $U = 173, p = 0.8$, whole inflorescence: mean $\pm$ SD: $0.53 \pm 0.69, n = 19$, scent: $0.63 \pm 1.06, n = 19$) (Figure 1). Wasps are obviously attracted from a distance by the flower’s fragrance. The results of our
behavioral experiments support the primacy of olfactory cues in the long distance attraction of wasps. The floral scent offered without visual cues clearly attracted the wasps to visit flowers.

Olfactory cues for long distance attraction were also found to be important in other wasp-pollinated orchids. The Chinese orchid *Dendrobium sinense* is a typical wasp flower, which fools its pollinator. While smelling like a honeybee, a typical prey item of the wasps, the orchid attracts prey-hunting wasps for pollination [4].

The scent of the orchid as the key role for wasp attraction

Gas chromatography coupled with an electroantennographic detector (GC-EAD) in combination with gas chromatography coupled with a mass spectrometer (GC-MS) was used to identify those compounds in the complex flower scent that are perceived by the antennae of worker wasps, a technique that proved to be effective for the detection and structure elucidation of attractant volatiles in other wasp-pollinated orchids [3, 4]. In headspace samples collected from *S. satyrioides* flowers, we detected six compounds each inducing an electrophysiological response by the antennae of workers of *V. vulgaris* (Figure 2). Using GC-MS, we identified ethanol, methyl methoxyacetate, 1,8-cineol, 2-ethylhexan-1-ol, benzaldehyde, and acetophenone. Ethanol, a common plant volatile [7], which is produced by fermenting fruits, could play a role in the long-distance attraction of the wasps. It is known that certain alcohols are attractive to wasps [8]. The only known occurrence of methyl methoxyacetate as a plant volatile is from the laksa plant (*Polygonum hydropiper* L.) [9]. The monoterpene 1,8-cineol is a common floral volatile [7, 10, 11] and has already been found in orchid fragrance [12]. 2-Ethylhexan-1-ol, as well as benzaldehyde have been found as volatile constituents of the venom sac of three Ropalidia wasp species [13], but the function of these compounds has not been experimentally tested. Acetophenone has been described as a compound of floral volatiles [7, 14, 15], however, interestingly it is also an alarm
pheromone component of various wasp species [16, 17]. It may have been responsible for the aggressive behavior observed in the wasps that visit flowers of *S. satyrioides* (Brodmann and Ayasse, personal observations). The wasps were found to bite into the flowers, and in addition we observed that visiting wasps also sting individual flowers of an inflorescence thereby showing the typical behavior of wasps attacking prey [18] or enemies [19].

Figure 2. Electrophysiologically active compounds.
Simultaneous recordings of GC (FID) and EAD signals obtained with headspace samples of *S. satyrioides* flowers by using the antenna of a *V. vulgaris* worker. The GC analyses were performed on a polar DB-Wax capillary column. We found six EAD-active compounds.

Does the scent of orchid flowers elicit alarming behavior in wasps?
In order to confirm our hypothesis that the scent of the orchid plays a key role in attracting wasps and in stimulating aggressive behavior, we performed further behavioral experiments. In a Y tube olfactometer test, the workers of *V. vulgaris* significantly preferred the odor of flowers of *S. satyrioides* (sign test, \( p = 0.01, n = 20 \)) as well as the synthetic mixture of the EAD active compounds of the orchid (sign test, \( p = 0.05, n = 20 \)) compared to the empty control and the solvent pentane (Figure 3). We also observed aggressive behavior of the wasps in this experiment in that they bit into the teflon tubes or filter papers impregnated with mixtures of synthetic compounds. To investigate the specificity of the floral signals that are
only pollinated by *V. vulgaris* and *Dolichovespula sylvestris*, we performed additional Y tube experiments with queens of *Polistes gallicus*, a wasp species that is not described as a pollinator of the orchid. We offered flowers of *S. satyrioides* against the empty control. *Polistes gallicus* queens were neither attracted by the floral scent (sign test, *p* > 0.05, *n* = 14) nor did they show aggressive behavior, indicating a highly specific attraction to *Vespula* spp.

In order to test the effect of the floral compounds as an alarm pheromone we performed a dual choice experiment in which we placed two dummies in front of the nest entrance of a *V. vulgaris* colony, one of them impregnated with a mixture of synthetic EAD active compounds of *S. satyrioides*, the other one with solvent only. Shortly after offering scent impregnated dummies close to the nest entrance, many alerted wasps left the nest and circled around the impregnated dummy. In the wasps the odor impregnated dummy elicited a significantly higher number of approaching, touching, stinging and biting events as compared to a solvent control (Figure 4; Wilcoxon Signed Rank Test, *n* = 7, approaches: mean 32.4 ± 2.2 SE, *p* = 0.017, *Z* = -2.3; touches: mean 17.5 ± 2.2 SE, *p* = 0.018, *Z* = -2.3; stings and bits: mean 8.8 ± 1.5 SE, *p* = 0.018, *Z* = -2.3). After the 2 minutes observation time the wasps frequently tried to pull the dummy with the orchids’ scent into their nest (Brodmann, personal observation). The conclusion is that floral volatiles elicit typical alarming behavior in pollinating wasps.

**Figure 3.** Attraction of wasps to various odor samples.

Comparison of the attractiveness of the odor from an orchid flower, and the synthetic mixture of the EAD active substances of *S. satyrioides* in a Y tube olfactometer (sign test, *p* ≤ 0.05, *n* = 20 for each test).
Figure 4. Dummy experiments with wasps. Comparison of the attractiveness of a prey-dummy impregnated with a synthetic mixture of the GC-EAD active compounds of *S. satyrioides* or pentane (control) in a dual choice experiment in the field. Registered reactions of *V. vulgaris* wasps towards the dummies that were put in the surrounding of a nest entrance of the wasps were approaches, touches and stinging and biting into the impregnated dummy. (Wilcoxon Signed Ranks Test, \( *p \leq 0.05, n = 7, \) observation time for each test 2 min). Bars represent mean number of behavioral events ± standard error of the mean.

Do the orchid flowers mimic alarm pheromone components of wasps?

Chemically mediated alarming behavior has been reported from several species of social Vespidae. The source of alarm pheromones in social wasps is typically the venom gland, although the head is implicated as an additional source of alarm pheromone for *V. vulgaris* [20] and *V. squamosa* [21]. In behavioral experiments workers of *V. vulgaris*, *V. germanica*, and *Dolichovespula saxonia* and the European hornet *Vespa crabo* showed alarm reactions if secretions of the venom gland were presented at the nest entrance [22]. However, so far alarm pheromones have been isolated and identified in only three species of social wasps: *Vespa crabo*, *Vespula squamosa*, and *V. maculifrons*. 2-Methyl-3-butene-2-ol was identified in the venom gland of *V. crabo* and elicited aggressive behavior in the wasps [23]. N-3-methylbutylacetamide released alarming behavior in *Vespula squamosa* [24] and was also found in extracts of venom sacs of *V. vulgaris* [20], though its function in this species has not been tested experimentally. A variety of volatiles, amongst them acetophenone, have been identified as components of the alarm pheromones in hornets [16, 17], but until now no behavioral experiments have performed to confirm that they elicit aggressive behavior in the wasps. In our chemical analyses except benzaldehyde none of the compounds mentioned so far have been identified in the venom sac or in the mandibular gland of *V. vulgaris* wasps. The only described alarm pheromone compounds which we also found in the orchids is benzaldehyde.

Why should an orchid produce alarm pheromone compounds of wasps. There is experimental evidence that *V. vulgaris* and *V. germanica*, while stinging their enemies, deposit a long lasting alarm pheromone on them and that this pheromone elicits alarming behavior and attacks in other workers in order to be able to repel even larger predators like vertebrates [19]. Released in front of a nest, this would focus attacks on the intruder and also
alert the colony if and when this predator approached the nest again [19]. Although stinging is generally reserved for defense purposes in social wasps [25], several authors have documented cases in which foraging social wasps used their sting when grappling with particularly large and active prey [18]. Odor impregnation of the prey may also help to find it again and while foraging on large prey items that cannot be transported to the nest by one individual alone. *Steveniella satyrioides* obviously mimics components of the alarm pheromone of other wasp species and thereby elicits a highly specific behavior in flower visiting wasps that results in removal or deposition of pollinia, and finally pollinating of a flower.

**Conclusion**

Because whole orchid flowers, as well as the scent alone, elicit aggressive behavior like stinging and biting in the wasps, we conclude that the flowers mimic compounds of the alarm pheromone of several wasp species to attract alerted wasps for pollination. Except benzaldehyde we did not find any compounds emitted from the orchids’ flowers in the venom of the pollinator *V. vulgaris*. However, acetophenone and 2-ethylhexan-1-ol, components that we found in the orchids’ scent, have been described to be present in the venom of other wasp species [13, 16, 17]. The aggressive behavior of the wasps we observed in our behavioral experiments with orchids’ scent strongly suggests that the wasps respond to the alarm pheromone compounds of other wasp species, too. Dummy experiments showed that wasps use optical cues while approaching flowers and prefer dark items. Olfactory cues reduce the threshold to attack, but induce attack by the wasps, a combination of optical and olfactory cues is needed [26]. The red color of the orchid flowers may contrast to the green grass and is probably seen by the wasps as a black spot [27]. In combination with the scent, which contains compounds of the alarm pheromone of wasps, the wasps attack the orchid flower, expecting an insect prey and thereby pollinate it. This is the first example of an orchid that exploits the social communication system of its pollinator in order to attract it for pollination.

**Experimental procedures**

**Volatile collection**

Floral scent emitted from *S. satyrioides* flowers was collected using dynamic headspace adsorption technique in May 2008 in Bolu, Turkey. Intact inflorescences were carefully enclosed in polyethylene terephthalate oven bags (Toppits®, Germany), and volatiles were trapped using an adsorbent tube containing a thin layer (1.5 mg) of activated charcoal (Gränicher & Quarters, Daumazan, France) by using a membrane pump, adjusted to a flow rate of 500 ml / min for ca. 9 h. The inflowing air stream was cleaned of atmospheric
Chemical mimicry in wasp-pollinated orchids

pollutants by an activated charcoal filter (Orbo 32 large, Supelco, Steinheim, Germany). The trapped volatiles in the adsorbent tube were eluted with 40 µl dichloromethane (HPLC grade, Sigma-Aldrich, Steinheim, Germany). After each sampling session, the sorbent tubes were cleaned three times, using subsequent extraction with ethanol, dichloromethane, and pentane.

Extracts of the venom sac, and the mandibular gland of workers of *V. vulgaris* wasps were collected 2010 in Ulm. Worker wasps were killed by freezing and the venom sac and the mandibular gland were dissected and extracted for 24 h in pentane and dichloromethane (HPLC grade, Sigma-Aldrich). The samples were subsequently stored at -20°C until used for chemical analyses.

**Chemical analyses**

Headspace samples of *S. satyrioides* (*n* = 6), as well as the extracts of the venom sac and the mandibular glands of wasps were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a polar DB-Wax capillary column (J&W, 30 m x 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml / min constant flow) was used as the carrier gas. The sample (1 µl) was injected splitless at an oven temperature of 40°C. After 1 min delay, the splitter was opened, and the oven temperature increased at a rate of 5°C / min to 240°C.

Coupled gas chromatography-mass spectrometry (GC-MS) was performed on a double focusing VG70/ 250 SE mass spectrometer (Vacuum Generators, Manchester, UK) linked to an HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, US) equipped with a fused silica column (FFAP, 50 m x 0.25 mm, operated at an initial temperature of 60°C and programmed to 220°C at a rate of 5°C / min). Structural assignments were based on comparison of the analytical data obtained with natural products, data reported in the literature [28], and those of synthetic reference compounds. The structures of candidate active compounds were verified by co-injection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as an internal standard.

**Electrophysiology**

Electrophysiological analyses of headspace samples of the orchid *S. satyrioides* were performed with an HP 6890 gas chromatograph (Agilent Technologies), equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae of *V. germanica* workers were tested. For each EAD, the tip of an excised antenna was cut off, and the antenna mounted between two glass-capillary electrodes filled with insect Ringer solution. The electrode at the base of the antenna was grounded via an Ag-AgCl wire, and the recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a computer. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 5°C / min. The effluent was split, and 30 ml / min of make-up gas (nitrogen) was added (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD = 1:3). The outlet of the EAD was placed in a cleaned and humidified airflow that was directed over the
antenna of the worker wasp. We classified a substance to be EAD active when it proved to be active in a minimum of 10 replicates.

**Behavior experiments**

Bioassays were performed in May 2009 in Bulo, Turkey with *V. vulgaris* queens that were collected with a net in the surrounding of our test location. In the first bioassay, the importance of visual versus olfactory cues of *S. satyrioides* flowers was examined in a flight cage experiment. A plant covered with a UV permeable quartz glass cylinder with two holes for the inlet and outlet of air (the latter enriched with flower volatiles) allowed to test the importance of optical versus olfactory cues of *S. satyrioides* flowers. With this setup we performed three test series: 1) To test the combination of visual and olfactory cues, the whole plant was presented. 2) To test the olfactory cues only, the cylinder was covered with an additional cardboard cylinder so that the wasps could not see but could smell the flowers. In these tests, the inflowing air stream (200 ml / min) was cleaned from atmospheric pollutants by a activated charcoal filter (Orbo 32 large, Supelco), passed the flowers, and left through the second hole. 3) To test the importance of visual cues alone, the holes of the cylinder were closed so that the wasp could only see an inflorescence but not smell it. For each test a new wasp was used. Before each test the wasp was 5 min in the flight cage to accommodate. Each test lasted 5 min and was performed 19 times with each time one *V. vulgaris* queen.

The olfactometer experiment involved a Y-tube olfactometer (length 22 cm, diameter 0.8 cm), horizontally fixed in a polystyrene box (18 x 18 x 16 cm). One inflorescence of *S. satyrioides* was put into a glass cylinder (length 25 cm, diameter 15 cm), and the control glass cylinder was empty. Both cylinders were connected to the short shanks of the Y tube using Teflon tube. The second openings of both glass cylinders were connected by equally long Teflon tubes to a motor pump (Volcraft, Laboratory Power Supply, PS-302A). Air pumped through each glass chamber (50 ml / min) through a single inlet was filtered and cleaned from atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (Orbo-32, Supelco). After having passed the glass chamber containing the orchid flowers or the blank control, the air streams were directed into the shanks of the Y tube. To test synthetic volatiles, 10 µl (representing 1 headspace equivalent) of the test mixture (synthetic test mixture of EAD active compounds of *S. satyrioides* consisting of 2 µg ethanol, 2.1 µg methyl methoxyacetate, 0.07 µg 1,8-cineol, 0.02 µg rac. 2-ethylhexan-1-ol, 0.04 µg benzaldehyde and 0.22 µg acetophenone dissolved in pentane) or of the pure solvent was applied on a piece of filter paper (3 x 0.5 cm) and placed at each end of the shorter Y-tube arms. In all tests, a worker wasp was released into the long arm of the Y-tube, and its choice was registered. A site was counted as “chosen” if the insect touched the filter paper baring it at the end of the tube. For each test, a new wasp, a new Y-tube, and new filter papers were used. To avoid any preference of the insects for one side of the Y-tube, the positions of shanks for treatment and blank control were shifted after every run. Each test was repeated 20 times.

The dummy experiments were performed in the Botanical Garden of the University of Ulm with two nests of *V. vulgaris*. Our dummies consisted of an oval brown plasticine model
(length 1.5 cm, diameter 0.5 cm). A filter paper impregnated with 20 µl of the synthetic mixture (see above) (representing 2 headspace equivalents) or pentane as a control was fixed below the plasticine model on a piece of polystyrene which served as a base. These two dummies were put in front of the entrance of a wasp nest close to the surface at a distance of 10 cm, and the reactions of the wasps to the dummies were recorded for 2 min. This test was repeated seven times. New dummies and impregnated filter papers were used for each test. As verified by GC analyses, the qualitative and quantitative composition of the synthetic mixtures was the same as found in the natural samples. Synthetic compounds were obtained from Sigma-Aldrich; purity ranged from 95-99%.

Data analysis

We compared the total number of approaches in the cage experiment by a Mann-Whitney U-test with a Benjamini-Hochberg correction [29]. For the statistical analysis of the Y-tube experiments we used the “Sign test”. For the dummy experiments we used the Wilcoxon Signed Ranks Test.

Acknowledgments

We also like to thank Rod Schneidmiller [Sterling International, Inc. (SII)] for financial support. Q.-H.Z. is a SII employee but not a SII shareholder or officer, and thus has no financial conflict of interest as it related to this work.

References

Chemical mimicry in wasp-pollinated orchids


Pollinator attraction of the wasp-flower *Scrophularia umbrosa*

Jennifer Brodmann, Denise Emer, Manfred Ayasse

Institute of Experimental Ecology, University of Ulm, Germany
Abstract

The evolution of mutualism in plant-pollinator interactions are from high interest, and topic of several research studies. Certain species of *Scrophularia* (*Scrophulariaceae*), like *S. nodosa* and *S. umbrosa* are mainly pollinated by social wasps and are consequently described as wasp-flowers. Plants attract their pollinators with the help of various floral cues, like floral odour or / and optical cues to their flowers (Knudsen et al. 2006; Vogel 1983).

The aim of this study was to investigate the role of olfactory and visual floral signals responsible for the wasp attraction in *S. umbrosa*. Using a combination of chemical (GC, GC-MS) and electrophysiological analyses (GC-EAD) we could identify the compounds in the complex floral odour bouquet that are detectable by the wasps. We identified the aldehydes octanal, nonanal, decanal, and benzaldehyde, the alcohols hexan-1-ol, Z-3-hexen-1-ol, and 1-octen-3-ol, the acetates Z-3-hexenyl acetate, and geranyl acetate, and the ketone 6-methyl-5-hepten-2-one in the floral odour bouquet of *S. umbrosa*. As well as in the wasp-flower *Epipactis helleborine* we found so called “green leaf volatiles” (GLVs) in the floral odour (Brodmann et al. 2008). “Green leaf volatiles” (GLVs), mostly six-carbon aldehydes, alcohols and acetates and other volatile organic compounds (VOCs), are emitted by many plants infested by herbivores, e.g. caterpillars (Whitman and Eller 1990). Behavioural experiments demonstrated that in contrast to the other investigated wasp-flowers (Brodmann et al. 2008; Brodmann et al. 2009), the floral odour of *S. umbrosa* seems to be less important for the wasp attraction, and that also visual cues are involved in pollinator attraction. The floral GLVs a tested alone are highly attractive, but together with the other EAD active compounds they lose their attractiveness. In contrast to other wasp-flowers, like the orchid *E. helleborine*, *S. umbrosa* attract a broader visitor spectrum, and hence is not so specialized in wasp attraction.

Introduction

The convergent evolution of plant-pollinator interactions formed the phrase of “pollination syndromes”, which is defined as groups of floral traits such as colour, morphology, scent and nectar characteristics, which are thought to be associated with certain groups of pollinators (Fenster et al. 2004). Therefore pollinators can be organized into functional groups. Robertson (1928) made a classification of the visitors into nine functional groups: long-tongued bees, short-tongued bees, other Hymenoptera, Diptera, Coleoptera, Lepidoptera, Hemiptera, Neuroptera, and birds. According to Stebbin’s “most effective pollinator principle” (1970), a plant should specialize on the most effective and / or most abundant pollinator when pollinator availability is reliable. Repeatedly the conceptual structure of pollination syndromes has been criticized based on the argument that flowers usually attract a broader spectrum of visitors than one might expect based on their syndromes and that flowers often diverge without excluding one type of pollinator in favour of another (Fenster et al. 2004).
Besides the well-studied pollination syndrome of bee-, bird- and bat-flowers, wasp-flowers, which are exclusively pollinated by social wasps (Hymenoptera: Vespidae), are a so far less well investigated syndrome. Until now little is known about the floral cues that are responsible for the wasp attraction. Wasp-flowers exhibit physiological and morphological adaptations for the visitation and pollination of social wasps. Amongst others the flowers are often described as “reddish brown” (Müller 1881), “dirty purple” (Werth 1956), and “dirty brown” (Schremmer 1962) coloured, and according to Wiefelspütz (1970) this colours are thought to attract the wasps. Schremmer (1962) furthermore noticed “relatively small, mostly bulbous flowers with a broad entrance” and “sucrose rich nectar” (Baker and Baker 1983). A prime example of a wasp-flower are the two Epipactis species (Orchidaceae) E. helleborine and E. purpurata (Müller 1873). Recent studies revealed that the floral scent plays the key role in wasp attraction, and that the orchid performs prey mimicry to attract prey-hunting social wasps for pollination. Substances produced by green plant tissue, when infested by herbivores insect, so called “green leaf volatiles” (GLVs), are exposed to be essential for the wasp attraction (Brodmann et al. 2008).

Further examples of wasp-flowers are described in the genus Scrophularia (Scrophulariaceae), which is represented by approximately 268 species, mainly distributed in the holoarctic, with its primary diversification centre in Asia (Ortega Olivencia and Devesa Alcaraz 1992; Stiefelhagen 1910). The flowers offer pollen and nectar as a reward to pollinators (Ortega Olivencia and Devesa Alcaraz 1992). Scrophularia nodosa (Sprengel 1793) and S. umbrosa (Müller 1881) are thought to be wasp-flowers. With its small, bulbous rust-brown or greenly-brown coloured flowers (Haeupler and Muer 2000; Sebald et al. 1996), and the exposed nectar, S. umbrosa exhibits several characters of a typical wasp-flower. However, in contrast to other wasp-flowers, like the orchid E. helleborine, S. umbrosa shows a broader visitor spectrum. Expect social wasps, also other flower visitors could be found, amongst them especially honeybees (Faegri and Van der Pijl 1979), and Syrphids (Schremmer 1958).

Plants attract their pollinators with the help of various floral cues to their flowers. One of these floral cues to attract their pollinators is odour, which is known as an important signal in many plants (Knudsen et al. 2006). In sexually deceptive orchids the odour is even the most important cue to attract pollinator males. The flowers mimic female sex pheromones and thereby attract only male insects, which pollinate the flowers in an attempted copulation or precopulatory routine (Ayasse 2006; Schiestl 2005; Schiestl et al. 2003). Also in the deceptive wasp-pollinated orchid Dendrobium sinense the floral odour plays the key role in wasp attraction. The nectarless orchid mimics the scent of honeybees, a common prey item of the pollinator, and thereby attracts prey-hunting wasps for pollination (Brodmann et al. 2009). But besides the floral odour also the optical floral cues are involved in pollinator attraction. It is shown that experienced honeybees are able to discriminate between dummy flowers on the basis of colour patterns (Hempel de Ibarra et al. 2002), or that bumblebees are directed by floral guides to their flowers (Lunau et al. 2006). Besides olfactory and optical cues of the flower, nectar plays a crucial role in plant reproduction by rewarding the pollinators (Simpson and Neff 1983). The main ingredient of the nectar in the majority of plants is sugar, mainly glucose, fructose and sucrose (Baker and Baker 1983). Apart from sugar, nectar contains
amino acids, lipids, organic acids, as well as various vitamins, enzymes, antioxidants, mineral ions and secondary metabolites (Baker 1975; Galetto et al. 1998). It is known that diverse pollinators species prefer different sugar compositions of the nectar, and wasp-flowers are found to be sucrose dominated (Baker and Baker 1983).

The aim of this study was to determine the floral signals of the wasp-flower *S. umbrosa*, which are responsible for the wasp attraction. Because in other wasp-flowers it is known, that scent plays the key role (Brodmann et al. 2008; Brodmann et al. 2009), we investigated the flower odour via chemical (GC, GC-MS) and electrophysiological analyses (GC-EAD). Furthermore we performed behavioural experiments to study the relative importance of the floral signals to foraging wasps. We furthermore conduct nectar analysis to analyze the role of nectar sugar composition in *S. umbrosa*.

**Materials and Methods**

**Volatile collection**

Floral scent emitted from *S. umbrosa* flowers (cultivated in the Botanical Garden of Ulm) was collected using dynamic headspace adsorption techniques in the years 2007 and 2008. Intact inflorescences were carefully enclosed in polyester oven bags (Toppits®, Germany), and volatiles were trapped in an adsorbent tube containing a thin layer of activated charcoal (CLSA, 1.5 mg, Gränicher & Quarterso) by using a membrane pump, adjusted to a flow rate of 500 ml / min for ca. 8 h. The inflowing air stream was cleaned of atmospheric pollutants by a charcoal filter (activated charcoal, Supelco, Orbo 32 large). The trapped volatiles in the adsorbent tube were eluted with 40 µl dichloromethane (Sigma-Aldrich, HPLC grade). After each sampling session, the sorbent tubes were cleaned using ethanol, dichloromethane, and pentane.

**Chemical analyses**

Headspace samples were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a polar DBWax capillary column (J&W, 30 m x 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml / min constant flow) was used as carrier gas. One µl of the sample was injected splitless at 40°C. Subsequently, the splitter was opened and the oven temperature increased at a rate of 5°C / min to 240°C.

Coupled gas chromatography-mass spectrometry (GC-MS) was performed with a double focusing VG70/ 250 SE mass spectrometer (Vacuum Generators, Manchester, England) linked to a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, US) that was equipped with a fused silica column (FFAP, 50 m x 0.25 mm, operated at an initial temperature of 50°C and programmed to 220°C at a rate of 5°C / min). Structural assignments were based on comparison of analytical data obtained with natural products, and those of synthetic reference compounds. Structures of candidate active compounds were verified by co-
injection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as internal standard.

**Electrophysiology**

Electrophysiological analyses of headspace samples from *S. umbrosa* flowers were performed on a HP 6890 gas chromatograph (Agilent Technologies) equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae from workers of *V. vulgaris* were tested. For each EAD the tip of an excised antenna was cut off and the antenna mounted between two glass-capillary electrodes filled with insect ringer solution. The electrode at the antenna’s base was grounded via an Ag-AgCl wire, and the recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split valve and programming to 240°C at 10°C / min. The effluent was split and 30 ml / min of make-up gas (nitrogen) was added (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD=1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the female wasp’s antenna. We considered a substance to be EAD active when it proved to be active in a minimum of ten replicates.

**Behavioural experiments**

**Field experiments**

To investigate the relative importance of floral signals to pollinating wasps, we compared the attractiveness of naturally inflorescences (optical and olfactory cues), inflorescences covered with a quartz glass cylinder (optical cues), and natural scent of *S. umbrosa* (olfactory cues) which were offered to workers of *V. vulgaris* in a field experiment. The experiments were performed on the campus of the University Ulm in August and September of the years 2003 and 2004. The equipment was located on a table with radius of 50 cm near an area were many wasps were active. During a time period of 15 min the number of wasps, which flew inside the radius (approach) and pounced against the glass cylinder (pouncing), were recorded. The following test series were performed: (1) In order to test a combination of visual and olfactory cues, the whole inflorescence was presented to the wasps. (2) To test the importance of optical cues alone, the holes of the cylinder were closed. (3) For testing of the olfactory cues only, the cylinder was covered with an additional cardboard cylinder so that wasps could not see but smell the flowers. In these tests, the inflowing air stream (100 ml / min) was cleaned from atmospheric pollutants by a charcoal filter (activated charcoal, Supelco, Orbo 32 large), passed the flowers, and left through the second hole. Each test lasted 15 min and was performed at least 7 times.

**Y tube experiments**

The olfactometer experiment involved a Y tube olfactometer (length 22 cm, diameter 0.8 cm), horizontally fixed in a polystyrene box (18 x 18 x 16 cm). To avoid visual disturbance, the only light resource was a cold light lamp (Schott KL 1500 LCD, 2950K) placed above the centre of the Y tube. 20 flowers of *S. umbrosa* were put into a glass cylinder (length 25 cm, diameter 15 cm). An empty glass cylinder served as a control. Both glass cylinders were
connected by equally long Teflon tubes to a motor pump (Volcraft, Laboratory Power Supply, PS-302A). Air forced into each glass chamber (50 ml / min) through a single inlet was filtered and cleaned from atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (Orbo-32, Supelco). After having passed the glass chamber containing the test flowers or a blank control, the air streams were directed into the shanks of the Y tube. To test synthetic volatiles which were found to be electrophysiological active, 10 µl (representing 5 headspace equivalents) of the test mixture (the composition is given below) or of the pure solvent was applied on a piece of filter paper (3 x 0.5 cm) and placed into one of the two shorter Y tube shanks. In all tests, a *V. vulgaris* wasp was released into the long shank of the Y tube, and its choice was registered. A site was counted as “chosen” if the wasp touched the filter paper at the end of the tube. For each test, a new wasp, a new Y tube, and new filter papers were used. To avoid preferences of the wasps for one side of the Y tube, the positions of shanks for treatment and blank control were shifted after every run. Each test series was repeated 20 times.

The following samples were used in the Y tube tests: (1) 20 flowers of *S. umbrosa*, (2) synthetic mixture of EAD active compounds of *S. umbrosa* consisting of 0.03 µg octanal, 0.1 µg Z-3-hexenyl acetate, 0.05 µg 6-methyl-5-hepten-2-on, 0.14 µg hexan-1-ol, 0.12 µg Z-3-hexen-1-ol, 0.26 µg nonanal, 0.17 µg 1-octen-3-ol, 0.1 µg decanal, 0.03 µg benzaldehyde, and 0.1 µg geranyl acetate, (4) synthetic mixture of EAD active compounds without the GLVs consisting of 0.03 µg octanal, 0.05 µg 6-methyl-5-hepten-2-on, 0.26 µg nonanal, 0.17 µg 1-octen-3-ol, 0.1 µg decanal, 0.03 µg benzaldehyde, and 0.1 µg geranyl acetate.

**Analysis of sugars**

Nectar samples from *S. umbrosa* (5 samples from various plants) were sampled using 1 and 5 µl microcapillary tubes (Hirschmann) in the year 2007 and immediately transferred in a 70% ethanol dilution (diluted by sterilized water) and stored in the freezer at -40°C until chemical analyses were performed. Ethanolic samples were vacuum centrifuged, diluted in pure ion-free water, filtered (VWR Centrifugal Filter, 0.45 µm pores) and divided for separate sugar analysis by high performance liquid chromatography (HPLC; Waters autosampler+, CHM column heater module). Sugar analysis was performed with an isocratic pump (Waters 510), Sentry Guard column (high performance carbohydrate, 3.9 x 20 mm), Waters high performance carbohydrate column (4.6 x 250 mm), solvent (72 : 28 acetonitrile : water mix) and refractive index detector (Waters 410; flow rate 1.4 mL/min). HPLC was controlled and data obtained using Waters Millenium 3.0 software. The sugars glucose, fructose and sucrose were used as standards every 10 samples.

**Data analysis**

We compared the number of approaches and pouncings in the field experiment by a Mann-Whitney U-Test with a Benjamini-Hochberg correction (Benjamini and Hochberg 1995). For the statistical analysis of the Y tube experiments we used the “Sign test”.

- 71 -
Results

Electrophysiological and chemical analyses of the scent of S. umbrosa

We found ten electrophysiologically active compounds detected by wasps. By using gas chromatography coupled with mass spectrometry (GC-MS) we identified the aldehydes octanal, nonanal, decanal, and benzaldehyde, the alcohols hexan-1-ol, Z-3-hexen-1-ol, and 1-octen-3-ol, the acetates Z-3-hexenyl acetate, and geranyl acetate, and the ketone 6-methyl-5-hepten-2-one (Fig. 1). To estimate the absolute amount (µg / inflorescence) of these substances we performed gas chromatographic analyses (GC) with the headspace samples S. umbrosa. The aldehyde nonanal is with 0.26 µg / inflorescence the main component in the EAD active compounds of the floral odour bouquet of S. umbrosa (Tab. 1).

![Figure 1. Electrophysiologically active compounds with headspace samples of S. umbrosa. Simultaneous recordings of GC (FID) and EAD signals by using the antenna of a V. vulgaris worker. The GC analyses were performed on a polar DBWax capillary column. We found ten EAD active compounds. Z-3-hexenyl acetate and nonanal could not be separated with the GC parameters used.](image-url)
Table 1. Mean (± SE) absolute amount (µg / inflorescence) of EAD active compounds of headspace sample of S. umbrosa (n = 7).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Absolute amount (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octanal</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Z-3-Hexenyl acetate</td>
<td>0.10 ± 0.50</td>
</tr>
<tr>
<td>6-Methyl-5-hepten-2-one</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Hexan-1-ol</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Z-3-Hexen-1-ol</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>Nonanal</td>
<td>0.26 ± 0.70</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>0.17 ± 0.49</td>
</tr>
<tr>
<td>Decanal</td>
<td>0.10 ± 0.20</td>
</tr>
<tr>
<td>Benzaldehyde</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Geranyl acetate</td>
<td>0.10 ± 0.15</td>
</tr>
</tbody>
</table>

**Behavioural Experiments**

*Field experiments*

The results of our field experiments showed that the natural inflorescence, as well as the optical cues alone showed a tendency to release more pouncings than the olfactory cues alone (Fig. 2; Mann-Whitney U test; naturally inflorescence/optical cues: $p = 0.5$, $U = 53$, naturally inflorescence/olfactory cues: $p = 0.3$, $U = 35$; naturally inflorescence: mean ± SE: 5.7 ± 0.8, n = 14, optical cues: 7.8 ± 1.9, n = 9, olfactory cues: 4.0 ± 1.0, n = 7).

*Y tube experiments*

In Y tube experiments, we tested the attractiveness of the floral odour of S. umbrosa flowers and of mixtures of synthetic compounds. The experiments showed that neither the odour of whole flowers (Fig. 3; sign test, $p = 0.5$, n = 20), nor the synthetic mixture of all EAD active compounds of S. umbrosa (sign test, $p = 0.1$, n = 20) were significantly more attractive to the wasps than the solvent control. In a further Y tube experiment the synthetic mixture of the GLVs Z-3-hexenyl acetate, hexan-1-ol, and Z-3-hexen-1-ol was significant more attractive than the solvent control (pentane) (sign test, $p = 0.02$, n = 20). A synthetic mixture containing the rest of the EAD active compounds, without the GLVs, was not attractive to the wasps (sign test, $p > 0.5$, n = 20).
Figure 2. Comparison of the attractiveness of the whole inflorescence of *S. umbrosa* (optical + olfactory cues), the inflorescence covered with a quartz glass cylinder (optical cues), and the natural scent of *S. umbrosa* (olfactory cues), which were offered to workers of *V. vulgaris*. Different letters indicate significant differences within the test groups (Mann-Whitney U test, \( p < 0.05 \), \( n = 7 \)). Registered reactions were approaches and landings on the flowers / glass cylinder. Bars represent mean number of behavioural trades ± standard error of the mean.

Figure 3. Comparison of the attractiveness of the odour from *S. umbrosa* flowers, synthetic mixture of EAD active substances of *S. umbrosa*, synthetic mixture only with the three GLVs, synthetic mixture without the GLVs to social wasps in a Y tube olfactometer (Sign test, * \( p = 0.01 \), \( n = 20 \)).
Analysis of sugars
In the nectar of S. umbrosa sucrose is dominated with a mean of 150 ± 45 SD µg/µl. Fructose was present with a mean of 19 ± 9.2 SD µg/µl. No glucose was found in the samples.

Discussion

Optical versus olfactory floral signals for pollinator attraction
Wasps are, without any doubt, the most frequent and numerous visitors, attracted by the nectar rich flowers of S. umbrosa (Faegri and Van der Pijl 1979). However, our investigations support the finding of Emer (2005), that S. umbrosa is less specialized than other wasp-flowers, for example the orchid E. helleborine which is exclusively pollinated by social wasps. Whilst in E. helleborine, the flower scent plays the key role in wasp attraction (Brodmann et al. 2008), our study revealed that in S. umbrosa both olfactory and optical cues are important for the wasp attraction.

In the floral odour of S. umbrosa we identified the same compounds, namely GLVs, responsible for the wasp attraction than in the wasp-flower E. helleborine (Brodmann et al. 2008). Behavioural experiments with GLVs identified in S. umbrosa confirmed that they are highly attractive to wasps, but in combination with the other electrophysiological active floral odour compounds found in S. umbrosa, they were less attractive to wasps. To ensure its reproductive success, S. umbrosa may produce GLVs for wasp attraction and additional floral compounds to attract other insects for pollination. This other compounds might mask the attractiveness of the wasp attractive GLVs. In conclusion in S. umbrosa their might be a trade-off between wasp attraction and the attraction of other pollinators.

Except the GLVs the other compounds we identified are typical floral odour compounds (Knudsen and Tollsten 1993). For instance benzaldehyde is a common compound and occurs in many bee-pollinated plants, but its behaviour modulating function has up to now mostly been found in moths (Huber et al. 2005; Knudsen et al. 2006; Schiestl and Marion-Poll 2002). Furthermore the alcohol 1-octen-3-ol is also known as an attractant for various Dipterans (Blackwell et al. 1996; French and Kline 1989; Kline and Mann 1998), which could declare the occurrence of syrphids on the flowers.

It was shown that the lips at the lower part of the inflorescence of S. umbrosa reflect UV light, while the flowers of the upper parts absorb UV light (Kugler 1963; Rosen and Barthlott 1991). It is well-established that insects are able to see ultraviolet radiation (Richards 1986). Optical cues, like bicoloured flowers and UV are known to be important for luring honeybees to flowers (Vöth 1999). The presence of this optical cues, in combination with the common floral odour compounds could explain, why the nectar rich flowers of S. umbrosa are attractive for many other flower visitors, like honeybees, solitary wasps, and syrphids and is less specialized in wasps as pollinators.
The role of nectar contents

*S. umbrosa* offers exposed nectar which is usually exploited by a wide range of short- and long-tongued insects, resulting in generalized pollination systems (Johnson and Steiner 2000; Waser et al. 1996). Like in many wasp-flowers (Baker and Baker 1983), we found the nectar of *S. umbrosa* sucrose dominant.

Flowering time and habitat choice

The flowers of the *Scrophularia* spp. are dichogamous, specifically protogynous (Olivencia and Alcaraz 1993), a phenomenon which has been viewed to favour cross-pollination and limit self-fertilization (Proctor et al. 1996). Müller (1883) studied the pollination of *S. nodosa* and found that when the pollinating wasps were absent, the flowers tend to self-pollination. In *S. umbrosa* we could not confirm these observations. We found a broad pollinator spectrum that may optimize the pollination success via pollinating insects before the flowers conduct self-pollination, thereby increasing the number of produced seeds per capsule in comparison to self-pollination (Stiefelhagen 1910). In *S. umbrosa* the flowering time does not completely overlap with the peak of the wasp density. In contrast to many deceptive orchids, which take advantage in attraction of a specific pollinator, in *S. umbrosa*, it could be profitable to attract a broader spectrum of pollinators. Orchids store all their pollen in pollen packets, so called pollinaria, and a flower visitor removes the whole pollen content. For the non-orchid *S. umbrosa* a specific pollinator is not so essential and pollen is available for a longer time period.

Furthermore the choice of the habitat may influence which insects can be expected to visit a flower. Whilst the specialized *E. helleborine* prefers the border of a forest or dark forest understory, where besides wasps other flower visitors are rare, *S. umbrosa* mostly grows on sunny exposed places, where many insect species occur.

Conclusion

In contrast to other investigated wasp-flowers, where floral odour plays the key role in wasp attraction (Brodmann 2008; Brodmann 2009), in *S. umbrosa* optical cues are also important for pollinator attraction. The flowers were found to produce the same GLVs than *E. helleborine*. In behavioural experiments they were highly attractive to wasps, in combination with the other EAD active compounds of *S. umbrosa*, however, this GLVs lose their attractiveness for wasps. Probably a combination of optical cues of the flowers and floral odour is responsible for the attraction of a broad range of flower visitors, which also serve as pollinators, and thereby increase the reproductive success of a plant. The fact that the non-orchid *S. umbrosa* produces the same compounds, namely GLVs, than the wasp-pollinated orchids of the genus *Epipactis* leads us to the conclusion that there was a convergent evolution of these pollinator attractive substances.
Chemical mimicry in wasp-pollinated orchids

References

Baker HG (1975) Sugar concentrations in nectars from hummingbird flowers. Biotropica 7:37-41
Chemical mimicry in wasp-pollinated orchids


Müller H (1873) Die Befruchtung der Blumen durch Insekten und die gegenseitigen Anpassungen beider (Leipzig, Germany: Engelmann Verlag)

Müller H (1881) Alpenblumen, ihre Befruchtung durch Insekten und die gegenseitigen Anpassungen beider (Leipzig, Germany: Engelmann Verlag)


Olivencia AO, Alcaraz JAD (1993) Floral rewards in some Scrophularia species (Scrophulariaceae) from the Iberian Peninsula and the Balearic Islands. Plant Systematics and Evolution 184:139-158


Richards A (1986) Plant breeding systems. Allen and Unwin, Winchester, MA

Robertson C (1928) Flowers and insects. Lists of visitors to four hundred and fifty-three flowers. C. Robertson, Carlinville, Illinois, USA


Schremmer F (1962) Wespen und Hornissen (Wittenberg, Lutterstadt, Germany: A. Ziemsen Verlag)


Sprengel CK (1793) Das entdeckte Geheimnis der Natur im Bau und in der Befruchtung der Blumen. Berlin: Frederick Vieweg


Werth E (1956) Bau und Leben der Blumen. Ferdinand Enke Verlag, Stuttgart
Multifaceted ways of attracting various pollinators in the orchid genus *Epipactis*

Jennifer Brodmann, Manfred Ayasse

Institute of Experimental Ecology, University of Ulm, Germany
Abstract

Various plants use different floral cues to attract various pollinator species to their flowers. From two wasp-pollinated *Epipactis* species (Orchidaceae) is known, that the flower scent plays the key role in pollinator attraction, and that the orchid mimics prey to attract prey-hunting social wasps for pollination (Brodmann et al. 2008). In order to answer the question, how various *Epipactis* species attract their individual pollinators, we compared the olfactory and optical cues of the orchid flowers, as well as the nectar sugar composition of the flowers of two wasp-pollinated with three non wasp-pollinated species. By using a combination of chemical (GC, GC-MS) and electrophysiological (GC-EAD) analyses, we could show differences in the odour production of the flowers, as well as odour perception in various pollinators of the investigated *Epipactis* species. The optical measurements of the flowers pointed out differences in the colour and UV reflection between the species. In all investigated *Epipactis* species the nectar analysis exhibit a sucrose dominance. In behavioural experiments with potential pollinators we could show, that different *Epipactis* species attract their individual pollinators with the help of various floral cues. Our results confirm the hypothesis that in different species of the same genus different strategies are adopted to avoid competition for pollinators and thereby ensure their reproductive success by exploiting different pollinator niches.

Introduction

The coevolution of plants and their pollinators formed the phrase of “pollination syndromes”. Pollination syndromes are groups of floral traits such as colour, morphology, scent and nectar characteristics, which are thought to be associated with certain groups of pollinators (Fenster et al. 2004). Therefore pollinators can be organized into functional groups, like long-tongued bees, short-tongued bees, other Hymenoptera, Diptera, Coleoptera, Lepidoptera, Hemiptera, Neuroptera, and birds (Robertson 1928). Various species of plants attract their pollinators in a variety of ways, by releasing specific attractants or offering rewards, like nectar (Proctor et al. 1996). For instance bird-attracting flowers are typically large, showy and robust, having red, orange or bright yellow colours, no scent and copious amount of dilute nectar (Faegri and Van der Pijl 1979; Proctor et al. 1996), whereas nocturnal hawkmoths-pollinated flowers are typically white and release a strong floral odour during nighttimes (Waser and Ollerton 2006). Floral odour is known to play a major role in pollinator attraction (Vogel 1983), such as in the sexually deceptive orchids, where floral scent is even the most important cue to attract pollinators. The flowers mimic female sex pheromones and thereby attract only male insects, which pollinate the flowers in an attempted copulation or precopulatory routine (Ayasse 2006; Schiestl 2005; Schiestl et al. 2003).

All Hymenoptera so far tested have the ability to learn colours as a stimulus associated with a reward (Chittka and Lunau 1992; Dukas and Real 1991; Frisch 1914; Mazokhin-Porshniakov 1962; Menzel 1979; Menzel and Backhaus 1991), and hence optical floral cues
Chemical mimicry in wasp-pollinated orchids

are also involved in pollinator attraction. It is shown that honeybees are able to discriminate between dummy flowers on the basis of colour pattern (Hempel de Ibarra et al. 2002), or that bumblebees are directed by floral guides to their flowers (Lunau et al. 2006).

Besides olfactory and optical cues of the flower, nectar plays a crucial role in plant reproduction by rewarding the pollinators (Simpson and Neff 1983). The main ingredient of the nectar in the majority of plants is sugar: glucose, fructose and sucrose (Baker and Baker 1983). It is established that diverse pollinator species prefer different sugar compositions of the nectar (Baker and Baker 1983), like hummingbirds which prefer relatively high sucrose concentrations, whereas birds exploit only simple hexose sugars, such as glucose or fructose (del Rio 1990).

Orchids display a remarkably diverse range of intriguing pollination mechanisms, including some of the most extraordinary examples of adaptation to insect visitors (Proctor et al. 1996). Although 5 % of the known orchids are pollinated by wasps (Pijl and Dodson 1966), the syndrome of wasp-pollination was nearly overlooked so far. A long time the dull coloration of the flowers are thought to be responsible for the wasp attraction (Wiefelspütz 1970). Recent investigations revealed that in wasp-pollinated orchids specific odour compounds, accountable for prey recognition (Brodmann et al. 2008; Brodmann et al. 2009), or food driven odours (Cheng et al. 2009), are responsible for pollinator attraction. To answer the question, why wasp-flowers are nearly almost ignored from other potential flower visitors, Baker & Baker (1983) showed that wasp-pollinated flowers are sucrose dominated, and they expected that there could be a repellent sugar like cellobiose and gentiobiose in the nectar (Baker and Baker 1983), or nectar may even contain toxic alkaloids, amino acids or other unpleasant substances (Baker 1975; Baker and Baker 1977; Baker et al. 1978) that repel non-wasps visitors and nectar thieves.

The orchid genus *Epipactis* is represented by about 18 species and 20 subspecies in Europe (Baumann 2005), which are known to be pollinated by various insects. While some species have a broad pollinator spectrum and are, amongst others, visited by bees (Vöth 1999) and solitary wasps (Nilsson 1978), others are very specialised. *Epipactis helleborine* (L.) Crantz is the most common and widely distributed species of the genus (Wiefelspütz 1970), and is a prime example of a wasp-flower, as well as *E. purpurata*, because both are mainly pollinated by social wasps (Hymenoptera: Vespidae), like *Vespula vulgaris* and *V. germanica* (Müller 1873). Their flowers exhibit physiological, morphological and ecological adaptations for the attraction of pollinating social wasps (Müller 1873). As noted by Darwin (1888), this species are almost entirely overlooked by other potential pollinators, despite a large nectar reward, a mystery that was disclosed recently (Brodmann et al. 2008). The study revealed that the wasp-pollinated *E. helleborine* and *E. purpurata* produce certain volatile compounds, namely GLVs, and that this compounds are responsible for the wasp attraction (Brodmann et al. 2008). “Green leaf volatiles” (GLVs), mostly six-carbon aldehydes, alcohols and acetates and other volatile organic compounds (VOCs), are emitted by many plants infested by herbivores, e.g. caterpillars (Whitman and Eller 1990).

Besides the wasp-pollinated *Epipactis* species we find other species, like *E. atrorubens*, a species that is mainly pollinated by bumblebees, or *E. palustris*, which exhibits a broad
spectrum of visitors, but is effectively pollinated by honeybees (Vöth 1999) and solitary wasps (Nilsson 1978).

In our study we compared various floral cues, e.g. odour, optical signals and nectar sugar composition, of different *Epipactis* species to answer the question, how different *Epipactis* species attract different pollinator groups by using diverse floral cues. In addition we involved the autogamous *E. muellerie* (Baumann 2005) in the analyses, to reveal, if a species that does not have to attract any pollinator nevertheless exhibit floral cues. Recent investigations revealed that the wasp-pollinated orchids *E. helleborine* and *E. purpurata* produce GLVs in the floral odour, and that this volatile compounds are responsible for the wasp attraction (Brodmann 2008), therefore we hypnotized that the non wasp-pollinated species produce less or no GLVs, and instead produce other compounds important for the attraction of other pollinator groups. Furthermore we assumed that the autogamous species *E. muellerie* produce less scent, because it does not have to allure any insect pollinator. Besides the floral odour we compared the optical cues of various *Epipactis* species. Because it is known that optical cues, like bicoloured flowers and UV are important for luring honeybees to flowers (Vöth 1999), we assumed that the bee-pollinated *E. palustris* should reveal optical cues. Besides the odour and the optic we had a look at the nectar sugar composition of the different *Epipactis* species, because we hypnotized that the wasp-pollinated species are dominated by sucrose or that a repellent compound would be present in the nectar to repel unsuitable flower visitors (Baker and Baker 1983).

**Materials and methods**

**Plant material**

Plants of *E. helleborine* (4 plants Blaubeuren, Germany; 3 plants Geislingen, Wasserberg/Herrenberg, Germany), *E. purpurata* (4 plants, Arnegg, Germany), *E. atrorubens* (4 plants, Geislingen, Wasserberg/Herrenberg, Germany), *E. palustris* (3 plants, Gruibingen, Germany), and *E. muellerie* (3 plants, Geislingen, Reichenbach, Germany) were dig out in the years 2007, 2008 and 2009, potted and hold in the Botanical Garden of Ulm, Germany.

**Volatile collection**

Floral scent emitted from flowers of different *Epipactis* species was collected using the dynamic headsace adsorption technique. Intact inflorescences were carefully enclosed in polyester oven bags (Toppits®, Germany), and volatiles were trapped in an adsorbent tube containing 5 mg Super Q (Waters Devisioon of Millipore) by using a membrane pump, adjusted to a flow rate of 500 ml / min for ca. 7 h. The incoming air stream was cleaned of atmospheric pollutants by a charcoal filter (activated charcoal, Supelco, Orbo 32 large). The trapped volatiles in the adsorbent tube were eluted with 40 μl dichloromethane (Sigma-Aldrich, HPLC grade) and the samples were stored at -20°C in the freezer. After each
Chemical mimicry in wasp-pollinated orchids

sampling session, the sorbent tubes were cleaned using dichloromethane, diethylether, and pentane.

**Chemical analyses (gas chromatography, mass spectrometry)**

Headspace samples of the orchids (*E. helleborine* n = 6, *E. purpurata*, *E. atrorubens* n = 4, *E. palustris* n = 3, and *E. muellerie* n = 3) were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a polar DBWax capillary column (J&W, 30 m x 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml / min constant flow) was used as carrier gas. One µl of the sample was injected splitless at 40°C. Subsequently, the splitter was opened after one minute and the oven temperature increased at a rate of 5°C / min to 240°C.

Coupled gas chromatography-mass spectrometry (GC-MS) was performed with a double focusing VG70/ 250 SE mass spectrometer (Vacuum Generators, Manchester, England) linked to a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, US) that was equipped with a fused silica column (FFAP, 50 m x 0.25 mm, operated at an initial temperature of 60°C and programmed to 220°C at a rate of 5°C / min). Structural assignments were based on comparison of analytical data obtained with natural products, data reported in the literature (McLafferty 1989) and those of synthetic reference compounds. Structures of candidate active compounds were verified by co-injection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as internal standard.

**Electrophysiological analyses (GC-EAD)**

Electrophysiological analyses of headspace samples from *Epipactis* flowers were performed on a HP 6890 gas chromatograph (Agilent Technologies) equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae from workers of *V. germanica* were tested with headspace samples of *E. helleborine*, *E. purpurata*, *E. atrorubens*, and *E. palustris*. Antennae from workers of *A. mellifera* were tested with headspace samples of *E. helleborine*. For each EAD the tip of an excised antenna was cut off and the antenna mounted between two glass-capillary electrodes filled with insect Ringer solution. The electrode at the antenna’s base was grounded via an Ag-AgCl wire, and the recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. The gas chromatograph was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 10°C / min. The effluent was split and 30 ml / min of make-up gas (nitrogen) was added (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD=1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the female wasp’s antenna. Natural samples (collected scent) and synthetic compounds (identified upon GC/MS-analyses) were run under the same conditions. A compound was appointed as EAD active when it showed an amplitude in a minimum of 10 runs.
**Behavioural experiments**

The olfactometer experiment involved a Y tube olfactometer (length 22 cm, diameter 0.8 cm). To avoid visual disturbance, the Y tube was horizontally fixed in a box and the only light source was a cold light lamp placed above the centre of the Y tube. A glass cylinder (length 10 cm, diameter 2.5 cm) containing five orchid flowers and an empty control glass cylinder were connected with silicon tubing to the Y tube. Both glass cylinders were connected by equally long silicon tubes to a motor pump (Volcraft, Laboratory Power Supply, PS-302A). Air forced into each glass chamber (50 ml / min) through a single inlet was filtered and cleaned from atmospheric pollutants by a cylindrical borosilicate glass cartridge packed with activated charcoal (Orbo-32, Supelco). After having passed the glass chamber containing the orchid flowers or a blank control, the air streams were directed into the arms of the Y tube. To test synthetic volatiles, 10 µl (containing one headspace equivalent) of the test mixtures (the composition is given below) or of the pure solvent was applied to a piece of filter paper (3 x 0.5 cm), which was placed at each end of the shorter Y tube arms. In all tests, a wasp or honeybee worker was released into the long arm of the Y tube, and its choice of tube arm was registered. A site was counted as “chosen” when the insect touched the filter paper at the end of the tube. For each test, a new insect, a new Y tube, and new filter papers were used. To avoid preference of the insects for one side of the Y tube, the positions of the shanks for treatment and blank control were changed after every run. Each test series was repeated 20 times. The following samples were used in the Y tube tests, tested with wasp workers: 1) flowers of *E. helleborine*, *E. purpurata*, *E. palustris* and *E. atrorubens*, 2) one flower equivalent of a synthetic mixture of EAD active compounds of *E. helleborine* consisting of 0.03 µg hexyl acetate, 0.09 µg octanal, 0.04 µg Z-3-hexenyl acetate, 0.01 µg Z-3-hexenol, 1.28 µg nonanal, 0.22 µg decanal and 0.37 µg benzaldehyde dissolved in pentane. Flowers of *E. helleborine*, as well as the synthetic mixture of EAD active compounds of *E. helleborine* were also tested with honeybee workers. The qualitative and quantitative composition of the synthetic mixture was the same as those found in the flowers, as verified by GC analyses. Synthetic compounds were obtained from Sigma-Aldrich; purity ranged from 95-99%.

**Optical measurements**

Spectral reflectance of *E. helleborine*, *E. palustris* and *E. atrorubens* flowers was measured using an Ocean Optics (Dunedin, Florida, USA) S2000 spectrophotometer as described by Johnson and Andersson (2002). In each case the labellum of four flowers of at least two different plant individuals have been measured.

**Analysis of sugars**

Nectar samples from *E. helleborine* (14 samples from 7 plants), *E. purpurata* (3 samples from 2 plants), *E. palustris* (3 samples from 3 plants), *E. atrorubens* (5 samples from 3 plants), and *E. muellerie* (5 samples from 3 plants) were sampled using 0.5, 1, and 5 µl microcapillary tubes (Hirschmann) in the years 2006 / 2007 and immediately transferred in a 70% ethanol dilution (diluted by sterilized water) and stored in the freezer at -40°C until chemical analyses were performed. Ethanolic samples were vacuum centrifuged, diluted in
pure ion-free water, filtered (VWR Centrifugal Filter, 0.45 µm pores) and divided for separate sugar analysis by high performance liquid chromatography (HPLC; Waters autosampler+, CHM column heater module). Sugar analysis was performed with an isocratic pump (Waters 510), Sentry Guard column (high performance carbohydrate, 3.9 x 20 mm), Waters high performance carbohydrate column (4.6 x 250 mm), solvent (72 : 28 acetonitrile: water mix) and refractive index detector (Waters 410; flow rate 1.4 mL / min). HPLC was controlled and data obtained using Waters Millenium 3.0 software. The sugars glucose, fructose and sucrose were used as standards every 10 samples.

Statistical analyses

Comparison of the absolute amount of EAD active compounds released by the flowers of various *Epipactis* species was done with the Mann-Whitney U-Test with a Benjamini-Hochberg correction (Benjamini and Hochberg 1995). For the statistical analysis of the Y tube experiments we used the “Sign test”. For the analysis of the nectar composition between the species we used the Mann-Whitney U-Test + Benjamini-Hochberg correction (Benjamini and Hochberg 1995).

Results

Electrophysiological and chemical analyses of the orchids' scent

In order to detect the compounds of the orchids' odour bouquet which can be detected by wasps and honeybees, we performed gas chromatography coupled with an electroantennographic detector (GC-EAD) with the headspace samples of the different *Epipactis* species. We found seven electrophysiologically active compounds detected by worker wasps in all *Epipactis* species and also detected by honeybees in *E. helleborine*. By using gas chromatography coupled with mass spectrometry (GC-MS) we identified the aldehydes octanal, nonanal, decanal and benzaldehyde and the GLVs hexyl acetate, Z-3-hexenyl acetate, and Z-3-hexenol, which were present in all investigated *Epipactis* species (Fig. 1). We performed gas chromatographic analyses with the headspace samples of the different *Epipactis* species and compared the absolute amount (µg / inflorescence) of the seven EAD active compounds (Fig. 2). The analyses showed that the GLVs are in significant higher amount in the two wasp-pollinated species *E. helleborine* (absolute amount 0.09 ± 0.02 SE) and *E. purpurata* (absolute amount 0.074 ± 0.02 SE) in comparison to *E. atrorubens* (absolute amount 0.005 ± 0.001 SE, Mann-Whitney U test, p = 0.011, U = 0.0), *E. palustris* (absolute amount 0.002 ± 0.0009 SE, Mann-Whitney U test, p = 0.046, U = 0) and *E. muellerie* (absolute amount 0.001 ± 0.001 SE, Mann-Whitney U test, p = 0.046, U = 0); and *E. purpurata* (absolute amount 0.07 ± 0.01 SE) in comparison to *E. atrorubens* (absolute amount 0.005 ± 0.001 SE, Mann-Whitney U test, p =0.021, U = 0), and *E. palustris* (absolute amount 0.002 ± 0.0009 SE, Mann-Whitney U test, p = 0.034, U = 0), whereas the aldehydes are dominated in *E. palustris*. The bumblebee-pollinated *E. atrorubens* showed a high amount of benzaldehyde (absolute amount 0.166 ± 0.071 SE). In comparison to the
insect-pollinated *Epipactis* species the autogamous *E. muellerie* produce few amounts of compound in the odour bouquet (Fig. 2).

**Figure 1.** Electrophysiologically active compounds with headspace samples of *E. purpurata* flowers. Simultaneous recordings of GC (FID) and EAD signals by using the antenna of a *V. germanica* worker. The GC analyses were performed on a polar DBWax capillary column. We found seven EAD active compounds.

**Figure 2.** Comparison of absolute amount (µg / inflorescence) ± 2 SE of the five *Epipactis* species *E. helleborine*, *E. purpurata* and *E. atrorubens*, and *E. palustris*. Different letters indicate significance differences within the test groups (Mann-Whitney U test with a Benjamini-Hochberg correction (Benjamini and Hochberg 1995), p ≤ 0.05).
Behavioural experiments

In Y tube olfactometer experiments we tested the attractiveness of the flower odour of different Epipactis species to wasps and honeybees. The experiments showed that the scent of the wasp-pollinated E. helleborine is highly attractive for the wasps (Fig. 3; Sign test, p = 0.01, n = 20). In a choice experiment the wasp-pollinated species E. helleborine was significantly more attractive to wasps than the non-wasp pollinated E. palustris and E. atrorubens (Sign test, p = 0.01, n = 20). The two wasp-pollinated species, E. helleborine and E. purpurata are equally attractive to wasps (Sign test, p > 0.05, n = 20), as well as the two non wasp-pollinated species E. atrorubens and E. palustris (Sign test, p > 0.05, n = 20) (Fig. 3).

Further Y tube experiments proved, that the natural scent (Sign test, p = 0.01, n = 20), as well as the synthetic mixture of the EAD active compounds (Sign test, p = 0.001, n = 20) of the wasp-flower E. helleborine were highly attractive for the wasps (Fig. 4). The synthetic mixture showed the same attractiveness than the volatiles emitted from natural flowers to wasps, indicating that there are all important compounds for the wasp attraction included in the synthetic mixture (Sign test, p > 0.05, n = 20). Honeybees, who are not the pollinators of the wasp-flower E. helleborine, are not attracted by the floral scent of E. helleborine (Sign test, p > 0.05, n = 20) nor of the synthetic mixture of the EAD active compounds (Sign test, p > 0.05, n = 20) (Fig. 4).

Figure 3. Comparison of the attractiveness of the odour from flowers of the two wasp-pollinated E. helleborine (E. helle) and E. purpurata (E. purp) and the non wasp-pollinated E. palustris (E. palu) and E. atrorubens (E. atro) flowers, to the wasps V. germanica in a Y tube olfactometer (Sign test, * p ≤ 0.05, n = 20).
Optical measurements

The flowers of the wasp-pollinated *E. helleborine* are cryptic reddish-brown, whereas the flowers of *E. atrorubens* are dark purple, with the reflectance peaks at about 450 nm and 700 nm (Fig. 5). The flowers of *E. palustris* are white with orange-yellow spots in the middle of the labellum. The optical analyses of the flower lips of the orchid species showed (Fig. 5) that only *E. palustris* exhibit a reflectance in the UV area on its labellum (300-400 nm) (Chittka et al. 1994), whereas *E. helleborine* and *E. palustris* flowers showed no reflectance on this wavelength (Fig. 5).

**Figure 4.** Comparison of the attractiveness of the synthetic mixture of the EAD active compounds of *E. helleborine* (*E. helle*) against the solvent as a control and *E. helleborine* flowers to social wasps and honeybees in a Y tube olfactometer (Sign test, * p ≤ 0.05, ** p ≤ 0.01, n = 20).
Chemical mimicry in wasp-pollinated orchids

Analysis of sugars
Glucose, fructose, and sucrose were the only sugars identified in the nectar of the investigated *Epipactis* species with a dominance of sucrose. *E. helleborine* was the only species where we could find traces of glucose (Tab. 1). In all other species we identified sucrose and fructose only, except *E. palustris* which had only sucrose in the nectar (Tab. 1). The two wasp-pollinated species *E. helleborine* and *E. purpurata* showed significant differences in the proportions in sucrose and fructose (Mann-Whitney U test, \( p = 0.003, U = 0 \)). In *E. helleborine* the relative amount of fructose was significantly lower than in *E. purpurata* (Mann-Whitney U test, \( p = 0.003, U = 0 \)) and in *E. muellerie* (Mann-Whitney U test, \( p = 0.001, U = 7.5 \)).

![Figure 5. Comparison of the relative reflectance (%) of the labellum of the three species *E. helleborine*, *E. atrorubens* and *E. palustris*. The area from 350 - 400 nm show UV reflectance.](Image)

Table 1. Relative amount (%) ± SE of the three sugars sucrose, fructose, and glucose in *E. helleborine*, *E. purpurata*, *E. atrorubens*, *E. palustris*, and *E. muellerie*. N is the sample size. Mann-Whitney U test, \( p \leq 0.05 \) + Benjamini-Hochberg correction. Different letters indicate differences between the test species.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Sucrose (%) ± SE</th>
<th>Fructose (%) ± SE</th>
<th>Glucose (%) ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. helleborine</em></td>
<td>14</td>
<td>99.8 ± 0.2 ( ^{a} )</td>
<td>0.1 ± 0.1 ( ^{a} )</td>
<td>0.1 ± 0.1 ( ^{a} )</td>
</tr>
<tr>
<td><em>E. purpurata</em></td>
<td>3</td>
<td>56.9 ± 4.6 ( ^{bc} )</td>
<td>43.0 ± 4.6 ( ^{bc} )</td>
<td>0.0 ( ^{a} )</td>
</tr>
<tr>
<td><em>E. atrorubens</em></td>
<td>5</td>
<td>94.1 ± 3.6 ( ^{a} )</td>
<td>5.8 ± 3.6 ( ^{a} )</td>
<td>0.0 ( ^{a} )</td>
</tr>
<tr>
<td><em>E. palustris</em></td>
<td>3</td>
<td>100 ± 0 ( ^{ad} )</td>
<td>0.0 ( ^{ad} )</td>
<td>0.0 ( ^{a} )</td>
</tr>
<tr>
<td><em>E. muellerie</em></td>
<td>5</td>
<td>79.4 ± 8.0 ( ^{cd} )</td>
<td>20.5 ± 8.0 ( ^{cd} )</td>
<td>0.0 ( ^{a} )</td>
</tr>
</tbody>
</table>
Discussion

The orchids' scent

In wasp-pollinated *Epipactis* species floral odour, more precisely GLVs, play the key role in pollinator attraction (Brodmann et al. 2008). The results of our chemical analyses of the floral odour proved our assumption that the non wasp-pollinated species do not produce such high amount of GLVs like the wasp-pollinated *E. helleborine* and *E. purpurata*, because GLVs are not attractive for other pollinators. Behavioural experiments clearly showed the attractiveness of the floral scent of the wasp-pollinated *E. helleborine* for social wasps. In contrast, honeybees that are able to perceive the same compounds in the odour bouquet of *E. helleborine*, are not attracted in a behavioural experiment (Brodmann et al. 2008). Our investigations support former findings that GLVs are only produced in higher amounts in wasp pollinated species of *Epipactis*. In contrast, the non wasp-pollinated *Epipactis* species produce higher amounts of the aldehydes octanal, nonanal, decanal, and benzaldehyde in the floral odour bouquet than the wasp-pollinated species. In *E. atrorubens*, mainly pollinated by bumblebees, we found benzaldehyde as the major EAD active compound in the floral odour. Benzaldehyde is known to be an important substance with a pollinator attracting role in plant-insect relationships. It occurs in many bee-pollinated plants (Knudsen et al. 2006), which could explain the attractiveness of *E. atrorubens* to bumblebees.

Our chemical analyses of the floral odour of *E. palustris* confirmed the findings by Godfrey (1933) that the white flowers are almost scentless. Recent investigations showed that *E. palustris* mostly occurs together with other strong scented flowers. It may use a scent producing magnet species, the orchids *Gymnadenia conpoposa* and *G. odoratissima* to attract potential pollinators from a distance (Schuffenhauer 2009). The odour of *Gymnadenia* sp. proved to be highly attractive to honeybees (Schuffenhauer 2009). After reaching the close surrounding of *E. palustris* plants visual cues like the UV reflecting labellum and pollen mimicry may be responsible for attracting pollinators to visit the flowers. After finding a nectar reward the pollinators may proceed to visit further flowers (Schuffenhauer 2009). In the non-rewarding orchid *Anacamptis morio* it was demonstrated that in specimen that are associated with nectar producing plants, the pollination success of the orchids increase (Johnson et al. 2003).

In the autogamous species *E. muellerie* we found only trace amounts of floral scent compounds. Therefore our hypothesis that this species should not produce GLVs, because it is autogamous and is not depended in attracting pollinators is confirmed by our investigations.

Visual signals of the flowers

The flowers of the wasp-pollinated *E. helleborine* possess dull brown, dirty-red to washed yellow-green colours, which are for long time though to be responsible for the wasp attraction (Wiefelspütz 1970). This assumption was recently revised, and the floral odour is proved to be more important than the optical cues in attraction of social wasps (Brodmann et al. 2008). The inconsiderable coloration of the wasp-pollinated *Epipactis* species may play a role in limiting visual attraction to insects that are morphologically unsuitable as vectors of the
pollinaria or which exploit nectar without pollinating. Interestingly, the bumblebee-pollinated *E. atrorubens* has dark purple flowers. Bumblebees are thought to have a favourite colour corresponding to the blue range of wavelengths (Lunau and Maier 1995). Because the landing reaction of bumblebees is known to be released by olfactory and by optical signals (Lunau 1990; Lunau 1991), we assumed that the dark purple colour of *E. atrorubens* plays an essential role in pollinator attraction in combination with the strong vanilla scent of the flowers (Vöth 1999), which was recently shown in behavioural experiments (Appel 2008). It is known that optical cues, like bicoloured flowers and UV are important for luring honeybees to flowers (Vöth 1999). Many bee-pollinated flowers are bi-coloured, so that they send out different visual signals from different areas (Lunau et al. 2006). Because optical measurements revealed that *E. palustris* exhibit a UV reflectance on the labellum of the flowers, also described by Vöth (1999), we assumed that the optical cues of the flowers could be responsible for the attractiveness for honeybees. Our hypothesis was confirmed by behavioural experiments, which showed that honey bees are more attracted to the optical cues than the olfactory cues of *E. palustris* (Appel 2008).

The role of nectar in pollinator attraction

In pollinator-plant interactions many attention has been given on the sugars in the nectar of flowers, because they are the basis of the energy reward that flower-visitors receive while sucking nectar from flowers. The *Epipactis* species involved in our study offer exposed nectar, and flowers with exposed nectar are usually exploited by a wide range of short- and long-tongued insects, resulting in generalized pollination systems (Johnson and Steiner 2000; Waser et al. 1996). Because the species *E. helleborine* and *E. purpurata* only attract social wasps for pollination, and are almost ignored by other potential flower visitors (Darwin 1888), we hypnotized a specific sugar composition, a repellent sugar or other unpleasant substances in the nectar to deter nectar thieves. In contrast to the hypothesis of Baker & Baker (1983) that wasp-pollinated flowers are sucrose dominated, our analyses revealed that the nectar of all investigated *Epipactis* species, amongst them non wasp-pollinated species, possess a sucrose dominance. Furthermore, we did not find other sugars with a possible function as repellent compounds. Therefore our data do not confirm the hypothesis of Baker & Baker (1983), which indicates that there could be a repellent sugar like cellobiose and gentiobiose in the sugar of wasp-pollinated flowers to repel nectar thieves. In order to answer the question, if the nectar may even contain toxic alkaloids, amino acids or other unpleasant substances (Baker 1975; Baker and Baker 1977; Baker et al. 1978), further nectar analyses of the wasp-pollinated *Epipactis* species are necessary. Ehlers and Olesen (1997) showed, that in the wasp-pollinated species *E. helleborine* the nectar is toxic and could makes the visiting wasps “sluggish”. A recent study revealed that the nectar of the wasp-pollinated milkweed *Pachycarpus grandiflorus* is unpalatable to honeybees but palatable to the wasps, and thus serve as filter to repel unwished nectar thieves (Shuttleworth and Johnson 2009).
Conclusion

Our investigations support the hypothesis that in the orchid genus *Epipactis* various pollination syndromes evolved. The wasp-pollinated *Epipactis* species emit specific odour compounds, GLVs, in order to mimic the presence of prey items, and thereby attract prey-hunting wasps for pollination (Brodmann et al. 2008). Behavioural experiments demonstrated that the floral odour of the wasp-pollinated *E. helleborine* is highly attractive to wasps, but not to other flower visitors, like honeybees. By producing this wasp specific scent in combination with a cryptic coloration of the flowers, the orchid is able to avoid the attraction of other potential flower visitors. In the mainly bumblebee-pollinated *E. atrorubens* the dark purple colour, in combination with the release of benzaldehyde may be responsible for pollinator attraction. In contrast to the wasp specific floral odour of the wasp-pollinated species benzaldehyde is well known in many flowering plants (Knudsen et al. 2006). Trapping experiments showed that benzaldehyde attracts, amongst others, noctuid moths (Meagher 2002), flesh flies (Sarcophagidae), and ladybeetles (David 2005). Therefore the visitor spectrum is broader than in the wasp-pollinated species, which may mean a loss of pollen for the orchid to unsuitable flower visitors. The nearly scentless *E. palustris* seems to lure its pollinators with the help of UV patterns on the flowers, which is shown to be an important attractant for honeybees (Vöth 1999). The autogamous species *E. muellerie* almost completely reduced scent production and does not show a flashy colouration, thereby reducing costs for pollinator attraction. But besides the investigated floral cues also the morphology of the flowers could have an effect of the choice on the pollinator. It has been shown that wasp-flowers exhibit physiological and morphological adaptations for the pollination by social wasps (Müller 1873a). For instance the head of the wasp fits optimally in the flower shape, which benefits an optimal pollination. In general, flowers visited by social wasps tend to have a dull coloration and exposed nectar (Müller 1881; Schremmer 1962; Werth 1956). Furthermore the choice of the habitat surely also plays a role in pollinator attraction. The two wasp-pollinated species grow in shadow habitats in the edge of forests, where the occurrence of bees is rare, whereas the bee-pollinated species prefer open, sunny habitats.

In conclusion our results support the hypothesis that in various species of the genus *Epipactis* different pollinator attracting signals in combination with the occupation of different niches evolved in order to avoid competition for pollinators.

Acknowledgments

We thank Werner Hiller and Manfred Kalteisen who helped us to collect *Epipactis* plants. Furthermore we thank Peter Zindl (Botanical Garden of the University of Ulm) for cultivating the orchids, and Hans Malchus (Institute of Systematic Botany and Ecology) for helping us with the nectar analysis. We gratefully acknowledge the FAZIT Foundation for financial support.
Chemical mimicry in wasp-pollinated orchids

References


Baker HG (1975) Sugar concentrations in nectars from hummingbird flowers. Biotropica 7:37-41


Baumann H (2005) Die Orchideen Deutschlands. Arbeitskreise Heimische Orchideen (Bad Hersfeld, Germany: Hoehl-Druck)


Darwin CR (1888) The various contrivances by which orchids are fertilised by insects, 2nd edition (London: John Murray)


del Rio C (1990) Sugar preferences in hummingbirds: the influence of subtle chemical differences on food choice. Condor 92:1022-1030


Godfrey MJ (1933) Beobachtungen an *Epipactis purpurata* Sm. und ihrer chlorophyllfreien Form.
Chemical mimicry in wasp-pollinated orchids


Lunau K (1990) Colour saturation triggers innate reactions to flower signals: Flower dummy experiments with bumblebees. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 166:827-834


Mazokhin-Porshniakov GA (1962) Farbmetrischer Beweis der Trichromasie des Farbensehens der Bienen (am Beispiel der Hummel). Akad Nauk USSR Bioflzika 7:221-227


Müller H (1873) Die Befruchtung der Blumen durch Insekten und die gegenseitigen Anpassungen beider (Leipzig, Germany: Engelmann Verlag)


Robertson C (1928) Flowers and insects. Lists of visitors to four hundred and fifty-three flowers. C. Robertson, Carlinville, Illinois, USA


6.

The role of innate and learnt floral odour preferences in the pollinator attraction of the wasp-pollinated orchid *Epipactis helleborine*

Jennifer Brodmann, Manfred Ayasse

Institute of Experimental Ecology, University of Ulm, Germany
Abstract

A fascinating example of chemical mimicry in the pollination strategy of orchids was recently revealed in the orchid genus *Epipactis*. In the orchid *Epipactis helleborine*, exclusively pollinated by social wasps (Hymenoptera: Vespidae), it was shown that the floral odour plays the primacy function in the wasp attraction, more precisely so called “green leaf volatiles” (GLVs) (Brodmann et al. 2008). By emitting this volatiles that are usually emitted transiently by wounded plants infested by herbivores and, thus, deceptively indicating the presence of prey, the flowers are capable of attracting their pollinators. It could be shown that parasitic wasps use them to locate its prey (Whitman and Eller 1990), and predatory social wasps are highly attracted to this compounds as well (Brodmann et al. 2008). Due to the fact that these GLVs are very common and widespread in nature, we hypnotized that, besides this common occurring compounds, the orchid should also produce more specific floral odour compounds, that would modulate, in combination with the nectar reward, the behaviour of visiting wasps and would bring them to visit further flowers of the same species. This may finally guarantee pollination and pollen flow.

In this study we used electrophysiologically active compounds of flowers of *E. helleborine* that have been identified in headspace samples and solvent extracts, and tested two different syntethic mixtures of the complex flower odour bouquet of the orchid, first a mixture of unspecific GLVs, and secondly more specific floral odour compounds that were identified in solvent extracts of flowers in behavioural learning experiments. We examined the attractiveness of both mixtures of volatiles for naive wasps and for wasps that were 48 h conditioned with the same odours in combination with a sugar water reward in dual-choice experiments in a wind tunnel.

The results demonstrated that social wasps have innate preferences for the GLVs, whereas the attractiveness of the more specific floral odour compounds increased after learning. Therefore we assume that the volatile GLVs are responsible for the wasp attraction from a distance in the orchid naive wasps, which expect to find prey for its brood. But instead of a prey item the wasps discover nectar that is also needed as a carbon hydrate source for themselves and the larvae. While drinking the first time nectar, associating the low volatile floral odour with a nectar reward, the wasp learns the more specific compounds of the flower odour. The combination of innate and learnt behaviour of the pollinating wasps on different odour compounds of *E. helleborine* assuring a highly specific and effective pollination of the orchid flowers, because the positive association encourages the wasps to visit more flowers of this species.
Introduction

Over the past few decades, researchers have shown that insects are capable of associative learning, defined as the ability to acquire a neural representation of a new association between a stimulus and an environmental state that may affect fitness (Dudai 1989). Olfactory learning in insects has been studied most intensively in honeybees, using the insect proboscis extension reflex (PER), a technique pioneered by studies on bees (Bitterman et al. 1983; Kuwabara 1957; Menzel 1999). Besides honeybees, well-studied model systems are fruit flies, *Drosophila melanogaster*, and parasitic wasps. Parasitic wasps are known to exploit numerous chemical cues to locate hosts and food and it is shown that associative learning of different odour cues are involved in this learning (Lewis and Tumlinson 1988; Olson et al. 2003; Turlings et al. 1993). It could be demonstrated that they are able to form associations between volatile odorants from the faeces of hosts and other host specific non-volatile waste products with the presence of the host (Lewis et al. 1991), as well as between host-plant volatiles, such as GLVs, and host location/oviposition (Kaas et al. 1990; Kerguelen and Carde 1996; Zanen and Carde 1991). In comparison to the broad knowledge of parasitic wasps, comparatively less is known about social wasps and the capability of olfactory learning while foraging. It could be shown experimentally that foragers of the social wasp *Polybia sericea* in Brazil use visual and olfactory prey cues to relocate a foraging area and that at close range olfactory prey cues were more likely to elicit landing on stationary prey than were visual cues (Raveret Richter and Jeanne 1985). Other studies confirmed that social wasps are able to learn various fruit-like odours after a reward (Jander and Rudolf 1998).

Also in plant-insect interactions olfactory learning is from high interest, and besides visual cues floral volatiles are of major importance (Vogel 1983). From honeybees it is shown that they learn odours faster than colours (Koltermann 1969). There is some evidence that learning influences flower handling time in bumblebees (Laverty and Plowright 1988), honeybees (Sanderson et al. 2006), as well as the nectar-foraging behaviour of butterflies (Lewis 1989; Lewis 1993), and moths (Cunningham et al. 1998; Lewis 1989; Weiss 1997). Communication between flowering plants and their pollinators involves innate and learnt sensory signals. In this context the question, how the pollinating insect finds the flowers in the first place, and which cues have to be learnt while rewarding with flower nectar became interesting. For example flower-naive honeybees (Giurfa et al. 1995b) and the bumblebee *Bombus impatiens* (Lehrer et al. 1995) have unlearned colour and pattern preferences and they do not learn any stimuli in the absence of a reward (Simonds and Plowright 2004).

Over centuries more insects specialized to visit one or a few plant species, and during co-evolution between pollinator and flower the insects evolved the ability to learn olfactory and colour cues of the flowers to recognize their host plants. One example of such a specialization is the wasp-pollinated orchid *Epipactis helleborine* (L.) Crantz, which is mainly pollinated by social wasps (Hymenoptera: Vespidae) like *Vespula vulgaris* and *V. germanica* (Müller 1873). As noted by Darwin (1888), this species is almost entirely overlooked by other potential pollinators, despite a large nectar reward. A long time the mechanism for the attraction of pollinating social wasps was something of a mystery. In contrast to other flower
visitors, like bees, social wasps feed their larvae on small arthropods, like caterpillars (Schremmer 1962). In order to locate its prey, they are known to use a combination of visual and olfactory cues (Cornelius 1993). In parasitic wasps it is known, that they use volatiles (e.g. green leaf volatiles) emitted by green plant tissue to locate insect prey (Paré and Tumlinson 1999; Turlings et al. 1990). By using a combination of behavioural experiments, electrophysiological investigations, and chemical analysis it could be demonstrated that the wasp-flower *E. helleborine* emits “green leaf volatiles” (GLVs), which are attractive to foragers of the social wasps *V. germanica* and *V. vulgaris*. Furthermore it could be shown that the two wasp-pollinated species *E. helleborine* and *E. purpurata* produce a higher amount of GLVs than the non-wasp pollinated *E. atrorubens*. This results proved that these floral GLVs definitely have a key function in the wasp attraction (Brodmann et al. 2008). Because these GLVs are very common and widespread occurring compounds in nature, and wasps always get in touch with them, we addressed the question, how the orchid *E. helleborine* stays interesting for the wasps, and thereby ensures its reproductive success. The bluffed wasp lands on the flower, however, it does not find any prey item, but instead receives a nectar reward. We hypnotized that the orchid needs to produce a more specific floral odour cue that is learnt by the pollinator while the first flower visit. In former investigations low volatile electrophysiologically active compounds were identified, but the function of these compounds was so far unclear (Hölzler 2003). In our study we wanted to answer the question, which odour compounds of the complex flower odour bouquet of the orchid *E. helleborine* is recognized by naive wasps and which ones are learnt while a wasp is visiting a flower for the first time and do have a function to modulated the behaviour of the pollinating social wasp.

**Methods and Materials**

**Volatile collection**

The volatile compounds of the flowers of the orchid *E. helleborine* were collected using dynamic headspace adsorption techniques. Intact inflorescences were carefully enclosed in polyester oven bags (Toppits®, Germany), and volatiles were trapped in an adsorbent tube containing 5 mg Super Q (Waters Devison of Millipore) by using a membrane pump, adjusted to a flow rate of 500 ml / min for ca. 7 h. The incoming air stream was cleaned of atmospheric pollutants by a charcoal filter (activated charcoal, Supelco, Orbo 32 large). The trapped volatiles in the adsorbent tube were eluted with 40 µl dichloromethane (Sigma-Aldrich, HPLC grade). After each sampling session, the sorbent tubes were cleaned, using diethyl ether, dichloromethane, and pentane. Furthermore we collected low volatile compounds on the flower surface. Single flowers were put in a vial with 1 ml pentane and volatiles were extracted for 24 h at room temperature (25°C) in darkness. Afterwards the flowers were removed and the samples were concentrated (30 µl / blossom equivalent) and stored at -30°C in the freezer.
Chemical analyses

Headspace samples were analyzed on a Thermo Trace gas chromatograph (Thermo Electron, Waltham, Massachusetts, USA) equipped with a DB-Wax capillary column (J&W, 30 m x 0.25 mm) and a flame ionization detector (FID). Hydrogen (2 ml / min constant flow) was used as carrier gas. One µl of the sample was injected splitless at 40°C. Subsequently, the splitter valve was opened and the oven temperature increased at a rate of 5°C / min to 240°C. Flower extracts were analyzed on a Hewlett Packard 6890 gas chromatograph equipped with a DB5ms column (J&W, 30 m x 0.25 mm). Helium was used as carrier gas. Coupled gas chromatography-mass spectrometry (GC-MS) was performed with a double focusing VG70/250 SE mass spectrometer (Vacuum Generators, Manchester, England) linked to a HP 5890 gas chromatograph (Hewlett Packard, Palo Alto, US) that was equipped with a fused silica column (FFAP, 50 m x 0.25 mm, operated at an initial temperature of 60°C and programmed to 220°C at a rate of 5°C / min). Structural assignments were based on comparison of analytical data obtained with natural products, data reported in the literature (McLafferty 1989) and those of synthetic reference compounds. Structures of candidate active compounds were verified by co-injection. For quantitative analyses, defined amounts of n-octadecane (Sigma-Aldrich) served as internal standard.

Electrophysiology

Electrophysiological analyses of headspace samples, as well as flower extracts from *E. helleborine* flowers were performed on a HP 6890 gas chromatograph (Agilent Technologies) equipped with an FID and an EAD setup (Syntech, Hilversum, Netherlands). Antennae from workers of *V. germanica* and *V. vulgaris* were tested. For each EAD the tip of an excised antenna was cut off and the antenna mounted between two glass-capillary electrodes filled with insect Ringer solution. The electrode at the antenna’s base was grounded via an Ag-AgCl wire, and the recording electrode at the tip of the antenna was connected via an amplifier to a signal interface board (Syntech, Hilversum, Netherlands) of a PC. The gas chromatograph equipped with a DBWax capillary column for the headspace samples or rather a DB5ms column for the flower extracts was operated splitless at 50°C for 1 min, followed by opening of the split and programming to 240°C at 10°C / min. The effluent was split and 30 ml / min of make-up gas (nitrogen) was added (variable outlet splitter (SGE, Darmstadt, Germany); split ratio FID:EAD=1:3). The outlet for the EAD was placed in a cleaned and humidified airflow that was directed over the female wasp’s antenna. Natural samples (collected scent) and synthetic compounds (identified upon GC/MS-analyses) were run under the same conditions.

Behavioural experiments

Dual-choice flight chamber experiments were carried out at the University of Ulm, Germany, in a wind tunnel with a Perspex flight chamber (L x W x H: 2 x 0.73 x 0.78 m) that was equipped with a diffuse light source from the top. Air was directed through the flight chamber with a speed of 4.5 ms-1. A clean air stream was maintained by passing the incoming air through an activated charcoal filter and a dust filter before allowing entering the chamber. A
wasp nest in a wooden box that contained circa 50 - 100 individuals was connected to the tunnel so that the wasps, while foraging for food, were trained to fly in the wind tunnel. We offered the wasps sugar water ad libitum, freshly killed honeybees and wood as building material in the wind tunnel. We marked all the wasps with a colour spot on their thorax, and let them acclimatize in the wind tunnel for around 5 - 7 days. Once a day the wind tunnel was switched on for 30 min in the intention to get the wasps used to the conditions while the behavioural experiments. For the tests only new emerged, flower and odour naive, wasps were used.

For the dual-choice experiments we used a similar Piezoelectric ultrasound sprayers setup as described in El-Sayed et al. (1999). The odorant solution was released using a motor-driven syringe pump (SP101i, WPI) that pushes the odorant solution from an 1 ml syringe to the tip of a glass capillary. The capillary tip is excited to oscillate at ~120 kHz. This releases one or two microtroplets of odorant solution at each oscillation and results in an aerosol of the solution with 36.5 µl / min. To protect the capillaries from the wasps they were covered with an air permeable box.

In order to test if naive wasps would be attracted to EAD active compounds in headspace samples or flower extracts we offered to newly emerged wasps in a dual-choice experiment synthetic mixtures of biologically active compounds and pentane as a control using two ultrasonic sprayers. During a time period of five minutes the number of wasps landing on the box in front of each of the two sprayers was counted. The test was repeated for seven times in the case of the synthetic mixture of the GLVs and 14 times for the synthetic mixture of the flower extract with a total of two respectively five nests. After these tests with odour naive wasps we conditioned them using mixtures of the EAD active odour compounds in headspace samples and flower extracts that were added into the sugar feeder. The wasps were allowed to drink the perfumed sugar solution (sugar : water 1 : 1) for 48 h ad libitum. After this conditioning phase we repeated the test with the odour experienced wasps.

The synthetic mixture of the EAD active GLVs consisted of 0.03 µg hexyl acetate, 0.04 µg Z-3-hexenyl acetate, and 0.01 µg Z-3-hexen-1-ol dissolved in 2.5 ml pentane. The synthetic mixture of the EAD active compounds of the flower extracts contained 1.42 µg heptanal, 0.03 µg pentadecanal, 0.06 µg hexadecanal, 1.86 µg hexacecan-1-ol, and 3.8 µg vanillin dissolved in 2.5 ml pentane. 10 µl of each mixture represent one headspace or rather one flower equivalent and was used for the behavioural experiments, whereas the sprayer released 3.65 flower equivalent / min.

Data analysis
In the dual-choice experiments we registered the amount of landings of the wasps on the boxes in front of the two sprayers. For statistical analysis we compared the percentage of landings between the synthetic mixtures and the control pentane and between the test groups before and after conditioning by the paired samples t-test.
Results

Chemical and electrophysiological analyses

In headspace samples collected from *E. helleborine* flowers we found seven compounds inducing an electrophysiological response in antennae of workers of *V. germanica* and of *V. vulgaris*. Using gas chromatography coupled with mass spectrometry (GC-MS) we identified, amongst others the GLVs hexyl acetate, Z-3-hexenyl acetate and Z-3-hexen-1-ol in inflorescences (Brodmann et al. 2008). In the flower extract five EAD active compounds could be identified, namely heptanal, pentadecanal, hexadecanal, hexacecan-1-ol, and vanillin (Hölzler 2003).

Behavioural experiments

In the behavioural experiments, naive wasps as well as experienced ones preferred the GLVs in comparison to the solvent control (Fig. 1 A; paired samples t-test, n = 7; GLVs naive: p < 0.001, t = 7.48, GLVs experienced: p < 0.001, t = 12.00). However, after the conditioning phase there was no significant increase in the attractiveness of GLVs that were equally attractive for naive and experienced wasps (Paired samples t-test, n = 7 p = 0.726, t = -0.367). Naive wasps, as well as experienced wasps choose the GLVs with approx. 93% against the solvent control. The results of the tests in which we used a synthetic mixture of the EAD active compounds of the flower extracts in contrast revealed, that after the conditioning phase a significantly higher number of wasps landed on the sprayer that released EAD active compounds of the flower extracts (Fig. 1 B; paired samples t-test, n = 14, p = 0.003, t = -3.58). In the dual-choice experiment 56% of the naive wasps and 84% of the experienced ones preferred the sprayer emitting EAD active compounds of the flower extract. Furthermore, naive wasps in contrast to the experienced ones did not prefer the synthetic mixture towards the control pentane (Fig. 1 B; paired samples t-test, n = 14; experienced wasps: p < 0.001, t = 11.38).
Figure 1. Reactions of naive and experienced wasps to the synthetic mixtures of the GLVs in headspace samples (A) and the flower extract (B) in dual-choice experiments in a flight chamber experiment. Bars represent percentage of landings (%) ± standard error of the mean. Different letters indicate significance differences within and beyond the test groups (Paired samples t-test, n = 7/14 , p < 0.05).

Discussion

It is known that hymenopteran pollinators apply innate search images for selecting potential food sources (Giurfa et al. 1995a; Menzel 1985) or locations of food on flowers (Lunau 1991). Compared to visual cues, olfactory cues are predicted to play a more crucial role in the discrimination of flowers by bees (Kunze and Gumbert 2001). In several flowers, exclusively pollinated by social wasps, floral odour is proved to play the key role in wasp attraction (Brodmann et al. 2008; Brodmann et al. 2009; Cheng et al. 2009).

Recent investigations revealed that the wasp-pollinated orchid *E. helleborine* produce certain volatile compounds, namely GLVs, and that this compounds are responsible for the wasp attraction (Brodmann et al. 2008). The results of our study demonstrated that these GLVs are highly attractive for naive as well as for experienced social wasps, indicating that wasps have a strong preference for the highly volatile GLVs. In parasitic wasps, which use certain compounds, amongst them GLVs, for prey location (Turlings et al. 1993; Whitman and Eller 1990), it has already been shown, that naive wasps orient to certain GLVs the first time they encountered the odours (Whitman and Eller 1990). In conclusion this implies that parasitic as well as predatory wasps have a highly refined, innate, and genetically based proclivity to respond to these substances and that there is a long evolutionary adaptation of the wasps to react extremely sensitive on GLVs. But besides these GLVs, which proved to release searching behaviour in naive wasps, we also identified low volatile compounds in the orchids` odour, for example vanillin, which modulate the behaviour of wasps by experience.
Behavioural experiments showed that naive wasps do not react on these low volatile compounds, but experienced ones did, indicating that this compounds are learnt while the first flower contact. Because the volatile floral GLVs, which release an innate searching behaviour in wasps, are very common and widespread in nature, the orchid produces more specific floral odour compounds in combination with a nectar reward, and the visiting wasps learn the more specific floral odour compounds. The main compound, vanillin, is for instance, known as a very common compound in orchid fragrance (Kaiser 1993), and is, amongst others, known to attract male Euglossina bees to orchid flowers (Nemésio 2005). Because common compounds are assumed to be easier to learn by the pollinator than rare ones, we suggested that this compound is learnt by the wasps during their first flower visit. Learning flower odours while visiting a flower is a very common phenomenon. In honeybees it is known that associative learning becomes an essential component of the foraging behaviour (Gould 1993; Menzel 1985). Thus, for instance, forager honeybees learn visual and chemical cues to search and recognize their foraging targets (von Frisch 1965), or learn floral odours while the act of throphallaxis in the honeybee hive (Farina et al. 2005).

The results of our study supposed our assumption, that there are more common, widespread and extremely volatile compounds in the floral odour which activate the innate behaviour of the wasps and more specific compounds which are learnt while the first flower visit. The combination of the innate GLVs and other substances learnt by experience, guarantee the flowers an successful reproduction, because the wasps rewarded with nectar surely will visit other flowers of the orchid and thereby pollinate it.

References

Darwin CR (1888) The various contrivances by which orchids are fertilised by insects 2nd edition John Murray, London


Müller H (1873) Die Befruchtung der Blumen durch Insekten und die gegenseitigen Anpassungen beider. W. Engelmann Verlag, Leipzig
Schremmer F (1962) Wespen und Hornissen. Die neue Brehm-Bücherei
Zanen PO, Carde RT (1991) Learning and the role of host-specific volatiles during in-flight host-finding in the specialist parasitoid Microplitis-Croceipes. Physiological Entomology 16:381-389
Publications from this thesis


Contributions of co-authors

Manfred Ayasse as my supervisor. He is co-author on all my manuscripts derived from this thesis.

Wittko Francke, Robert Twele, Stefan Franke, and Joachim Ruther. They all did the structure elucidation of the odour compounds.

Gerald Hölzler did a part of the experiments on *Epipactis helleborine*, and is therefore co-author on manuscript 1.

My Chinese collaborators Luo Yi-bo and Song Xi-quiang. They give me the possibility for my work on the orchid *Dendrobium sinense* on the Chinese island Hainan, and are therefore co-authors on the manuscript 2.
Mein herzlicher Dank geht an

Prof. Dr. Manfred Ayasse, meinem Betreuer, für die Ermöglichung und herzliche Betreuung meiner Doktorarbeit, sowie für die schönen Sammelreisen in der Türkei

Prof. Dr. Harald Wolf, für die Erstellung des Zweitgutachtens meiner Arbeit

Den Chemikern aus Hamburg, sowie Prof. Dr. Joachim Ruther für die Identifizierung der chemischen Substanzen in meinen zahlreichen Duftproben

Der FAZIT Stiftung, Robert-Bosch Stiftung, Minerva-Stiftung, sowie Rescue Sterling, USA, für die finanzielle Unterstützung meiner Arbeit

Der Feuerwehr Neu-Ulm, für die Bereitstellung abgehängter, gemeingefährlicher, Wespennester. Und allen Leuten, die mir bei der Beschaffung von Wespennestern geholfen haben

Dem Botanischen Garten Ulm, und ganz besonders Peter Zindel, für die Mühe mit all meinen Pflanzen

Hans von der Bio 5, für die Hilfe bei den Nektaranalysen

Den Orchideenspezialisten Herr Hiller und Herr Kalteisen, für das Aufspüren der Orchideen

Meinen tapferen Helfern in China im Dschungel, mit den Orchideen und den Hornissen

Prof. Dr. Amots Dafni für seine tatkräftige Unterstützung in der Türkei und in Israel

Den Kollegen der Abteilung Bio 3, und ganz speziell der Arbeitsgruppe Chemische Ökologie, für das gute Arbeitsklima

Den österreichischen Landsleuten Stefan, der bei jedem Problem mit Rat zur Seite stand, und ganz besonders Johannes für seine Freundschaft in all den Jahren und die schöne Forschungsreise in Israel

Sebastian, der mir meinen Computer mehr als einmal gerettet hat

Dem Frauenzimmer (Ann-Marie, Hannah, Julia, inkl. Dauergast Kirsten) für die gute Stimmung und die schöne Zeit hier

Ann-Marie und Kirsten für ihre Freundschaft und ihre tatkräftige Unterstützung in allen Lebenslagen

Meinen Freunden, und ganz besonders meiner Familie.
Curriculum vitae
Jennifer Brodmann
University of Ulm, Institute of Experimental Ecology
Albert-Einstein-Allee 11, 89069 Ulm, Germany
email: jennifer.brodmann@uni-ulm.de
phone: 0731 - 5022662 / mobil: 0176 - 82083656

• Personal Information
  Name       Jennifer Brodmann
  Date of birth      11.10.1981
  Place of birth     Tettnang
  Citizenship       Germany

• Education
  1988 – 1992      Basic school Tettnang
                    high school diploma

• University Education
  Sep 2002 - Apr 2007    Studies in biology (Diplom), University of Ulm
  Sep 2006 - Apr 2007    Diploma thesis: “Pollinator-attracting semiochemicals of
                        the wasp flower Epipactis helleborine”
  Since Apr 2007         PhD student
                        Doctoral thesis: „Pollinator attraction in wasp-flowers“, in
                        the Institute of Experimental Ecology, working group
                        Chemical Ecology, Prof. Dr. Manfred Ayasse
                        (financial support by the Fazit Foundation and Sterling
                        Rescue, USA)
  Since 2008             Skill enhancement in university education
                        (Hochschuldidaktikzertifikat Baden-Württemberg, HDZ)
• Teaching Experience

2006 - 2009 Tutor in the animal define course in zoology
2006 - 2009 Tutor in the practical course in zoology
2007 - 2010 Tutor in the ecology course „Learning in bees“
2007 - 2010 Tutor in the “insect-plant interactions” course
2007 - 2009 Tutor in the “chemical ecology” course
2008 Supervision of the Diploma thesis from Kathrin Appel
„Bestäuberanlockende Blütensignale der Orchideenarten Epipactis atrorubens und E. palustris,“

• Field Trips

Aug / Sep 2007 + 2008 Field trip to China
(financial support by the die Robert-Bosch-Foundation and Fazit Foundation)
May 2008 + 2009 Field trip to Turkey
March / Apr 2009 Field trip to Israel
(financial support by the Minerva Foundation)

• Scientific and Industrial Cooperation

Chemical analysis in cooperation with Prof. Dr. Wittko Francke, University of Hamburg
and Prof. Dr. Joachim Ruther, University of Regensburg
Chinese cooperation with Prof. Luo Yi-bo, State Key Laboratory of Systematic and Evolutionary Botany
Cooperation with Dr. Stökl and Dr. Hanssen from the Max-Planck-Institute for Chemical Ecology, Jena
and Prof. Dr. Amots Dafni, University of Haifa, Israel
Industrial cooperation with Sterling Rescue®, USA, biological pest control
• **Publications**


Brodmann J., Ruther, J., Ayasse M. (in prep.) Orchid mimics alarm pheromone of the pollinator to attract alerted wasps for pollination.

Brodmann J., Twele R., Francke W., Ayasse M. (in prep.) Multifaceted ways of attracting various pollinators in the orchid *Epipactis*.

Brodmann J., Emer D., Ayasse M. (in prep.) Pollinator attraction of the wasp-flower *Scophularia umbrosa*.

Brodmann J., Ayasse M. (in prep.) The role of innate and learnt floral odour preferences in the pollinator attraction of the orchid *Epipactis helleborine*.

• **Conference Contributions and Awards**

- Travel award -

- Conference poster price (rang 3) -


- Conference poster price (rang 1) -


**Referents**

Prof. Dr. Manfred Ayasse, University of Ulm, Germany
Email: manfred.ayasse@uni-ulm.de
Tel.: 07315022663

J. Brodmann

Ulm, 27.07.10
Hiermit erkläre ich, die vorliegende Dissertationsarbeit selbständig angefertigt und keine anderen als die in der Arbeit angefuhrten Hilfsmittel verwendet zu haben.

Ulm, den 27.07.10