Communication in the Hymenoptera

Chemistry, Ecology and Evolution

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Thomas Schmitt

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Contents

1 General Introduction

1.1 Communication

All social interactions between organisms, be it courtship, species and kin recognition, division of labour or any other form of coexistence, involve communication. Animal signals evolved in a diversity of forms constrained by the environment in which the signal is propagated and the physiological equipment of sender and receiver. This generated different sensory modes of communication: chemical, auditory, visual and tactile communication. (Dusenbery 1992; Bradbury & Vehrencamp 1998; Greenfield 2002). However, how a signal in a certain modality evolved its characteristics is not known in most cases. This study contributes to the understanding of the evolution of communication.

But what is communication? To understand the evolution of communication it is necessary to define the different types of communication. However, the definition of communication is still under debate and definitions given by leading scientists in the field of animal communication studies vary considerably (Hölldobler 1984). Wilson (1975) defined biological communication as "action on the part of one organism (or cell) that alters the probability pattern of behaviour in another organism (or cell) in an adaptive fashion. By adaptive I mean that the signalling, or the response, or both have been genetically programmed to some extent by natural selection". This definition is more general than ones, which limit "true communication" to situations where the signal is generated by the sender to provide information and the response is adaptive for the receiver (e. g. Dusenbery 1992, p. 37). Dusenbery's definition would confine "true communication" to signals within a species, beneficial to the receiver. However, taking into account the conflicts even between the sexes within a species, communication that is equally adaptive for both signaller and receiver is likely to be rare. Dawkins (1995), in contrast, gave a more simple definition: "communication occurs when one animal's behaviour can be shown to have an effect on the behaviour of another. “Signals” are the means by which these effects are achieved” (Dawkins 1995, p. 73).

This shows that communication is one of the most contentious issues in animal behaviour and there is no commonly accepted definition. However, nearly all authors agree that communication involves the provision of information (cues or signals) by a sender, and the subsequent use of that information by a receiver in deciding how to respond (Bradbury & Vehrencamp 1998, p. 2). This definition allows to include deception by the sender and eavesdropping or other kinds of signal exploitation by the receiver. It is therefore useful to distinguish between cases of cooperative signalling, in which both, sender and receiver, benefit and of non-cooperative signalling, in which only the sender or only the receiver benefits (Harper 1991; Johnston 1997).

1.2 Signal Evolution

To understand the evolution of communication, one has to consider two sets of selective pressures. First, selective pressures acting on signallers, second, selective pressures acting on receivers. Thus, natural selection favour signallers to be more effective in manipulating the receiver and having lower fitness costs by, for example, choosing less costly signals or avoid eavesdropping (Wiley 1983, 1984; Endler 1992, 1993). On the other hand, selection favours receivers who can adequately adjust their behaviour in response to the information provided by signals (Johnston 1997). Besides natural selection one has to consider other potentially important nonadaptive forces such as developmental and phylogenetic constraints, pleiotropy and genetic drift (Phelan 1992).

Yet another important evolutionary factor are preadaptations, which evolve in other contexts than communication and signal design. Scenarios for signal origin also fall into two categories: sender preadaptations and receiver preadaptations. The classical ethological view is that signals evolve from sender preadaptations such as behavioural, physiological, or morphological traits (Otte 1974). If the presence of the trait provides useful information to receivers that are able to detect it, receivers will evolve to pay attention to the trait and use it to make behavioural decisions in response. Signals that evolve from this type of source contain information from the very beginning because the sender's incipient signal is linked to its condition. Both, sender and receiver might gain benefits from this type of communication. However, considering the conflict that exists between sender and receiver in the majority of cases, even in a cooperative signalling mode, it is conceivable that either the sender or receiver gains larger or exclusive benefits (Smith & Harper 2003).

Thus, cues or signals can also evolve to deceit the receiver or to manipulate the receiver's response. From the signaller's perspective, a signal is a means of manipulation. It serves to influence the receiver's behaviour in a way that benefits the signaller. Whether or not the receiver also benefits does not necessarily play a role for the sender (Johnston 1997). Any form of mimicry, for example, is a signal used by the sender to increase its own benefit and is frequently associated with costs for the receiver. Thus, selection favours individuals, whose signals are more effective at eliciting beneficial responses of receivers (Wiley 1983, 1984; Endler 1992, 1993).

Likewise the capability to recognise cues emitted by a sender does not have to evolve in a cooperative way. For a receiver, cues are a potential source of information, which benefits the receiver, but does not necessarily benefit the sender. Recently, receiver preadaptations have been found to play an important role in signal evolution. Preexisting perceptual biases in the receiver can act as a filter on signal form by favouring those signal characteristics that transmit most effectively in a given environmental and social context, a concept called sensory drive (Endler 1992). More extreme hypotheses, sensory exploitation (Ryan 1990; Ryan & Rand 1990, 1993) or sensory trap (Christy 1995), propose that signal evolution starts with receiver preadaptations, which are used by the sender to evolve a more effective signal design (Bradbury & Vehrenkamp 1998).

1.3 Chemical Communication

Chemical receptors are probably the evolutionary oldest sense organs and so chemical signals, or semiochemicals are believed to be the oldest type of signal. Chemical cues are used to locate food, potential prey and host as well as in social interactions (Bell & Cardé 1984; Harborne 1988; Cardé & Bell 1995; Vander Meer 1998). Chemical signals are derived from compounds originally having other uses or meanings, for example hormones, cuticular hydrocarbons or chemicals released in another context of communication (Signoret 1970; Albone et al. 1977; Perry et al. 1980; Stacey et al 1986; Hölldobler & Wilson 1990; Stanley-Samuelson & Nelson 1993; Sorensen & Stacey 1999). There is selection for functional signal features such as longevity and specificity. There is also evolution in the sensory system and response of the receiver. If detection of a particular chemical cue leads to a greater reproductive success or survival of the receiver, selection favours receptors to become more sensitive to it or to be developed in larger numbers. A pheromone communication system might be established which benefits both, sender and receiver. However, any pheromone or chemical recognition system could be exploited by other organisms, be it conspecifics or predators and parasites, and turned into a non-cooperative communication system.

The terminology of infochemicals takes into account whether signalling is cooperative or non-cooperative. Pheromones for example are a subclass of semiochemicals that mediate communication between conspecifics. Pheromones were originally defined as "substances secreted to the outside by an individual and received by a second individual of the same species in which they release a specific behaviour (releaser pheromone) or developmental process (primer pheromone)" (Karlson & Lüscher 1959). Semiochemicals that are transmitted and detected between individuals of different species are called allelochemicals and are further divided into three categories. The distinction between the three categories is made according to which organism benefits in the interaction: the emitter (allomone), the receiver (kairomone) or both (synomone) (Nordlund & Lewis 1976; Nordlund 1981).

The study of the structure, function, origin and significance of naturally occurring compounds that mediate inter- and intraspecific interactions between organisms is often challenging because of the very small quantities of the semiochemicals used by most insect species. A maximum amount of information has to be gained from a minimum amount of chemicals. Although general chromatographic and spectroscopic techniques are used to isolate and identify the compounds, specialized techniques had to be developed for handling these very small quantities. Usually identification of semiochemicals should be accompanied by an effective bioassay to understand their behavioural and evolutionary significance (Millar & Haynes 1998; Haynes & Millar 1998).

1.4 Outline

As mentioned above, chemical communication plays a crucial role in many social interactions of insects. Although the study of chemical communication of insects developed rapidly in the past 30 years, it is not well understood how these signals or cues have been shaped by natural or sexual selection. The present studies of a solitary wasp species, its brood parasitoid and three species of social Hymenoptera give an insight into the evolution of a pheromone communication system and the use of chemicals to transmit information or to deceit.

1.4.1 Signal evolution

Chapters 2 – 4 present the results of a test of the role of the sensory exploitation hypothesis (Ryan 1990; Ryan & Rand 1993) in the evolution of the sex pheromone communication system of the European Beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). The sensory exploitation hypothesis predicts that male sexual signals evolve according to sensory abilities of the females that have been evolved in a non-sexual context. Beewolf females hunt exclusively honeybees as provisions for their progeny. Males mark territories with a pheromone to attract females. A previous study by Schmidt et al. (1990) showed that the major compound in the pheromone is (Z)-11-eicosen-1-ol. This long-chain alcohol was also found in large amounts in the alarm pheromone of *Apis mellifera*. The co-occurrence of (Z)-11-eicosen-1-ol in the male pheromone and in the alarm pheromone of honeybees suggests that males might exploit a preexisting sensory ability of females.

The results of our studies support a three-step scenario for the evolution of the male pheromone. First, (Z)-11-eicosen-1-ol can be found on the cuticles of honeybees and in the air surrounding foraging honeybees. Thus, foraging honeybees smell of (Z)-11-eicosen-1-ol and beewolf females could use this characteristic odour as a kairomone to locate and identify their prey (Chapter 2). Second, bioassays show that olfactory cues are responsible for eliciting attacks on honeybee prey. Most interestingly, (Z)-11-eicosen-1-ol is crucial for the identification of the honeybees (Chapter 3). Third, a reanalysis of the beewolf sex pheromone revealed an extensive congruence between the marking secretion of male beewolves and the cuticular hydrocarbons of honeybees (Chapter 4). This congruence strongly supports the hypothesis that beewolf males evolved a pheromone that exploits the females' pre-existing sensory sensitivity.

1.4.2 Signal information

Cuticular hydrocarbons are the key compounds for recognition in Hymenoptera. Even for solitary Hymenoptera the recognition of kin might have a positive effect on their fitness. Due to the complicated sex determination in the Hymenoptera females should discriminate against brothers to avoid inbreeding that would result in a higher proportion of unfertile diploid sons. In *Philanthus triangulum*, the male sex pheromone shows a significantly higher similarity among brothers than among non-related individuals. Such a genetic component of a male sex pheromone, which enables the females to discriminate between kin and non-kin, has not yet been described from aculeate Hymenoptera (Chapter 5).

The recognition of nestmates and kin is essential to maintain the integrity of the social structure in colonies of eusocial Hymenoptera. Potential intruders have to be kept out of an intact colony. In honeybees, guard bees assume the task of colony defence by discrimination between nestmates and non-nestmates. There is evidence that volatiles emanated from the cuticle can be used for nestmate or even kin recognition. Potential cues for nestmate recognition from the headspace of undisturbed foraging honeybees were identified (Chapter 2).

Eusocial colonies are characterized by the reproductive division of labour between a breeding caste and their non-breeding helpers. Queens prevent their workers from reproducing by signalling their presence and fecundity. In the ant *Camponotus floridanus* queens mark their own eggs with such a signal. The cuticular hydrocarbons on the eggs inform the worker about the queen's presence and prevent them from reproducing (Chapter 7).

1.4.3 Signal deception

Host-parasite interactions are among the most important biotic relationships. Host species should evolve mechanisms to detect their enemies and employ counterstrategies. Parasites, in turn, should evade detection to maximise their success. Cuticular hydrocarbons are potential cues for hosts to recognise parasites. Parasites should be adapted to the cuticular hydrocarbon profile of their host to evade detection. Two examples of possible chemical mimicry were studied.

First, the congruence of the cuticular hydrocarbon composition between the host, females of the European beewolf *Philanthus triangulum*, and their primary brood parasitioid, the cockoo wasp, *Hedychrum rutilans*, was investigated. These highly specialized cockoo wasps enter beewolf nests to oviposit on paralysed bees that are aimed to serve as food for their progeny. The cockoo wasp larva kills the beewolf larva and feeds on it and the bees. Thus, *Hedychrum rutilans* can be a major cause of larval mortality in *Philanthus triangulum*. Beewolf females do not recognise the parasitoids when they encounter them in the nest, which was hypothesised to be the result of chemical cloaking. A comparison between the cuticular hydrocarbon composition of beewolf females, beewolf males, honeybees and cockoo wasp females provides evidence that the cockoo wasp exhibits chemical mimicry of the odour of its host (Chapter 6).

Second, the adaptation of the chemical signature in the social parasitic ant *Protomognathus americanus* to its *Leptothorax* host was investigated. Although this parasite is principally adapted to its hosts' cuticular hydrocarbon profile, the chemical adaptation varies between populations with host community composition, since the parasite faces a trade-off when confronted with more than one host species. In addition to adaptation of its own chemical signature, the slavemaker causes an adjustment of its slaves' cuticular hydrocarbons to its own profile. Therefore, potential host colonies could indeed discriminate between invading conspecific slaves, which commonly accompany slavemakers on raids, and "non slave" conspecifics (Chapter 8).

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2 Volatiles of foraging honeybees *Apis mellifera* L. (Hymenoptera: Apidae) and their possible role as semiochemicals and kairomones

2.1 Abstract

Nestmate and kin recognition play a major role in maintaining the integrity of social insect colonies. In the honeybee *Apis mellifera* guard bees are predominantly responsible for nestmate recognition. Although it has been suggested that recognition of nestmates by guard bees is mediated by contact chemoreception, there is evidence that volatiles emanated from honeybee workers might transmit recognition cues as well. These volatiles might also play a role as kairomones for honeybee predators. Females of the European beewolf *Philanthus triangulum* use volatiles from the cuticle of worker honeybees to identify their prey. We analysed both extracts of honeybee cuticles and volatiles that are emitted from undisturbed foraging bees. As expected, components with high volatility were overrepresented in the headspace compared to their abundance on the cuticle. Surprisingly, we found hydrocarbons with a chain length of up to 29 and some new minor compounds in the air surrounding foraging honeybees. Thus, even long chain hydrocarbons show a considerable volatility and might be used as olfactory recognition cues. (Z)-11-eicosen-1-ol occurred in small amounts both on the cuticle and in the headspace of honeybee workers and might, thus, function as a kairomone by females of the European beewolf. The significance of our results both for communication among honeybees and for hunting beewolf females is discussed.

2.2 Introduction

The cuticle of insects is coated with a mixture of hydrocarbons whose primary role is the prevention of desiccation (Hadley 1994, Buckner 1993). However, cuticular hydrocarbons have also been shown to play an important role as semiochemicals in social insects, particularly for species, nestmate, caste, and kin recognition (Howard and Blomquist 1982, Howard 1993, Breed 1998, Singer 1998, Vander Meer et al. 1998). In honeybees, cuticular hydrocarbons are involved in nestmate and kin recognition (Page et al. 1991, Breed and Stiller 1992, Arnold et al. 1996). The majority of compounds on the cuticle of honeybees are long-chain alkanes, branched alkanes, alkenes and esters (Blomquist 1980, Francis et al. 1985, 1989, Carlson 1988, Ogden et al. 1998). Minor compounds, that might function as pheromones have not been the focus of earlier studies.

Nestmate recognition and colony defence of honeybee hives is mainly executed by guard bees (Butler and Free 1952). They patrol the nest entrance, inspect entering bees, and exclude non-nestmates or other intruders probably using chemical cues (Moore 1987, Moritz et al. 1991, Breed et al. 1992, Beekman et al. 2002). Guard bees antennate approaching bees for identification. Therefore it has been suggested that the relevant compounds have a relatively low volatility and can only be perceived by contact chemoreception (Free 1987). However, a study by Kalmus and Ribbands (1952) has shown that foraging honeybee workers can distinguish between nestmates and non-nestmates at food sources without contact, suggesting that volatile compounds are involved. There is even evidence, that volatiles emanated from workers or groups of workers can be used for kin recognition (Getz et al. 1986, Moritz and Southwick 1987).

The colony odour on the cuticles of honeybees is a combination of cuticular hydrocarbons and compounds of the comb wax of the nest (Breed et al. 1988, 1998). The compounds from the comb wax include pheromones produced by workers and floral scents brought to the nest via pollen and nectar. Constituents with functional groups as fatty acids, esters, hydroxy alkyl esters and primary alcohols, as well as non-polar hydrocarbons such as hexadecane, octadecane and heneicosene, are likely to be the key components for nestmate recognition (Breed 1998, Fröhlich et al. 2001). In this study, we identify minor compounds of the cuticular hydrocarbon composition of honeybee workers and, for the first time, we analyse the composition of emanated substances in the headspace of foraging honeybees under undisturbed conditions.

The second aim of this study is to test particularly for the occurrence of (Z)-11-eicosen-1-ol on the cuticle and in the headspace of foraging honeybees. This alcohol is a major component of the honeybee alarm pheromone and has an alerting and attractive effect on nestmates (Free et al., 1982, 1983; Pickett et al., 1982). Females of the European beewolf *Philanthus triangulum* hunt honeybees and provision them as food for their progeny. There is evidence, that beewolf females use (Z)-11-eicosen-1-ol as an essential olfactory cue to identify their prey (Herzner et al., in prep). This secondary function as a kairomone requires that honeybee workers constantly smell of (Z)-11-eicosen-1-ol during foraging and not only during alarm conditions. Therefore, we focus on the detection of, at least, traces of (Z)-11-eicosen-1-ol on the cuticle and in the headspace of foraging worker honeybees.

2.3 Material and Methods

Composition of cuticular hydrocarbons of honeybees

Foraging honeybee workers were collected from colonies maintained by the apiary of the University of Würzburg. Only foraging workers of *Apis mellifera carnica* were caught and stored at –20°C. Five bees were individually soaked in 1 ml distilled hexane for 10 min. These extracts were evaporated to a residue of approximately 100 µl. We used 1 µl for GC-MS analyses. These were carried out on a HP GC System 6890 coupled to a MS 800 (quadrupole type) from Fisons Instruments. The GC was equipped with a DB-5 capillary column (0,25 mm ID x 30 m; film thickness 0,25 µm, J & W Scientific, Folsom, Ca, USA). Helium was used as a carrier gas with a constant pressure of 90 mbar. A temperature program from 60°C to 300°C with 5°C/min and finally 10 min at 300°C was employed. A split/splitless injector was used at 240°C and in the splitless mode for 60 sec. The mass spectra were recorded with an ionisation voltage of 70 eV and a source temperature of 220°C.

The software Xcalibur (ThermoFinnigan, Egelsbach, Germany) for windows was used for data acquisition. Identification of the components was accomplished by comparison with purchased chemicals and the use of a commercial MS database (NIST 4.0).

Volatiles in the headspace of foraging honeybees

Volatile chemicals from the headspace of foraging honeybee workers were collected using solid phase micro extraction (SPME). Because of the relative high proportion of nonpolar compounds on the honeybee cuticle we used a poly dimethylsiloxane coated (100 µm) fibre (SUPELCO, Deisenhofen, Germany). Honeybee workers were trained to forage on a sugar solution in an arena made of perspex (Figure 1). The SPME fibre was inserted into the arena (without contact to the honeybees), which was connected to a vacuum pump and an air stream passed the fibre (0.4 l per minute). A second identical arena, from which workers were excluded, served as a control and was run simultaneously to analyse the chemical background of the surrounding air and chemicals that were emitted by the arena. This experiment was run four times, for two hours each. The arena was established in a distance of around 20 meters from 10-15 beehives.

SPME fibres that were loaded with volatiles from foraging bees and control fibres were analysed immediately. The GC-MS system and temperature program were the same as for the extracts described above.

Pentadecanol, a major component emitted by perspex, was used as an internal standard and was not present in the air. This allowed us to calculate the relative amounts of all substances with reference to pentadecanol. To distinguish between background chemicals from the air and those that are emitted by honeybees we considered all compounds that occurred less than twice as much in the experimental arena than in the control arena to come from the surrounding air. These were not included in the analysis.

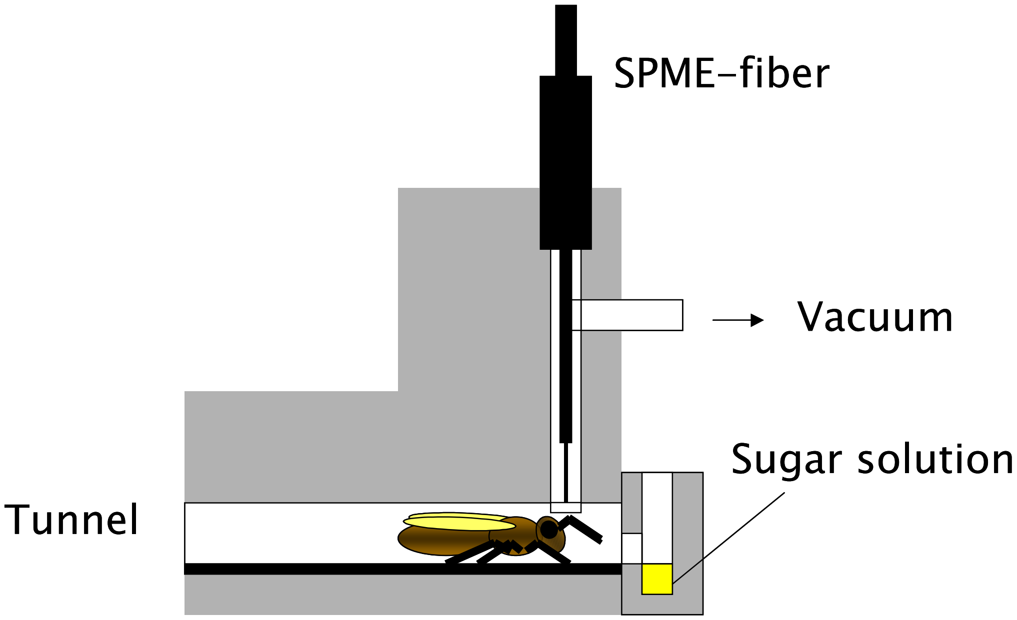

Figure 1: Schematic draft of the arena for the sampling of volatiles emitted from undisturbed foraging honeybee workers.

2.4 Results

(a) Composition of cuticular hydrocarbons of honeybees

Besides the known long-chain saturated and unsaturated aliphatic hydrocarbons and long chain esters (Francis et al., 1985; Salvy et al., 2001), we found traces or minor components of compounds, mainly with shorter chain lengths (alkanes, alkenes and on terpene) that have not yet been identified on the cuticle of honeybees (Table 1). Some substances on the cuticle could not be identified yet due to their small amounts. Geraniol and farnesol occurred on one of the five bees. In particular we identified (Z)-11-eicosen-1-ol as a new minor component of the cuticular hydrocarbon composition of foraging honeybee workers.

	Cuticular hydrocarbons	Headspace
Nonanal		**
Geraniol	**[1]	*[1]
Undecanal		*
Dodecanal		**
Pentadecane	*	***
Tridecanal		**
Hexadecane	*	**
Tetradecanal		***
Heptadecene		**
Heptadecane	*	***
Pentadecanal		***
Farnesol	*[1]	*[1]
Octadecane		*
Hexadecanal		***
Nonadecene	*	
Nonadecane	**	***
Heptadecanal		**
Eicosane	*	**
Heneicosene	*	
Heneicosane	**	***
Docosene	*	
Docosane	**	***
11-Eicosen-1-ol	**	*
Tricosene	***	***
Tricosane	****	****
Tetracosene	**	
Tetracosane	**	***
Pentacosene	***	**
Pentacosane	****	***
Hexacosane	**	
Heptacosene	***	
Heptacosane	****	**
Me-Heptacosane	**	
Octacosane	**	
Me-Octacosane	**	
Nonacosene	**	
Nonacosane	****	**
Me-Nonacosane	**	
Triacontane	**	
Hentriacontene	***	
Hentriacontane	***	
Me-Hentriacontane	**	
Tritriacontene	****	
Tritriacontane	***	

Table 1: Identified constituents of extractable cuticular hydrocarbons and volatiles in the headspace of foraging worker honeybees. The relative protions are given in four classes: * = 0 – 0.1%; ** = 0.1 – 1.0%; *** = 1.0 - 5.0%; **** = 5 - 100%. [1]These compounds occurred only in one of the five analysed bees and in one of the four trials.

(b) Volatiles in the headspace of foraging honeybees

In the headspace of foraging workers we found the major alkanes present on the cuticle up to a chain length of C29. Also minor alkanes as well as alkenes and in one of four trials geraniol and farnesol occurred in the air surrounding bees. However, the proportions of all components differed considerably from the cuticle extracts. Generally, highly volatile compounds are overrepresented in the air compared to the cuticle. Octadecan and aldehydes from C9 to C17, which were not found on the cuticle, occurred partially in high proportions in the headspace of the foraging bees. We could unequivocally identify traces of (Z)-11-eicosen-1-ol by the extracting ion mode using the characteristic masses 278 (M^+), 250, 109 and 96 of (Z)-11- eicosen-1-ol and taking its retention time into account (Table 1).

2.5 Discussion

Alkanes, methyl-branched alkanes, alkenes and alkadienes from chain length C19 to C35 had already been identified on the cuticle of honeybees (McDaniel et al. 1984, Francis et al. 1985, 1989, Martin et al. 2001). However, we found traces and minor constituents with shorter chain lengths and characterised them as alkanes, alkenes, alcohols and terpenes. As should be expected the more volatile compounds increased in their proportion in the headspace of foraging honeybees compared to the cuticle. Surprisingly, also long chain alkanes and alkenes up to C29 emanated from the cuticle of honeybee workers in detectable amounts although they have been assumed to be non-volatile (Free 1987). Arnold et al. (1996, 2000) have shown that 14 long-chain hydrocarbons from C23 to C33 might function as cues for kin recognition in honeybees. Hexadecane, octadecane, Z-9-heneicosene and Z-9-tricosene have already been tested for recognition activity in honeybees and yielded positive results (Breed 1998, Breed et al. 1992a, 1992b). We found minor amounts of (Z)-11-eicosen-1-ol on the honeybee cuticles. It is known as a major component of the honeybee alarm pheromone and it has been shown to have an alerting and attractive effect on nestmates (Free et al., 1982, 1983, Pickett et al., 1982). The occurrence of at least some of these compounds in the air surrounding foraging honeybees shows that they might have pheromonal activity and that recognition cues might be transmitted as volatile signatures, i.e. without direct contact.

The principal constituents of hydrocarbons with functional groups in the headspace of honeybee workers are aldehydes from chain length C9 to C17. The source of these aldehydes might be either the cuticular hydrocarbons of the bees or the comb wax (Blum et al. 1988). However, the large amounts of these aldehydes in the air surrounding honeybee workers is unlikely to originate from the traces of these aldehydes found on the cuticle. An alternative might be the degradation of alkenes and alkadienes caused by oxygen, heat and sunlight, a process that has been extensively studied with regard to rancidity of food oils (Frankel 1998). The oxidation of unsaturated hydrocarbons is also known from the cuticle of other Hymenoptera where the released volatiles are saturated and unsaturated aldehydes, which might function as sex pheromones (Bartelt et al. 1983a, 1983b, 2002, Swedenborg et al. 1992). Due to their high volatility these aldehydes might also play a role in olfactory kin or nestmate discrimination in honeybees. There are many more traces of compounds with functional groups are found on the cuticle and in the headspace of foraging honeybee workers that might have a function as semiochemicals. Their origin is unknown and some of them are still unidentified. It had been suggested that such compounds might play a role for recognition besides the predominant nonpolar hydrocarbons (e.g. Breed 1998).

Other hydrocarbon components such as geraniol and farnesol, that were found in only one trial of head space analysis, are constituents of the Nasonov gland (Free 1987). Their occurrence in the headspace of foraging bees is probably the result of the exposure of the Nasonov gland during foraging on the sugar solution.

We identified (Z)-11-eicosen-1-ol as a new component on the cuticle of almost all foraging honeybees and as traces in the surrounding air. It is not yet known why (Z)-11-

eicosen-1-ol is present on the honeybees’ cuticles. Possibly, this compound does also occur in the honeybees’ Dufour’s gland which seems to be slightly leaking (A. Hefetz, pers. comm.). We do not know yet whether the (Z)-11-eicosen-1-ol on the honeybees' cuticles has also a meaning for honeybees themselves in non alarm situations. Possibly it provides a cue to discriminate between conspecifics and other species.

Since (Z)-11-eicosen-1-ol is otherwise rare in nature it would also represent a reliable cue for the identification of honeybees by a specialized predator or parasite. In fact, we have already shown that beewolf females use it as an essential kairomone for the identification of honeybees (Herzner et al., in prep.). That this substance serves as a kairomone for beewolf females despite its very small amounts on the honeybee and its low volatility underlines the extraordinary sensitivity of insect olfaction and the potential meaning of trace components for insect communication purposes.

The present work provides a list of substances from the honeybee cuticle that might have pheromonal activity. The occurrence of the majority of these chemicals, even long-chain alkanes and alkenes, in the headspace of foraging honeybees might explain the ability of honeybee workers to discriminate between nestmates and non-nestmates as well as sisters and half-sisters without contact. Except (Z)-11-eicosen-1-ol the significance of their newly identified compounds as semiochemicals is not yet known. Further studies have to investigate their relevance for nestmate and kin recognition or as kairomones for potential predators or parasites.

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3 Prey recognition by females of the European Beewolf and its potential for a sensory trap

3.1 Abstract

The asymmetry in mating strategies between males and females may influence the evolution of mate-signalling systems. Exploitation of pre-existing female preferences for certain visual or acoustical stimuli by male courtship signals has been reported from a variety of species. However, information on chemical communication systems is comparatively scarce. The sensory trap model of sexual signalling suggests that female preferences originated and are maintained due to selective pressures in a non sexual context, e.g. prey recognition. We tested a key prediction from the sensory trap hypothesis for the evolution of the male sex pheromone in a solitary wasp, the European Beewolf *Philanthus triangulum*. Beewolf females hunt exclusively honeybees as provisions for their larvae. Males mark territories with a pheromone to attract females. The co-occurrence of an unusual long chain alcohol, (Z)-11-eicosen-1-ol, in the male pheromone and on the cuticle of honeybees suggests that, according to the sensory trap model, males might exploit the female preference for (Z)-11-eicosen-1-ol. In this study we focused on the question whether females of the European Beewolf use (Z)-11-eicosen-1-ol for prey recognition. Using behavioural assays with honeybee dummies we show, first, that beewolf females find and identify their honeybee prey by virtue of olfactory cues and second, that (Z)-11-eicosen-1-ol is an essential component of the prey recognition cue. This is remarkable since (Z)-11-eicosen-1-ol is only present on a honeybee in very small amounts. Thus, female European Beewolves have a rather high sensitivity for (Z)-11-eicosen-1-ol that probably evolved in the context of prey hunting. Therefore, males who have included this compound in their sex pheromone probably attracted more females and experienced a selective advantage according to the sensory trap model.

3.2 Introduction

The reproductive interests of males and females often differ dramatically. Females usually make larger parental investments than males, who invest more in mate attraction and mate encounter (Trivers 1972; Phelan 1992, 1997). To maximise their reproductive success females should evolve extraordinary sensory, neural, physiological, and physical capabilities to locate and accumulate resources needed for provisioning offspring. Males, however, should evolve a high efficiency to locate or attract females to maximise the number of matings and thereby their reproductive fitness. This fundamental asymmetry in reproductive strategies may be a major determinant for the evolution of courtship signals. Therefore, male sexual signals are expected to track the female response in evolutionary time (Phelan 1992, 1997).

Recognizing this asymmetry, the 'pre-existing biases' (Basolo 1990) and 'sensory exploitation' (Ryan 1990a, b) models of sexual signalling suggest that the evolution of male sexual signals is influenced by pre-existing characteristics of the females' sensory or neural systems. An expansion of this hypothesis is the sensory trap model (West-Eberhard 1984; Christy 1995) which takes into account how such pre-existing sensory sensitivities and female preferences may have evolved. It states that female preferences originate because they are selected for in at least one context outside mate choice, i.e. in a natural selection context like foraging. All three models propose that the female preference predates the preferred male trait and its use in sexual signalling.

There is a growing body of evidence that supports sensory traps as an important factor for the evolution of visual and vibrational male courtship signals (e.g. Proctor 1991; Clark & Uetz 1992; Rodd et al. 2002; Stålhandske 2002; Christy et al. 2003a, b; Madden & Tanner 2003). Although sexual signalling and mate choice frequently involve chemical communication, surprisingly little is known about possible evolutionary pathways of chemical communication systems and the role of sensory exploitation or sensory traps in shaping the composition of pheromones (Christy 1995;

Phelan 1992, 1997). Selection for prey detection or recognition is an example of how selective forces in a context other than mate choice can influence female mating preferences (Proctor 1991; Christy 1995; Rodd et al. 2002). If females use odours to locate their food or prey, male signals that are sufficiently similar to this odour to attract females will be favoured by sexual selection (Christy 1995).

This study tests a key prediction of the sensory trap model for the evolution of the sex pheromone of male European Beewolves, *Philanthus triangulum*. Female beewolves are strictly monophagous and hunt exclusively honeybee workers (*Apis mellifera*). They search for honeybees on flowers, paralyse them, and bring them to their nest as provisions for their offspring (Strohm 1995). Beewolf females' reproductive success is limited by the number of bees they can secure for their progeny (e.g. Strohm & Linsenmair 1997, 1998; Strohm & Marliani 2002). Females can therefore be expected to have evolved special sensory, neural, and physiological abilities to maximise their success in detecting and capturing honeybees. Tinbergen (1935) provided some evidence for the use of olfactory cues in the prey hunting behaviour of female European Beewolves, however, the chemical nature of these cues has not been analysed. Many hymenopteran species use chemical cues for the location and/or identification of food sources. Especially species that prey on or parasitize other species rely on chemical stimuli that are associated with their prey or hosts (Dicke & Sabelis 1992; Godfray 1994; Stowe et al. 1995; Quicke 1997; Hendrichs & Hendrichs 1998). These so-called “kairomones” can either be actively emitted signals intended for a different receiver, like e.g. pheromones (Dunkelblum et al. 1996; Hendrichs & Hendrichs 1998; Hoffmeister & Gienapp 1999; Millar et al. 2001), or inadvertently provided cues, like e.g. cuticular substances (Anton & Gnatzy 1998; Howard et al. 1998) or products like faeces (Steidle & Ruther 2000; Schaffner & Müller 2001). In any case, kairomones usually reliably indicate the presence and identity of the victims.

Male beewolves establish and scent mark territories with the secretion of a cephalic gland to attract females (Evans & O’Neill 1988; Schmitt et al. 2003). Remarkably, the major component of the males’ pheromone, (Z)-11-eicosen-1-ol, is one of the major compounds of the alarm pheromone of honeybees, the exclusive prey of the females (Free et al. 1982, 1983; Pickett et al. 1982). Based on the sensory trap model (West-Eberhard 1984; Christy 1995; Phelan 1992, 1997) we propose a three step scenario for the evolution of the male sex pheromone in *P. triangulum*: (1) Foraging honeybees should smell of (Z)-11-eicosen-1-ol. (2) Since successful prey hunting is the major factor influencing their reproductive potential, beewolf females evolved a high sensory sensitivity for this characteristic component to locate or identify honeybees. (3) Males have evolved (Z)-11-eicosen-1-ol as a pheromone component because of the high sensitivity of females for this substance. In the current study we focus on prediction two. Predictions one and three will be discussed elsewhere (unpublished data, T. Schmitt, G. Herzner, E. Strohm; unpublished data, E. Strohm, T. Schmitt, G. Herzner).

Here we tested, by means of behavioural assays, whether beewolf females use olfactory cues and in particular (Z)-11-eicosen-1-ol emitted by the honeybees, to detect and identify honeybees. Females of the European Beewolf are highly specialised and have probably evolved an accordingly highly specialized sensory-neural-motor system. In contrast to generalistic species this provides a good opportunity for a male to effectively exploit the female's sensory system and behavioural response. For the same reason, it will be more likely to identify the stimuli necessary for prey location and identification. Thus, due to the females' extreme specialisation, beewolves provide an exceptionally promising model system to test the sensory trap hypothesis.

3.3 Methods

3.3.1 Beewolves

In order to elucidate the role of olfaction, and (Z)-11-eicosen-1-ol in particular, in prey hunting of female European Beewolves, we established a bioassay to determine the response of beewolf females to different experimentally manipulated prey objects (test prey). Females were either collected at different field sites in or close to Würzburg or obtained from a laboratory population reared at the Biocenter of the University of Würzburg. They were brought into an environmental chamber (26/22°C day/night 14h/10h light/dark cycle) and individually placed in sand-filled breeding cages (60x18x18 cm) to which foraging partitions (15x18x18 cm) were attached that were lit by neon lamps. For five to seven days females were allowed to accustom to the laboratory conditions and provided with honey and honeybees ad libitum. During the following training and experimental period they were provided with honey only and confronted with differently manipulated honeybees or honeybee dummies.

3.3.2 Training

Beewolf females were trained to attack and paralyse honeybees that were offered at a specific spot in the foraging cage. For this purpose bees were anesthetized with CO_2 and attached to commercial hairgrips by clamping of the wings. Beewolf females that attacked the bees (which were then released from the hairgrips) were allowed to take them to their nests. After the females had reliably learned to accept the fixed honeybees (after approximately one week), freeze-killed and defrosted honeybees were offered. This step was included to eliminate the movement of the bees that could be a stimulus for prey detection by the females. Freezing does not alter the outer appearance or the odour bouquet of the bees (unpublished data, G. Herzner). Females that learned to accept the dead bees as prey were used for the bioassays described below.

During the initial training phase females that attacked the fixed live or frozen bees showed a characteristic behaviour. After the first perception and localisation of the bees (for which most likely olfactory as well as visual cues were responsible, see also Tinbergen 1935) they hovered in front of the prey at a distance of approximately 10 cm for a few seconds before they finally pounced at it and stung it. Based on this behavioural sequence the females' behaviour could be assigned to one of the following categories during the subsequent bioassays: females either (1) did not display the hovering at all, (2) hovered in front of the prey object but then did not attack it or they

(3) showed the hovering behaviour and finally attacked the prey. The first two categories were regarded as “no attack”, the latter as “attack”.

3.3.3 Experimental Blocks

The Role of Olfaction

We first investigated whether olfaction plays a role in prey hunting of beewolf females. We tested whether the characteristic honeybee “body odour”, comprised by the cuticular substances, is essential for releasing an attack. Therefore, we tested (1) odourless bees whose cuticular hydrocarbons were removed, (2) odourless bees that were re-scented by contact with life honeybees, and (3) odourless bees that were re-scented with a honeybee extract. To obtain odourless "bees" we soaked freshly freeze-killed honeybees in acetone for two days and subsequently dried them in a drying oven at 70°C for one day. In this manner the characteristic bee odour was removed (this was verified using gas-chromatography). After the initial training phase of the female beewolves (live bees, dead bees; see above) these odourless honeybees were offered. To attain the first group of re-scented bees, odourless bees were stored in a vial that contained 15 live honeybees for one day. The transfer of the cuticular substances to the odourless bees was again verified by gas-chromatography. The re-scented bees were taken out of the vial immediately preceding the test with a female and each re-scented bee was used only once. For the second group of re-scented bees the cuticular substances were reapplied to odourless bees using an extract of honeybees. A honeybee extract was achieved by soaking three freshly freeze-killed honeybees in 2 ml distilled hexane for 10 minutes (Bee extract). Each extract sample was reduced in volume to approximately 50 µl and applied to an odourless bee with a pipette immediately before each test to avoid a premature volatilization of substances. After the solvent had evaporated (after 1 min), the re-scented bees were used for the bioassay. As control 50 µl of pure hexane were applied on odourless bees and presented to beewolf females.

To further reduce visual stimuli, the odourless honeybees were replaced by honeybee dummies. The dummies were made of dark-grey Teflon and attached to thin wires. They were cylindrical in shape and had the approximate size of honeybees (1.5 x 0.6 mm). The dummies were scented as described above for the odourless bees, either by placing them in a vial with live bees or by the application of a honeybee extract. We compared the number of attacks on odourless and re-scented honeybees and odourless as well as scented honeybee dummies.

The Role of (Z)-11-eicosen-1-ol

To examine the role of (Z)-11-eicosen-1-ol in prey recognition, we conducted a second set of bioassays using the Teflon dummies. (Z)-11-eicosen-1-ol is not only a major component in the alarm pheromone of honeybees, but can also be found on honeybees' cuticles (unpublished data, T. Schmitt, G. Herzner, E. Strohm). To determine the natural amounts of (Z)-11-eicosen-1-ol on honeybee cuticles we analyzed honeybee extracts by combined gas-chromatography and mass-spectrometry (GC-MS). We found (Z)-11-eicosen-1-ol in varying amounts in all extracts. After the initial training phase of the females, three different kinds of scents were tested on dummies. First, the normal honeybee extract (Bee extract), second, the pure hydrocarbon fraction of the honeybee extract containing no (Z)-11-eicosen-1-ol (HC), and third, the hydrocarbon fraction of the bee extract to which (Z)-11-eicosen-1-ol was re-added (HC+Eicosenol).

To remove (Z)-11-eicosen-1ol from the mixture of hydrocarbons, ten honeybees were extracted in 3 ml distilled hexane for 10 min. The resulting extracts were loaded onto a silica gel column (Macherey and Nagel, Chromabond 500 mg) and eluted with 3 ml hexane. The eluted fraction contained the whole set of hydrocarbons (HC: alkanes, methylalkanes, and alkenes), but no (Z)-11-eicosen-1-ol. The HC-solution was partitioned into three aliquots that were reduced in volume to approximately 50 µl and each aliquot was used for one dummy. To obtain solutions of the purified HC fractions that contained (Z)-11-eicosen-1-ol we added commercially available (Z)-11-eicosen-1ol (ICN Biomedicals, Irvine, CA, USA) in the mean amount found on honeybees (HC+Eicosenol). The amount of (Z)-11-eicosen-1-ol in the Bee extracts, the absence of

(Z)-11-eicosen-1-ol in the HC, and the amount of (Z)-11-eicosen-1-ol in the HC+Eicosenol mix was determined by GC-MS. We compared the proportion of attacks on Bee extract dummies with the HC dummies solution as well as the proportion of attacks on HC dummies with the HC+Eicosenol dummies. In order to avoid pseudoreplication all individual prey objects were used only once.

3.3.4 Procedure

Hairgrips were thoroughly cleaned with acetone preceding all experiments. Every morning each focal female was first offered a normal live honeybee fixed to a hairgrip and allowed to paralyse it and take it to the nest. When the female left her nest to forage again, a test prey was offered for 2 min and the response of the female (attack/no attack) was recorded. When the female attacked the test prey, the latter was removed and replaced by a live honeybee that could be paralysed and brought to the nest. When the female did not attack the prey during the 2 min test phase, we immediately tested her motivation for foraging by offering a normal live honeybee. If the female attacked the bee within 2 min, she was considered to have been motivated during the bioassay and the previous test prey was categorized as 'not attacked'. If the female did not catch the live honeybee within 2 min, she was considered to have not been motivated for prey hunting and the previous trial was excluded from the analysis. In order to avoid pseudoreplication, each motivated female was tested only once with a particular test prey.

3.3.5 Chemical Analysis

Coupled capillary gas chromatography-mass spectrometry (GC-MS) was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with a RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID; df =

0.25µm; temperature programme: from 60°C to 300°C at 5°C/min and held for 1 min at 60°C and for 10 min at 300°C). Helium was used as the carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250°C and in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. The software MSD ChemStation for Windows was used for data acquisition. The identification of the alkanes, alkenes and (Z)-11-eicosen-1-ol was accomplished by comparing retention times and mass spectra of honeybee extracts with purchased substances or with data from a commercial library (NIST, Gathersburg, MD, USA) (see also Schmitt et al. 2003).

3.3.6 Data Analysis

The data were analyzed with Fisher's exact test (one-tailed) using the statistics program BIAS for Windows (#7.07). Sample sizes were limited by the number of beewolf females available for the tests, the very time consuming training of the females and the relatively long time span of four to five weeks needed for the bioassays (this period corresponds to the females' average life expectancy). Some females did not learn to attack the tethered bees or did not attack the dead bees (in 2001: 13 out of 44; in 2002: 10 out of 33; in 2003: 9 out of 28) and could thus not be used in the bioassays. Those that could be trained were not active outside their nests every day. Active females could usually be tested with only one or two prey objects during one day, since they spent much of their time feeding or in their nests. Some of the females died before their response to all prey objects could be tested. Therefore, sample sizes differ somewhat between different tested stimuli.

3.4 Results

The Role of Olfaction

Odourless honeybees (n=29) and odourless honeybee dummies (n=17) did not trigger the hovering behaviour and were (with one exception) not attacked (Fig. 1). By re-scenting the previously odourless bees the natural hovering and hunting behaviour was elicited. Prey objects that were re-scented via the contact with live honeybees, were recognised as prey and attacked in 82-90% of the tests (difference to odourless control: Fisher's exact test, $p<0.0001$; for bees: n=34; for dummies: n=20, Fig. 1). Likewise, the honeybee extracts applied to odourless bees and dummies elicited attacks in 75-81% of the tests (difference to odourless control: Fisher's exact test, $p<0.0001$; for bees: n=28; for dummies: n=17, Fig. 1). After contact with the re-scented bees, females displayed the final stinging behaviour. Scented dummies, on the other hand, did not evoke stinging attempts but were thoroughly and excitedly antennated by the females. Since the proportion of hovering flights and predation attacks (stinging behaviour not included) on scented honeybees and dummies was very similar and we wanted to reduce the influence of visual cues, we used only dummies for the subsequent tests.

The Role of (Z)-11-eicosen-1-ol

The chemical profile of honeybee cuticles is dominated by alkanes and alkenes (Francis et al. 1985; Salvy et al. 2001). (Z)-11-eicosen-1-ol is only a minor component (unpublished data, T. Schmitt, G. Herzner, E. Strohm). A typical total ion chromatogram of a honeybee worker extract containing (Z)-11-eicosen-1-ol and a chromatogram of this extract after removal of the (Z)-11-eicosen-1-ol is shown in Figure 2 (for orientation the identities of the major peaks are given in the chromatogram). (Z)-11-eicosen-1-ol could be completely removed from the hydrocarbon fraction of the honeybee extract as can be seen in the overlay of the two chromatograms. The pattern of all other components is, however, identical.

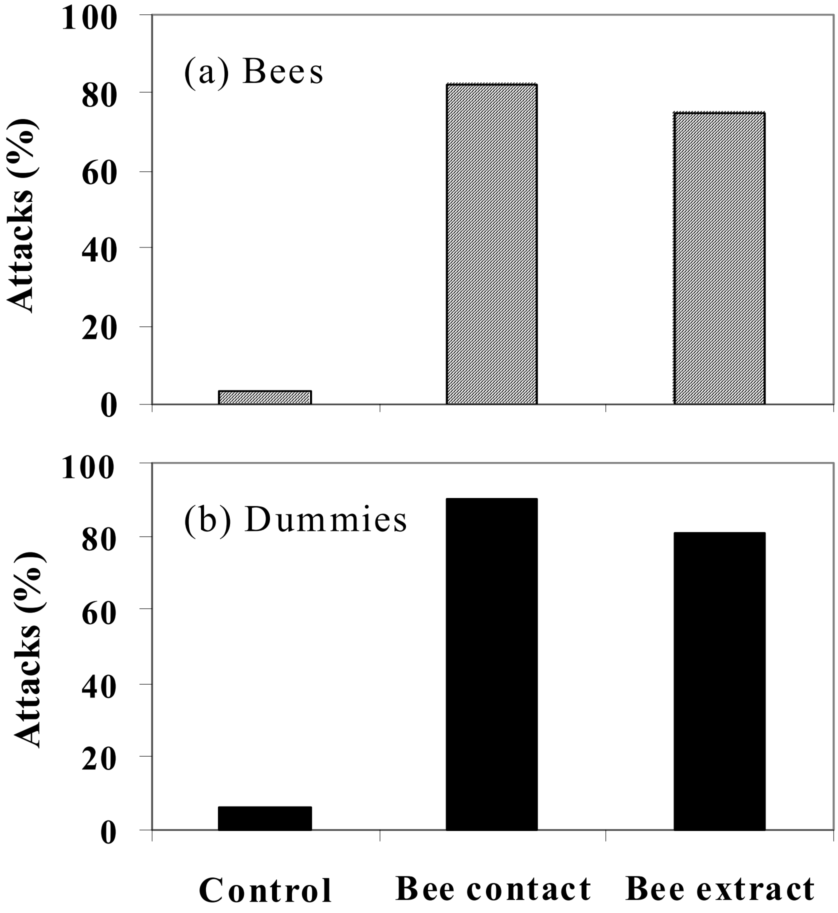

Figure 1: Proportion of attacks on differently treated prey objects. (a) Honeybees: Odourless control (n=29) bees were not attacked by beewolf females. Re-scented bees that had been in contact with live honeybees (bee contact, n=34) or were treated with cuticle extracts of honeybees (Bee extract, n=28) were readily accepted as prey and attacked by the beewolf females (difference to control: Fisher's exact test, $p<0.0001$ for contact and extract). (b) Honeybee dummies: Odourless control dummies (n=17) were not attacked. Scented dummies taken from a vial with live bees (bee contact, n=20) or dummies to which a honeybee extract was applied (Bee extract, n=17) were accepted as prey (difference to control: Fisher's exact test, $p<0.0001$ for contact and extract).

The results of the second set of bioassays are illustrated in Figure 3. In contrast to the Bee extract (n=19) HC (n=14) never elicited attacks on dummies (Fisher's exact test, $p=0.002$). HC was initially attractive to females; they displayed the hovering flights but did not attack the dummies. Notably, HC+Eicosenol (n=8) was about as attractive as the Bee extract and was significantly more attractive to hunting beewolf females than HC

(Fisher's exact test, p=0.002). The Bee extract and HC+Eicosenol triggered the normal sequence of the hunting behaviour comprising the hovering flight and the following attack.

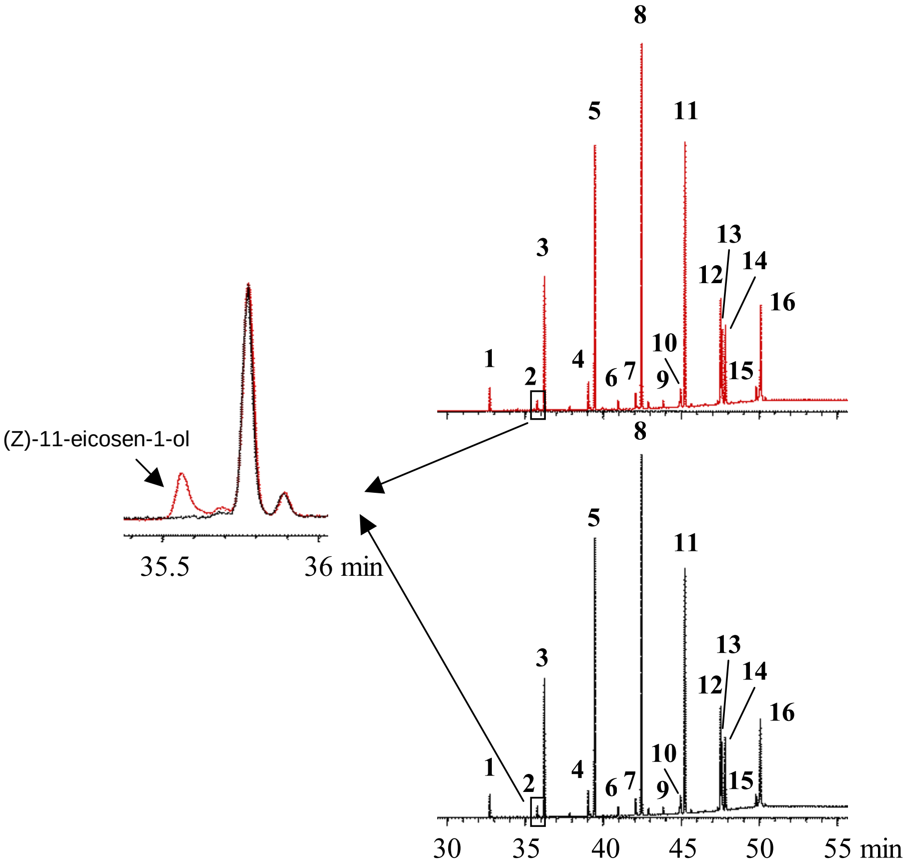

Figure 2: Comparison of the chromatograms of a cuticular extract of ten honeybees (red) and the hydrocarbon fraction of the same extract (black). The chart on the left shows a magnified section of an overlay of both chromatograms. Note that (Z)-11-eicosen-1-ol is only a minor peak of the chemical profile of honeybee cuticles and that it is absent from the hydrocarbon fraction (black). All other peaks are identical in both solutions. (For orientation: 1: heneicosane, 2: (Z)-9-tricosene, 3: tricosane, 4: (Z)-9-pentacosene, 5: pentacosane, 6: hexacosane, 7: (Z)-9-heptacosene, 8: heptacosane, 9: octacosane, 10: (Z)-9-nonacosene, 11: nonacosane, 12: (Z)-7-hentriacontene, 13: (Z)-9-hentriacontene, 14: hentriacontane, 15: (Z)-7-tritriacontene, 16: (Z)-9-tritriacontene.)

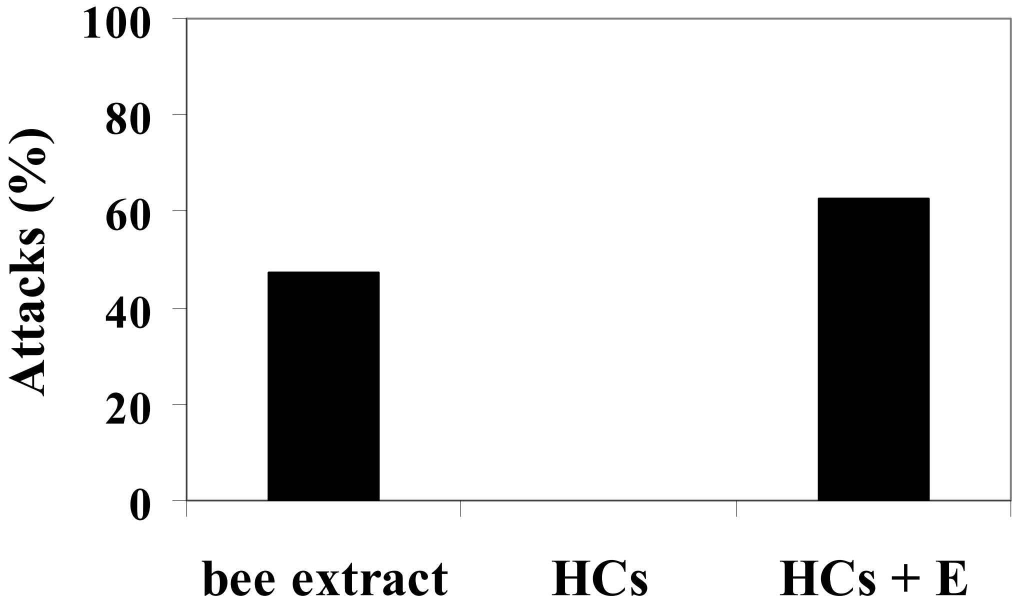

Figure 3: Proportion of attacks on honeybee dummies treated with differently processed honeybee extracts. In contrast to the natural honeybee extracts (Bee extract, n=19) the pure hydrocarbon extracts (from which (Z)-11-eicosen-1-ol had been removed by chromatography; HC, n=14) never elicited attacks on dummies (Fisher's exact test, p=0.002). After the re-addition of (Z)-11-eicosen-1-ol, the hydrocarbon solution was significantly more attractive to beewolf females (HC+E, n=8; Fisher's exact test, p=0.002).

3.5 Discussion

3.5.1 Prey Recognition in Female European Beewolves

The results of our behavioural assays clearly demonstrate that beewolf females use olfactory cues for prey identification. In accordance with Tinbergen (1935) we conclude that the hunting behaviour of beewolf females consists of three distinct steps and involves different sensory modalities. The hovering flight in front of the potential prey at a distance of approximately 10 cm seems to be an important step of the hunting sequence in which the female decides to attack or to ignore a potential prey. The hovering flight was elicited by Bee extracts as well as the hydrocarbon solutions (with or without (Z)-11-eicosen-1-ol). This implies that females rely on “bee like” odours for the first detection and localisation of the potential prey. White dummies treated with honeybee extract could not be localised and were not attacked by females (unpublished data, G. Herzner). Thus, both visual and olfactory cues are essential for initial prey detection.

The actual identification of the prey and the decision to attack seems to take place during the hovering and is obviously mediated by olfactory cues. Notably, the hydrocarbon fraction alone did not elicit attacks. Only honeybee extracts containing (Z)-11-eicosen-1-ol, as in either the Bee extract or in the HC+Eicosenol solution, elicited attacks. Thus (Z)-11-eicosen-1-ol is an essential cue for prey recognition and attack.

The final stimuli that evoke the stinging behaviour seem to be triggered by both gustatory and tactile cues. Re-scented honeybees were stung by beewolf females, indicating that all necessary cues were present. Dummies bearing the same odour were not stung, most probably due to the “wrong” shape and surface of the dummies. Such a multisensory detection, localisation, and acceptance of prey or hosts, involving visual, olfactory, gustatory and tactile cues, has been described from other hymenopteran species, like the digger wasp *Liris niger* (Anton & Gnatzy 1998) and two species of aphid parasitoids (Battaglia et al. 2000; Völkl 2000).

The sensory equipment responsible for prey detection and recognition in *P. triangulum* has not yet been investigated in detail. We found a high diversity and density of presumably olfactory and gustatory sensilla on the antennal flagella of European Beewolves (Herzner et al. 2003). One type of these sensilla, the multiporous large sensillum basiconicum is only present on the antennae of female beewolves. This sensillum type has been shown to play a role in the discrimination between potential prey species in the digger wasp *Liris niger* (Anton & Gnatzy 1998), and may serve a similar function in *P. triangulum*.

3.5.2 (Z)-11-Eicosen-1-ol as Reliable Prey Recognition Cue

Predators or parasitoids with a broad prey or host range usually use cues which are common to many potential prey or host species (Lewis et al. 1971; Schaffner & Müller 2001; but see Steidle & van Loon 2003). Specialised predators, like the European Beewolf, however, usually locate or identify their prey with the help of infochemicals (or mixtures thereof) that are more or less unique to the prey (Bargen et al. 1998; Bernays 1998; de Moraes et al. 1998; Powell et al. 1998; Al Abassi et al. 2000; Steidle & van Loon 2003).

Beewolf females flying through their hunting grounds are exposed to an enormous number of chemical stimuli. Due to their monophagy females must be able to reliably distinguish between honeybees and non-prey species. Alkanes, methylalkanes, and alkenes, which are the prominent compounds found on honeybee cuticles (Francis et al. 1985; Salvy et al. 2001), are widespread among the Hymenoptera (e.g. *Lasioglossum malachurum*: Ayasse 1991; several bumble bee species: Oldham et al. 1994; the leafcutter bee *Megachile rotundata*: Paulmier et al. 1999; *Andrena nigroaenea*: Schiestl et al. 1999; the almond seed wasp *Eurytoma amygdali*: Krokos et al. 2001; three species of decorator wasps *Eucerceris*: Clarke et al. 2001; and the wheat stem sawfly *Cephus cinctus*: Bartelt et al. 2002; *Polistes fuscatus*: Panek et al. 2001; the European hornet *Vespa crabro*: Ruther et al. 2002) and other insect orders (e.g. Diptera: Ishii et al. 2001; Coleoptera: Nelson et al. 2002; Lepidoptera: Guo & Blomquist 1991; Heteroptera: Drijfhout & Groot 2001). They can hence not easily be used as reliable cues for prey recognition. (Z)-11-eicosen-1-ol, however, is very scarce in Hymenoptera and has hitherto not been reported from non hymenopteran species. Besides its occurrence in *A. mellifera* and in the pheromone of *P. triangulum* males (Schmitt et al. 2003; unpublished data, E. Strohm, T. Schmitt, G. Herzner), it has been described as a major component of the venom of the Asian honeybee *Apis cerana* (Schmidt et al. 1997), the Dufour's gland secretion of the neotropical stingless bee *Frieseomelitta varia* (Patricio et al. 2003), and in the thoracic glands of male carpenter bees, *Xylocopa micheneri* from Arizona (Andersen et al. 1988). Thus, (Z)-11-eicosen-1-ol has not been described in species other than *Apis mellifera* in the distribution range of the European Beewolf

Philanthus triangulum and might hence be an ideal cue for a largely unequivocal prey recognition by beewolf females.

Removal of (Z)-11-eicosen-1-ol from the honeybee extracts rendered them unattractive to foraging females. It is a well known but little understood phenomenon that odour blends loose or change their information content by only slight changes in their composition. In several bee species, females become unattractive for males after mating due to the removal (Ayasse et al. 1999), addition (Schiestl & Ayasse 2000) or removal and addition (Simmons et al. 2003) of certain components from or to the odour bouquets. Although (Z)-11-eicosen-1-ol is only a very minor component of the chemical cuticular profile of honeybees, its presence is essential for prey recognition; it can thus be regarded as a discriminator or recognition substance (Hölldobler & Michener 1980).

3.5.3 (Z)-11-Eicosen-1-ol and the Sensory Trap

The very small amounts of (Z)-11-eicosen-1-ol and its low volatility suggest that beewolf females possess high sensory (olfactory) and neural abilities that evolved to maximise their success in detecting and identifying honeybees. The neural hypothesis (Bernays & Wcislo 1994; Bernays 1998, 2001) states that resource specialisation, which is usually associated with strong sensory and neural focusing, leads to more economic information acquisition and processing, which allows for faster and more effective search and recognition behaviours. Such a fast and accurate assessment and identification of the potential prey is crucial to a female's survival and its reproductive success. The resulting strong restriction to only one or a few very particular host cues by the females may act as an important selective force for the evolution of the males' sexual signals ("sensory drive", see e.g. Endler 1992). Thus, a highly specialised – and therefore highly sensitive – prey recognition mechanism should be more prone to exploitation by male signalling than a less fine-tuned system.

Our results clearly support our second prediction that follows from the sensory trap model. (Z)-11-eicosen-1-ol is used as an essential cue for prey recognition and has therefore a high potential to function as a sensory trap. Males who incorporate it in their pheromone may evoke an out-of-context feeding response of females to attract them (Christy 1995) thereby increasing their reproductive success.

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4 (*S*)-2,3-Dihydrofarnesoic acid, a new component in cephalic glands of male European beewolf *Philanthus triangulum*

4.1 Abstract

The chemical composition and functional significance of pheromones of solitary Hymenoptera is much less well known compared to social species. Males of the genus *Philanthus* (Sphecidae) are territorial and scent mark their territories to attract females. Because of inconsistent results of earlier studies we reanalysed the content of the cephalic glands of male European beewolves, *Philanthus triangulum* F. Besides a variety of alkanes and alkenes, four major compounds were found. Two of these, (*Z*)-11-eicosen-1-ol and (*Z*)-10-nonadecen-2-one, had been previously described as constituents of the cephalic glands. We identified 1-Octadecanol as a new component of the cephalic gland and a fourth compound, enantiopure (*S*)-2,3-dihydrofarnesoic acid, was identified for the first time in nature. Structural elucidation and enantiomeric analysis were performed by HRGC-MS and HRGC-FTIR as well as enantioselective gas chromatography and by means of authentic reference compounds. Occurrence and function of the four compounds in insect chemistry are discussed.

4.2 Introduction

Although pheromone communication has been extensively studied in social Hymenoptera with regard to chemical composition, functional significance and evolutionary aspects (reviewed by Vander Meer et al., 1998; Ayasse et al., 2001), much less is known about chemical communication in solitary Hymenoptera. Solitary wasps and bees exhibit a great complexity in the chemical composition of their glandular secretions but only a few sex pheromones have been identified completely and little is known about their evolution (Ayasse et al., 2001). Knowledge of chemical communication in solitary Hymenoptera might provide information about the situation in the predecessors of the highly evolved chemical communication of social species.

Many species of the Philanthinae, a subfamily of the Sphecidae (digger wasps), exhibit an unusual mating system among Hymenoptera (Thornhill and Alcock, 1983; Evans and O'Neill, 1988; Strohm and Lechner, 2000). Male philanthine wasps establish small territories, where they scent mark plants or other structures with cephalic secretions. Receptive females approach territories and most matings take place in the territory or on nearby vegetation (Evans and O'Neill, 1988). The volatiles probably attract receptive females and provide species recognition cues (Simon Thomas and Poorter, 1972; Alcock, 1975; Gwynne, 1978; O’Neill, 1979, 1983; Schmidt et al., 1985; Evans and O’Neill, 1988; Strohm, unpublished observations).

Previous studies on the male cephalic secretions of species of the philanthine genera *Eucerceris* and *Philanthus* have shown a broad variety of components (Schmidt et al., 1985; McDaniel et al., 1987, 1992; Clarke et al., 2002). The content of the marking glands of male European beewolves, *Philanthus triangulum* F. (Hymenoptera, Sphecidae) has been studied twice (Borg-Karlson and Tengö, 1980; Schmidt et al., 1990). These two studies reported completely different compositions. Therefore, we reanalysed the cephalic glands of male European beewolves. Our analysis of a population in Central Germany revealed considerable differences to both of these earlier studies. Furthermore, we identified a component that was hitherto unknown from secretions of any animal species.

4.3 Methods and Material

Insects and Sampling. Beewolf males were obtained from a field population nesting in the vicinity of the Biocenter of the University of Würzburg or from a laboratory population reared under controlled conditions (see Strohm and Linsenmair, 1997a, b; Strohm et al., 2001 for more details on the study site and rearing conditions). The males were killed and stored in a freezer at -20°C. Since anatomical analyses (E. Strohm, unpubl. data) suggest that the responsible gland in European beewolf males is not a mandibular gland (as suggested e.g. in Evans and O'Neill, 1988), we use the term cephalic gland. Three methods of extraction were used. First, the large cephalic glands were dissected and extracted in distilled hexane or dichloromethane for four hours. Second, entire heads of beewolf males were extracted in the same way. Known amounts of octadecane were added to all samples to provide an internal standard. Third, SPME fibres (SUPELCO, Deisenhofen, Germany; coated with a 100 µm polydimethylsiloxane film) (Arthur and Pawliszyn, 1990) were loaded by drawing the fibres through dissected cephalic glands.

Capillary Gas Chromatography-Mass Spectrometry (HRGC-MS). HRGC-MS was performed with a Fisons Instruments GC 8000 Series gas chromatograph (Fisons, Egelsbach, Germany) coupled to a Fisons Instruments MD 800 quadrupol mass detector. The GC was equipped either with a J & W DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25 µm; J & W, Folsom, CA, USA; temperature program: from 60°C to 310°C at 5°C/min and held for 10 min at 310°C) or with a J & W DB-1 fused silica capillary column (30 m x 0.25 mm ID; df =0.25 µm; J & W; temperature program: from 60°C to 150°C at 10°C/min, from 150°C to 350°C at 5°C/min and held for 10 min at 350°C). Helium was used as carrier gas at a constant pressure of 90 kPa. Injection was carried out at 250°C in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV and a source temperature of 220°C.

Chemical ionisation mass spectra (CI-MS) were obtained on the same GC-MS system using the J & W DB-5 column (temperature program as described above). Iso-butane with a pressure of 1 bar was used as ionisation gas and the source temperature was 150°C.

The software Xcalibur (ThermoFinnigan, Egelsbach, Germany) for windows was used for data acquisition.

Capillary Gas Chromatography-Fourier Transform Infrared Analysis (HRGC-FTIR). HRGC-FTIR spectra were obtained using a HP 5890 GC (Agilent Technologies, Böblingen, Germany) coupled to a FTS 575C Tracersystem (BioRad, Hercules, CA, USA). GC separation was performed using a DB-1 capillary column (30 m x 0.25 mm ID; df = 0.25 µm; J & W Scientific, Folsom, CA, USA). Temperature was programmed from 80°C to 270°C with 4 °C/min heating rate. Helium was used as carrier gas with a constant flow of 1-2 ml/min. Injection was carried out using a split/splitless injector at 250°C in the splitless mode for 60 sec. Injection volume was 0.1 µl.

IR spectra were recorded by scanning 256 times in a frequency range from 4000–700 cm^{-1} with a resolution of 1 cm^{-1}. Data system was a Dell Optiplex GX110-PC with BioRad WinIR Pro (Version 2.7) Tracer Software and Sadtler IRSearchMaster.

Enantioselective Capillary Gas Chromatography (enantio-HRGC). Enantio-HRGC was carried out with a Carlo Erba 5160 GC using a fused silica capillary column coated with 30% 2-methyl-3-ethyl-6-di-O-*t*-butyldimethylsilyl-β-cyclodextrin in (85-88%) dimethyl-(12-15%)-diphenylsiloxane copolymer silanol terminated (PS086) (25 m x 0.25 mm ID; df = 0.15 µm; temperature program from 50°C to 160°C at 2°C/min and from 160°C to 240°C at 5°C/min). Split injection (1:20) and an injector temperature of 230°C were employed. Hydrogen was used as carrier gas with an average linear velocity of 50 cm/s. The temperature of the FID detector was 250°C. Samples of authentic racemic and (*R*)-methyl 2,3-dihydrofarnesoate (Ho and Millar, 2001a,b) as well as a methylated sample of beewolf head extract in hexane were analysed.

Chemicals. Solvents (Fluka , Deisenhofen, Germany) were distilled and checked for purity by GC-MS prior to use. 11-Eicosen-1-ol was purchased from ICN Biomedicals (Irvine, CA, USA) and 1-octadecanol as well as the alkanes (C18 to C30) were purchased from Aldrich (Deisenhofen, Germany). Racemic methyl 2,3-dihydrofarnesoate and pure (*R*)-methyl 2,3-dihydrofarnesoate were kindly provided by Jocelyn G. Millar (Ho and Millar, 2001a,b). 10-Nonadecen-2-one was synthesised (see below).

Synthesis of (Z)-10-Nonadecen-2-one. This compound was synthesised from oleic acid and methyl lithium (Fluka, Deisenhofen, Germany) as described by Bestmann et al. (1975). To a solution of 5 g oleic acid in diethyl ether 2 g methyl lithium in diethyl ether was added slowly under nitrogen at 0°C and stirred for 4 h. The reaction mixture was then diluted with 5% sulfuric acid. After removing the organic layer, drying over sodium sulfate, filtering and evaporating the solvent, the residue was distilled. EI-MS (70 eV): *m/z* (%) 41 (67), 43 (100), 55 (87), 71 (87), 82 (63), 96 (61), 111 (26), 125 (42), 135 (13), 149 (7), 184 (4), 198 (3), 222 (3), 262 (2), 280 (2).

Dimethyl Disulfide (DMDS) Derivatisations. DMDS derivatisation was carried out to determine the position of double bonds according to the method of Dunkelblum et al. (1985).

Methylation. A hexane solution of a beewolf male head extract was carefully evaporated under a stream of nitrogen to dryness and redissolved in 50 µl methanol. Then, 50 µl trimethylsulfonium hydroxide (TMSH) (Aldrich) were added and 1 µl of the mixture was injected into the GC without further treatment.

4.4 Results

Qualitative differences between the extracts made with hexane, dichloromethane or SPME fibres were not found. We also did not find differences between males from the field population and those reared in the laboratory. However, the contents of the cephalic glands differed somewhat among individual males (Herzner et al., in prep.). In the following, minor components that were only found in some males will not be reported. The heads of 25 males from the laboratory population were individually analysed. A typical total ion chromatogram of a hexane extract of the glands of an individual male is shown in Figure 1. The mean (± SD) total amount of the entire pheromone was 353 ± 167 µg. The mean relative amount of each constituent and its standard deviation is given in Figure 2.

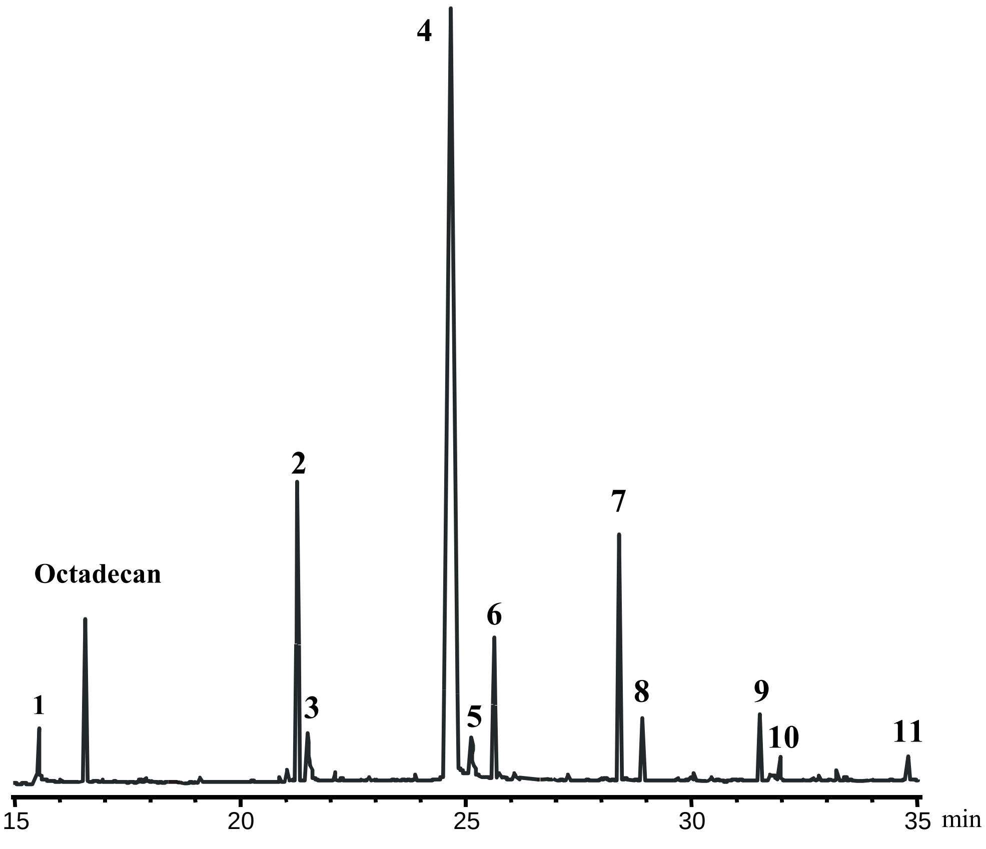

Figure 1: Gas chromatogram of a hexane extraction of the cephalic gland of an individual beewolf male: (**1** = (*S*)-2,3Dihydrofarnesoic acid , **2** = (*Z*)-10-nonadecen-2-one, **3** = 1-octadecanol, **4** = (*Z*)-11-eicosen-1-ol, **5** = (*Z*)-9-tricosene, **6** = tricosane, **7** = (*Z*)-9-pentacosene, **8** = pentacosane, **9** = (*Z*)-9-heptacosene, **10** = heptacosane, **11** = nonacosane).

(S)-2,3-Dihydrofarnesoic Acid. Compound **1** was identified as methyl 2,3-dihydrofarnesoate after methylation with TMSH by comparing the chromatographic and GC-MS as well as the GC-FTIR data with that of an authentic reference: *m/z* (%): 41 (71), 55 (16), 59 (15), 69 (100), 81 (18), 95 (18), 109 (56), 123 (18), 151 (2), 177 (6), 209 (18). In the untreated extract this compound showed the following EI-MS data: *m/z* (%): 41 (41), 55 (12), 69 (100), 81 (12), 95 (11), 109 (32), 123 (20), 135 (2), 151 (1), 177 (2), 195 (19), 223 (1). CI-MS confirmed the molecular mass of 238. HRGC-FTIR analysis revealed 1704 (-COOH) and 987 (*trans* band) (Attygalle et al., 1995). The stereochemistry was determined by HRGC enantioseparation of the methylated racemate (Bicchi et al., 2002). The first eluted peak was the (*R*)-enantiomer according to an authentic (*R*)-reference (Ho and Millar, 2001a,b). The methylated gland extract contained exclusively the methyl (*S*)-2,3-dihydrofarnesoate. Thus, compound **1** of the beewolf male pheromone was identified as enantiomerically pure (*S*)-2,3-dihydrofarnesoic acid (Figure 3).

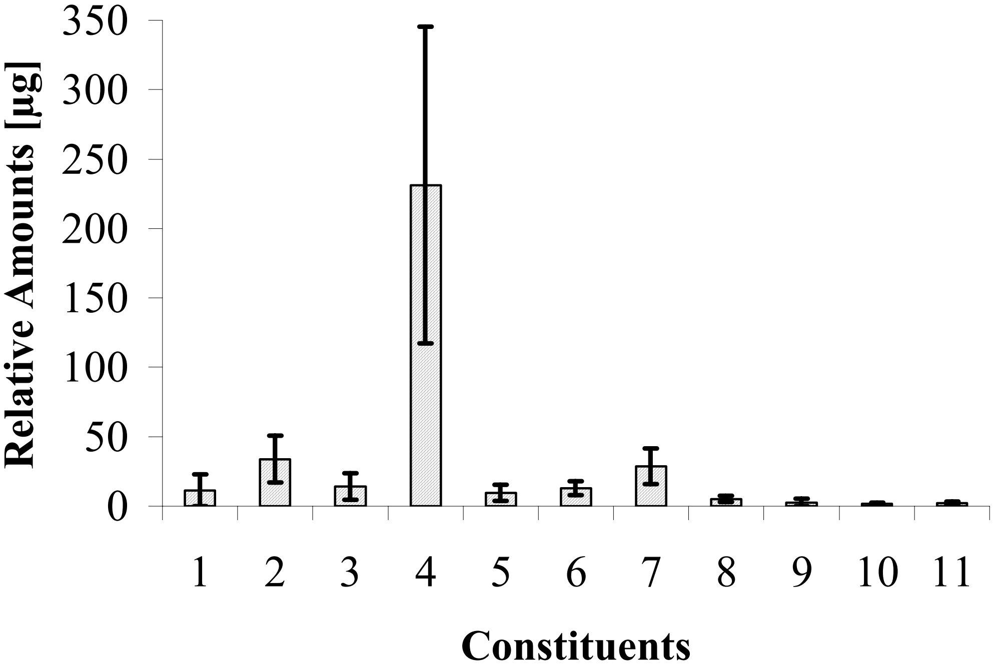

Figure 2: Relative amounts of the constituents of the male cephalic secretion of *Philanthus triangulum*, means (± SD) of 25 beewolf head extracts. Numbers of components are the same as in Figure 1.

(Z)-10-Nonadecen-2-one. Peak **2** of the cephalic gland extract was identified as (*Z*)-10-nonadecen-2-one. After derivatisation with DMDS it showed characteristic EI-MS peaks at 173 and 201 and M^+ at 374. The configuration of the double bond was determined by HRGC-FTIR (Attygalle et al., 1994). GC-FTIR: 3001 (cis compound of RCH=HCR'), 1703 (-C=O), 721 (cis compound of RCH=HCR').

1-Octadecanol. (**3**) *m/z* (%): 41 (62), 43 (82), 55 (100), 69 (88), 71 (41), 83 (97), 97 (84), 111 (42), 125 (19), 139 (6), 154 (3), 168 (2), 182 (1), 196 (1), 224 (2), 252 (1).

(Z)-11-Eicosen-1-ol. The major compound **4** in the extract was identified as (*Z*)-11-eicosen-1-ol. The position of the double bond was determined after DMDS derivatisation. M^+ of the derivatised compound was 390 and the major mass peaks were found at 173 and 217. The HRGC-FTIR data of the extract revealed the geometry of the double bond (Attygalle et al., 1994). *m/z* (%): 41 (58), 43 (45), 55 (99), 67 (61), 69 (65), 82 (100), 96 (75), 109 (30), 123 (17), 138 (9), 152 (4), 166 (2), 180 (1), 222 (1), 250 (1), 278 (3). GC-FTIR: 3326 (O-H), 3001 (cis compound of RCH=HCR'), 1054 ($-CH_2-OH$), 721 (cis compound of RCH=HCR').

Alkanes. All alkanes were identified by comparing retention times and mass spectra of the beewolf head extracts with a mixture of purchased alkanes.

(Z)-Alkenes. The corresponding alkenes were identified by their typical mass spectra. The position and the geometry of the double bond were determined by the same methods as described above. All alkenes have a double bond at position 9 with *cis* configuration.

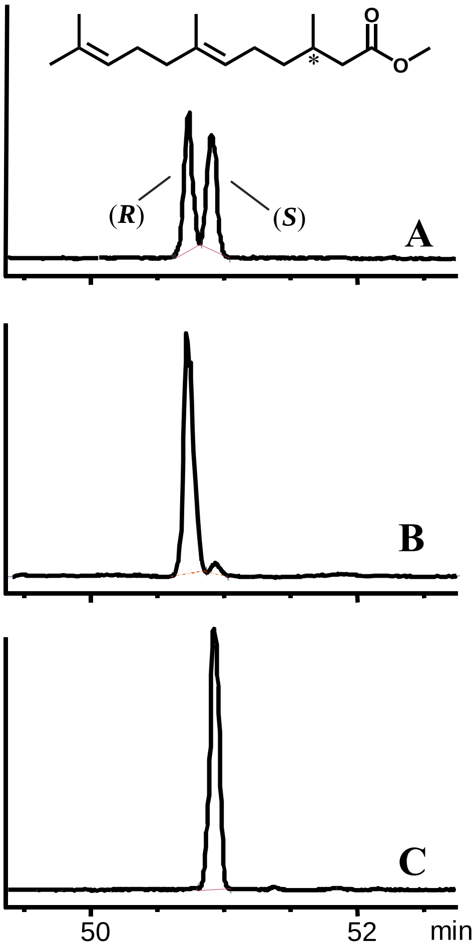

Figure 3: Assignment of the absolute configuration of methyl-2,3-dihydrofarnesoate by enantioselective gas chromatography. (A) racemic, (B) synthetic methyl-(*R*)- and (C) methyl-(*S*)-2,3-dihydrofarnesoate from male beewolf cephalic secretion.

4.5 Discussion

The cephalic glands of *Philanthus triangulum* males from a population at the Biocenter in Würzburg contain a complex mixture of at least 11 components. The major compound is (*Z*)-11-eicosen-1-ol, which is consistent with a previous study on beewolf males of a population from France, South of Bordeaux (Schmidt et al., 1990). Also in agreement with this study, we found (*Z*)-10-nonadecen-2-one. However, contrary to Schmidt et al. (1990) we did not find any nonadecenal, eicosenal, octadecenoic, or octadecanoic acid in the extracts. Another study, which was done on head extracts of a population from Öland, Sweden, found 2,5-dimethyl-3-propylpyrazine and 2,5-dimethyl-3-isopentylpyrazine in both males and females (Borg-Karlson and Tengö, 1980). We did not find these compounds either. The different results of these three studies might be partly due to different methods or might reflect differences among populations. Quantitative or qualitative differences in pheromone compositions among populations are known from several species of Lepidoptera (Löfstedt et al., 1986; Hansson et al., 1990; Toth et al., 1996; Kawazu et al., 2000), click beetles (Coleoptera, Elateridae) (Yatsynin et al., 1996) and the European pine sawfly (Hymenoptera, Diprionidae) (Anderbrant et al., 2000).

Schmidt et al. (1990) found another six compounds including three not further specified hydrocarbons. Detailed examination was not carried out because these compounds were ubiquitously found in extracts of head, thorax and abdomen of male and female wasps. The dissected cephalic glands analysed in our studies contained a variety of alkanes and alkenes from C23 to C29 (Figure 1).

The major compound (*Z*)-11-eicosen-1-ol (**4**) is well known from the honeybee, *Apis mellifera*. Its function as a component of the alarm pheromone and its attractiveness to conspecifics has already been shown (Pickett et al., 1982; Free et al., 1982; Free et al., 1983). Its occurrence in honeybees, which represent the exclusive prey of females of the European beewolf, as well as in the gland of male beewolves might have implications for the evolution of the male sex pheromone of *P. triangulum* (Schmitt et al., in prep.). Furthermore, (*Z*)-11-Eicosen-1-ol is a major component of the venom of *Apis cerana* (Schmidt et al., 1997) and has been detected in the thoracic glands of male carpenter bees (*Xylocopa micheneri*) (Andersen et al., 1988). In the two latter cases the function of (*Z*)-11-eicosen-1-ol is not known.

(*Z*)-10-Nonadecen-2-one (**2**) has only been found twice in arthropodes. It has been described as a trace constituent isolated from lipid fractions of the total extract of the ant *Iridomyrmex humilis* (Cavill et al., 1980) and as a component of the defensive secretion of the New Zealand tenebrionid beetle *Uloma tenebrionides* (Gnanasunderam et al., 1985).

The newly identified compound (*S*)-2,3-dihydrofarnesoic acid (**4**) has not been definitively identified in nature before. The occurrence of 2,3-dihydrofarnesoic acid was described from trichomes of *Lycopersicon hirsutum*, a wild relative of the tomato, but its stereochemistry was not established (Snyder et al., 1993). Interestingly, the methyl ester of (*R*)-2,3-dihydrofarnesoic acid is a component of the male sex pheromones of the stink bug species *Chlorochroa ligata*, *C. uhleri* and *C. sayi* (Ho and Millar, 2001 a,b). Closely related substances such as 2,3-dihydrofarnesal and 2,3-dihydrofarnesol have been found in secretions from labial glands of males of several bumblebees (*Bombus*) and are used for scent marking, possibly to attract mates (Bergström et al, 1967; Bergström and Svensson, 1973, Svensson and Bergström, 1977, 1979; Bergman and Bergström, 1997).

1-Octadecanol (**3**), another new compound in the cephalic glands of the European beewolf, is known as a minor constituent of the glands of the congener *P. barbatus*, a species from North America. This alcohol was also found as a component of the alarm pheromone of *A. mellifera* (Free, 1987; Free et al., 1989) and in the venom of *A. cerana* (Schmidt et al., 1997).

The classes of compounds found in the cephalic glands of male European beewolfs (alcohols, a terpenoid, ketones, alkanes, alkenes) differ somewhat from those in males of several North American *Philanthus* species. The evolutionary significance of this difference is not yet understood. Assuming the pheromone provides species recognition cues one would even expect stronger differences among the North American species that often occur sympatrically than between these and the European beewolf that is the only representative of the genus in most of its geographical range (e.g. Ayasse et al., 2001, Borg-Karlson et al., 2003).

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5 Brothers smell similar: Sex pheromone variation in a wasp and implications for inbreeding avoidance

5.1 Abstract

Female choice is thought to increase the fitness returns of females. The complementary choice model states that the best mate depends on the particular genotype of a female. Thus, females should choose males with a certain genotype to provide their progeny with an optimal allele combination. Aculeate Hymenoptera represent a special case of complementary female choice since males should be chosen on the basis of their similarity at the sex determination locus. The prevalent sex determination mechanism in bees and wasps (single locus sex determination) requires that in order to produce a daughter diploid offspring are heterozygous at the sex determination locus. Otherwise infertile diploid males result. Inevitably, the proportion of diploid males increases with inbreeding. In the European beewolf, a solitary sphecid wasp, the production of infertile diploid males is particularly disadvantageous. Male beewolves scent mark territories to attract mates. We hypothesized that the male sex pheromone varies in such a way that allows the separation of different families. This would be a prerequisite for females to discriminate against brothers and so avoid the detrimental effect of inbreeding. We analyzed the sex pheromone of male progeny of eight families using gas-chromatography and mass-spectrometry. We found a significantly higher similarity among brothers than among non-related individuals. Such a genetic component of a male sex pheromone has not yet been described from aculeate Hymenoptera. If beewolf females are only as good in discriminating among sib and non-sib as our analysis they might reduce the proportion of sib matings by up to 50-80%.

5.2 Introduction

Female choice for certain male characters is one of the most important forces driving evolutionary change (Andersson 1994, Boughman 2002). Whereas acoustical and visual male signals have received considerable attention (e.g. Alcock 2001, Andersson 1994, Burkhardt and de la Motte 1988, Klappert und Reinhold 2003, Ryan 1983), surprisingly little is known about female choice that is based on chemical signals (Eisner and Meinwald 1995, Moore 1997, Sappington and Taylor 1990 a, b, c, Van Dongen et al. 1998, Hine et al. 2002). However, chemical signals are probably the most important sensory cues for finding resources and mating partners in the vast majority of species. Due to the potential for variation in qualitative and quantitative features of semiochemicals along with the extreme sensitivity of olfactory systems of some species (e.g. Kaissling 1971, Angioy et al. 2003), chemical signals might convey much more information, e.g., about potential mates, than acoustical or visual signals. Here we study a species of solitary wasps that face a problem of female choice intrinsic to the Hymenoptera. We ask whether the chemical signal of males provides information for an adaptive female choice.

There are several models, how females can increase the fitness of their progeny by choosing the right mate (here we will only deal with indirect effects on female fitness). According to the good genes model, males with intrinsically superior genes are the best choice for all females (Andersson 1994, Hine et al. 2002, Johnstone 1995, Møller and Alatalo 1999, Tomkins and Simmons 1999, Wilkinson et al. 1998). By contrast, the model of genetic complementarity assumes that there is one particular "best" mate for each individual female (Colegrave et al. 2002, Johnsen et al. 2000, Reinhold 2002, Tregenza and Wedell 2000). Most studies on complementary female choice were concerned with post-copulatory cryptic female choice in polyandrous species (e.g., Birkhead and Møller 1998, Birkhead and Pizzari 2002, Colegrave et al. 2002, Eberhard 1996). In these polyandrous mating systems there are usually no obvious indicators that could convey information on male genotype prior to copulation (Colegrave et al. 2002, Tregenza and Wedell 2000, Zeh and Zeh 1997). In species where females mate only once no post-copulatory choice is possible. In these species complementary female choice requires indicators of male genetic equipment.

The aculeate Hymenoptera provide a particularly interesting group that, due to their sex determination mechanism, is predestined to evolve a means of complementary female choice. Hymenoptera are haplo-diploid with females developing from fertilized eggs and males usually developing from unfertilized eggs. In most Hymenoptera, there is a single sex-determining locus (e.g. Beye et al. 2003). Haploid individuals (which are necessarily hemizygous at the sex-determining locus) develop into males. Diploid individuals heterozygous at this sex-determining locus develop into females, whereas diploid individuals homozygous at the sex-determining locus develop into males (single-locus complementary sex determination, sl-CSD; Butcher et al. 2000 a, b, Cook and Crozier 1995, Crozier 1977, Kerr 1987, but see Haig 1998). If females share one sex determination allele with a male partner (so called “matched matings”), 50% of the fertilized eggs (presumptive daughters) will develop into diploid males (Cook and Crozier 1995, Godfray and Cook 1997, Ratnieks 1991). Usually, diploid males are either sterile or not viable at all (Godfray and Cook 1997, Petters and Mettus 1980, Woyke and Skowronek 1974). Inbreeding considerably increases the probability of matched matings and, thus, increases the proportion of such “futile” diploid males (with sibling matings the proportion of matched matings varies between 25 and 50% depending on whether the mother was outbreeding or was also inbreeding). Avoidance of mating with close relatives can thus be regarded as a special case of mate choice for genetic complementarity in Hymenoptera.

If kinship (or even the sex-determining alleles) could be assessed by females, the frequency of matched matings could be reduced. Cuticular hydrocarbons have been shown to be the primary chemical cue involved in kin recognition, but mainly in the context of nest mate recognition in social hymenoptera (Gamboa et al. 1986, 1996, Greenberg 1979, Howard 1982, Obin et al. 1993, Smith and Wenzel 1988, see also Ratnieks 1991). The composition of sex pheromones has been shown to exhibit individual variation in some insect species (Antony 1985, Collins and Cardé 1989, Löfstedt et al. 1985, Sappington and Taylor 1990 a, b, Sreng et al. 1989, see also Moore

1997, Svensson et al. 1997, Zhu et al. 1996) and could thus provide a basis for mate choice. In Hymenoptera, however, analyses of individual variability of pheromones are rare. In the sweat bee *Lasioglossum zephyrum,* the composition of the female sex pheromone is known to vary with kinship (Smith et al. 1985, Smith and Wenzel 1988). If male pheromones would show such genetically based variation females could avoid inbreeding and diploid sons by choosing unrelated, complementary mates. In this study, we test the hypothesis that the male sex pheromone of a solitary sphecid wasp, the European beewolf *Philanthus triangulum*, varies among families in a way that might enable females to discriminate close relatives from unrelated potential mates.

Males of the European beewolf establish small territories that do not contain any resources essential to the females. They scent mark these territories with a sex pheromone from a mandibular gland to attract females (Schmitt et al. 2003, Strohm 1995, Strohm and Lechner 2000). Receptive females alight in the territories, males immediately approach them and copulate without any further courtship behavior. Thus, the male pheromone most likely plays the predominant role for mate choice. Usually, several males establish territories in close vicinity to female nests, forming a lek that allows females to compare and choose between males with presumably low costs. Beewolf females mate only once and as a consequence they have to choose the optimal mating partner prior to copulation. Due to the frequent colonization of new habitats (e.g. Hirschfelder 1964) and the usually low population densities (at least in most of the distribution of the species), local beewolf populations are often rather small and there is a high potential of encountering siblings as mating partners.

Female beewolves hunt honeybees as provisions for their larvae (e.g. Tinbergen 1935) and prey hunting has been shown to be costly (Strohm and Marliani 2002). Sons are usually provided with two bees, daughters with four bees; thus, daughters are about twice as costly as sons (Strohm and Linsenmair 1997 a, b, 1998, 1999, 2000). In addition, the investment sex ratio is strongly biased towards males (Strohm and Linsenmair 1997 a, b, 1998, 1999). The production of diploid males would therefore vitiate a major part of maternal investment in *P. triangulum*. Consequently, beewolf females should avoid inbreeding through kin recognition mediated by the composition of the male sex pheromone. We investigated this hypothesis using gas-chromatography and mass-spectrometry to analyze the composition and variation of the sex pheromone of male European beewolves.

5.3 Material and Methods

5.3.1 Specimens

Females (mothers) were obtained from a locally restricted field population that existed for about four years close to the Biocenter of the University of Würzburg. Since the uniqueness of the nesting site might have implications for the interpretation of our results, we report some details here. Females are nesting in a large cage (5 x 5 x 4 m, mesh width 1 cm) that was used as an aviary. We believe that the aggregation was started by one or only few founder individuals that were probably brought there as cocoons in a pile of sand. Because of the mesh, the entering of the cage in flight is impeded. Due to their philopatry, beewolf females that emerged in the cage establish their nests there and learn to deal with the mesh. However, the mesh probably precluded immigration by foreign females. Therefore, the females of our study population are probably more closely related than individuals nesting in a more accessible site.

Females were kept individually in small breeding cages in a climate chamber at a 26/22°C day/night 14h/10h light/dark cycle and provided with honey and honeybees *ad libitum* until they died. The cages were then controlled at least twice every day for newly emerged males. These were caught, individually marked, and released into another climate chamber (240x180x210 cm; 26/22°C day/night and 14h/10h light/dark cycle) containing sand-filled buckets for nesting, artificial territories, beewolf females and honeybees. All animals were provided with honey *ad libitum*. Under these conditions males are induced to establish and scent mark territories (Strohm 1995). Seven days after emergence, males were caught and stored at -18°C until chemical analyses were conducted.

For analysis, animals were thawed, their heads were cut off and fixed by an insect needle. The mandibles were removed and the ventral cuticle on both sides of the mouth opening was cut to open the reservoir of the pheromone gland. Dissection was carried out on sheets of filter paper that were renewed for each male. All dissection instruments were cleaned in distilled hexane prior to the handling of the next specimen. The heads were extracted in distilled hexane (males of analysis group A (see below) overnight, males of group B for four hours). For quantification of pheromone components an internal standard (octadecane) was added to each extract. An aliquot of 1µl of each sample was analyzed by combined gas chromatography - mass spectrometry (see below).

The pheromone blends of 60 male *P. triangulum* belonging to eight families were compared. Due to technical constraints the chemical analysis could not be conducted for all eight families at the same time. The specimens had to be divided into two groups: analysis group A contained three families (family # 6, 7, and 22) with a total of 26 males (n = 10, 11, and 5); group B consisted of five families (family # 1, 4, 8, 12, and 31) with a total of 34 males (n=5, 7, 7, 10, and 5). The assignment of families to analysis groups was random.

5.3.2 Chemical Analysis

Capillary gas chromatography-mass spectrometry (GC-MS)-analysis was performed with a Fisons Instruments (Fisons, Egelsbach, Germany) GC 8000 Series coupled to a Fisons Instruments MD800 quadrupol mass detector. The GC was equipped with a J & W DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25µm) (J & W, Folsom, CA, USA), and the temperature program ramped from 60°C to 310°C with 5°C/min. The temperature was held constant at 310°C for 10 min. Helium was used as a carrier gas with a constant pressure of 90 mbar. A split/splitless injector was set at 240°C and was in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 220°C and an interface temperature of 315°C. The software Xcalibur for Windows was used for data acquisition.

The chemical identity of the individual pheromone peaks of male *P. triangulum* was determined by Schmitt et al. (2003) and is as follows (in the sequence of appearance in the chromatogram): *(S)*-2,3-dihydrofarnesoic acid; (Z)-10-nonadecene-2-one; 1-octadecanol; (Z)-11-eicosen-1-ol; (Z)-9-tricosene; tricosane; (Z)-9-pentacosene; pentacosane; (Z)-9-heptacosene; heptacosane; nonacosane.

5.3.3 Data Analysis

The peaks of 10-nonadecen-2-one and 9-heneicosene were not clearly separated in all chromatograms and were pooled and treated as one peak for the statistical analyses. (Z)-9-tricosene and nonacosane were present in negligible amounts in our specimens and were therefore not included in the analysis. This exclusion of some peaks decreases the possible variation among males and might mask differences. Thus, it is conservative with regard to the hypothesis tested.

For each individual pheromone blend, the total peak area was standardized to 100% and a multivariate analysis (using SPSS 11.0) was performed to estimate the divergence (or the similarity) of the chemical profiles of the different families. Because peak areas represent compositional data, the areas were transformed to logcontrasts (Aitchinson 1986) prior to the analysis. The peaks were subjected to a principal component analysis (PCA, with varimax rotation) to reduce the number of describing variables. The extracted PCA factors were then subjected to a discriminant analysis (DA) to assess whether males of different families can be discriminated on the basis of their pheromone profiles. A possible influence of male size and familial affiliation on overall pheromone amount was tested using an ANCOVA model with family as a random factor and male size as the covariate. When necessary, data were log-transformed to obtain normal distributions and equal variances.

The primary focus of our study was to test for family specific differences in pheromone blends. In addition, narrow sense heritability (h^2) was estimated for the amount of each pheromone component (peak area transformed as explained above) and the total amount of pheromone present in the head extracts of the families. We estimated the heritabilities (as well as standard errors) based on a General Linear Model with "family" as a random factor according to the formulas provided by Roff (1997; including a correction for the unequal numbers of brothers per family). As a consequence of methodological difficulties, e.g. with the breeding design, the heritability values for the amounts of individual pheromone components that we obtained can only be considered relatively rough estimates. We nevertheless report these data (appendix: Tables 3-5) for reasons of completeness and to provide a first insight into possible genetic influences on pheromone components in *P. triangulum*. However, we do not further discuss these data.

5.4 Results

Inspection of the chromatograms revealed considerable variation among males with regard to the relative amounts of different components. The results for analysis groups A and B are described separately.

	predicted family		
family	**6**	**7**	**22**
6	60	40	0
7	18	82	0
22	40	0	60

Table 1: Classification results of the discriminant analysis for analysis group A. Given are the proportions of classifications to the different families (in %).

Analysis group A: The PCA produced two principal components with eigenvalues larger than 1, explaining 77.8 % of the total variance. A DA on these principal components significantly differentiated the pheromone blends of male *P. triangulum* belonging to the three different families 6, 7, and 22 (Wilks'-λ = 0.356, χ^2 = 23.26, df = 4, $p < 0.0001$; Fig. 1). The families were mainly separated from each other on the basis of discriminant function 1, which explains 94.1% of the total variation extracted by the PCA. Families 6 and 7 were further separated by discriminant function 2 (5.9%). The classification reveals a 100% separation between families 7 and 22 (Table 1). In general, the classification shows that 60-82% (on average 67.3%) of the males were correctly assigned to the families by the DA (only 33% correct classifications would be expected by chance). The families of group A can therefore be separated from each other on the basis of quantitative differences in some of the pheromone components.

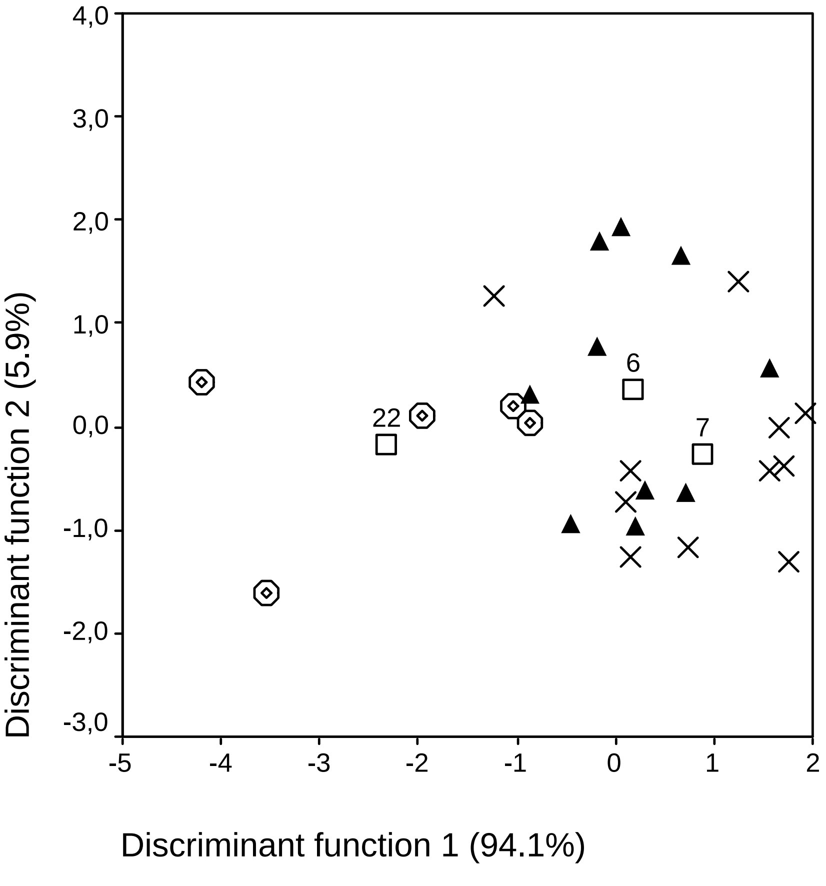

Figure 1: Discriminant analysis of analysis group A (3 families, 26 individuals). Despite some overlap, the families are separated significantly on the basis of the relative areas of eight pheromone peaks (Wilks'-λ = 0.356, χ^2 = 23.26, df = 4, $p < 0.0001$) (see also Table 1 and text for results of the preceding principal component analysis; ▲: family 6, n=10; ✕: family 7, n=11; □: family 22, n=5; □: family centroids).

The overall pheromone amount (the total of all eight components) was not significantly influenced by male size (ANCOVA: F = 1.75, df = 1, p = 0.2), but there was a significant difference in pheromone amounts among families (ANCOVA: F = 4.5, df = 2, p = 0.023). The general linear model revealed a statistically significant heritability for the overall pheromone amount ($h^2 = 0.48 \pm 0.6$; p = 0.043). Heritability estimates for the amounts of individual pheromone components are given in the appendix (appendix: Tables 3, 5).

	predicted family				
family	**1**	**4**	**8**	**12**	**31**
1	60	0	0	20	20
4	0	86	0	14	0
8	0	0	57	29	14
12	0	10	10	60	20
31	20	0	0	0	80

Table 2: Classification results of the discriminant analysis for analysis group B. Given are the proportions of classifications to the different families (in %).

Analysis group B: The PCA produced three principal components with eigenvalues larger than 1, explaining 82% of the total variance. As in group A, the DA on these three principal components significantly differentiated the pheromone blends of male *P. triangulum* belonging to the five different families 1, 4, 8, 12, and 31 (Wilks'-$\lambda = 0.246$, $\chi^2 = 40.73$, df = 12, p < 0.0001; Fig. 2). Discriminant function 1 accounts for 63.4% of the total variance extracted by the PCA and separated all but the families 4 and 8 from each other. The families 4 and 8 were separated by discriminant function 2 (31.9%). The third discriminant function (4.7%) did not further contribute to the separation of the families. Similar to analysis group A, the classification for analysis group B shows that 57-86% (on average 68.6%) of the males were correctly assigned to their families (only 20% correct classifications would be expected by chance, Table 2). The males belonging to the five families can therefore be separated from each other on the basis of quantitative differences in some of the pheromone components.

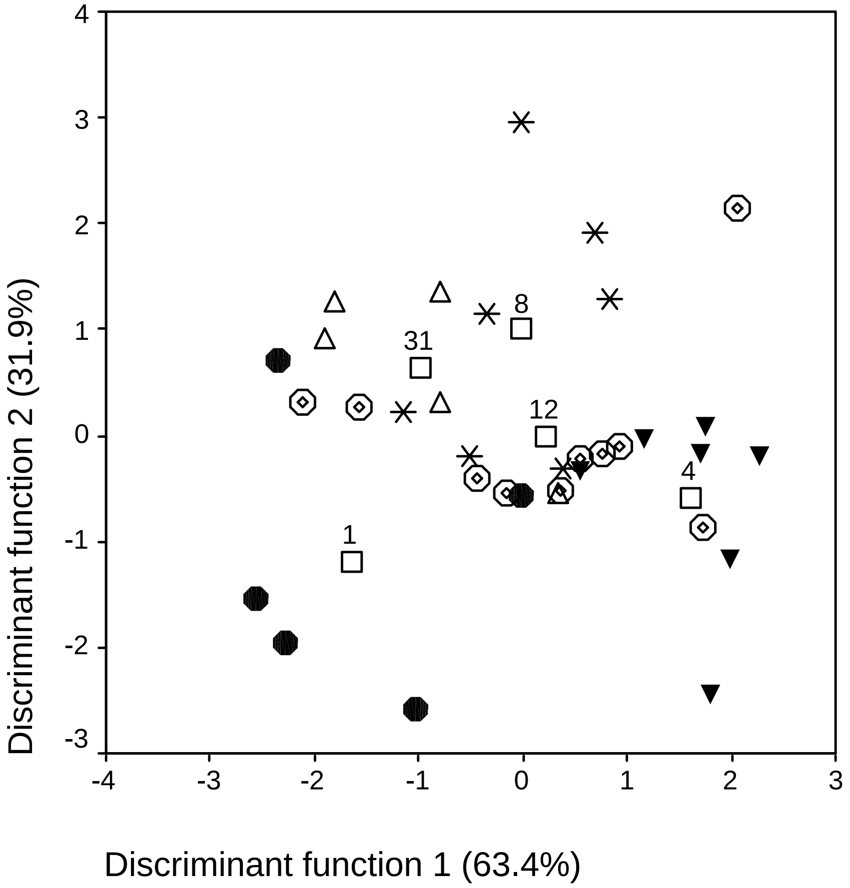

Figure 2: Discriminant analysis of analysis group B (5 families, 34 individuals). Despite some overlap, the families are separated significantly on the basis of the relative areas of eight pheromone peaks (Wilks'-λ = 0.246, χ^2 = 40.73, df = 12, $p < 0.0001$) (see also Table 1 and text for results of the preceding principal component analysis; ●: family 1, n=5; ▼: family 4, n=7; ✱: family 8, n=7; ◎: family 12, n=10; △: family 31, n=5; □: family centroids). For reasons of clarity discriminant function 3 is not shown.

As in analysis group A the overall pheromone amount was not significantly influenced by male size, although the effect is only marginally not significant (ANCOVA: F =

4.06, df = 1, p = 0.054). There was again a significant difference in pheromone amounts among families (ANCOVA: F = 3.4, df = 4, p = 0.022), and the general linear model revealed a statistically significant heritability for the total amount of pheromone ($h^2 = 0.54 \pm 0.5$; $p = 0.02$). Heritability estimates for the amounts of individual pheromone components are given in the appendix (appendix: Tables 4, 5).

5.5 Discussion

Our results indicate that the pheromone composition of male *P. triangulum* is significantly more similar among brothers than among unrelated individuals. Additive genetic variation among families might, thus, constitute a significant portion of the total individual variation in our study population of *P. triangulum*. This is, to our knowledge, the first evidence of a genetically based variability of a male sex pheromone in the Hymenoptera. According to our classification results, females could reduce the proportion of sib matings by 57-86 %.

The classification based on our analysis is not perfect, but this might not be expected due to different reasons. First, this analysis is based on a GC-MS analysis with subsequent statistical treatment using principal component and discriminant analysis. Beewolf females might have an olfactory system that is much more sensitive than a GC and they might use different algorithms that allow a much better distinction between brothers and unrelated males. Second, the partial overlap in pheromone composition between families could in part be due to a rather high relatedness among the mothers used in our study and the therefore still close relationship of their sons (see method section for the details of the population). Families collected from larger or different populations might be separated more clearly. Finally, there is probably an upper limit for variability of the sex pheromone composition in species where males are signaling. Due to the asymmetry of parental investment (females invest more in offspring, males invest more in mate finding and courtship) and sexual selection (females are generally the choosier sex), male-produced pheromones are tracking the female response in evolutionary time and can only vary within a range that reliably elicits female responses (Löfstedt 1990, Phelan 1992, 1997, Svensson 1996). On the other hand, the large number of components in the pheromone of beewolf males might provide an increased potential for variability.

The continuous variation in pheromone composition within families suggests a polygenic control of pheromone production (Collins and Cardé 1985). Brothers are more likely to share alleles than unrelated males, a complete match of the pheromone blends, however, cannot be expected. Within-family variation might enable females to exert a more finely tuned choice. There may be other more subtle deleterious effects of matched matings than the production of diploid males (e.g. higher susceptibility to parasites, see Gerloff et al. 2003), and females could optimize their fitness by choosing among those males carrying a compatible allele at the sex determination locus (Colegrave et al. 2002).

The overall pheromone amount produced by individual males differed significantly among families and showed significant heritability. Such an influence of familial affiliation on pheromone amount was also observed in several moth species (e.g. the Pink Bollworm Moth *Pectinophora gossypiella*: Collins et al. 1990, Collins and Cardé 1985; the Black Cutworm Moth *Agrotis ipsilon*: Gemeno et al. 2000; and the Cabbage Looper *Trichoplusia ni*: Gemeno et al. 2001). In our study species, this cannot be explained by between family differences in male size since size had no influence on overall pheromone amount. This surprising lack of a size effect is consistent with a former study that did not find evidence for size dependence of correlates of male mating success (Strohm and Lechner 2000). An alternative explanation for the differences in the amount of pheromone between families might be differences in basic physiological and metabolic capacities, allowing some families to produce larger pheromone amounts than others. In the honeybee *Apis mellifera*, for example, different genetic strains have been shown to differ in flight metabolic rate (Harrison and Fewell 2002).

As an Aculeate, the European Beewolf probably has single locus complementary sex determination (sl-CSD, Butcher et al. 2000 b, Cook and Crozier 1995). Females should therefore avoid inbreeding. Our results show that they could use the male pheromone as a precopulatory indicator for relatedness to discriminate among potential mates. Even though inbreeding avoidance does not preclude a matched mating and the production of diploid male offspring completely it will considerably reduce its prevalence. In *P. triangulum*, the avoidance of diploid males is especially important, because diploid male larvae cost twice as much as a haploid male (e.g. Strohm and Linsenmair 1999, 2000), but do most probably not contribute genetically to the next generation.

The mechanism by which beewolf females could recognize their brothers is unclear. There is evidence for inbreeding avoidance from a variety of animals (reviewed in Blouin and Blouin 1988, Pusey and Wolf 1996). In social species or those, where kinship can be deduced from familiarity, 'kin' recognition is often mediated by imprinting or learning of individuals that occur in the same nest or birth place (Fletcher 1987, Linsenmair 1972, 1985, Schildknecht et al. 1988, Greenberg 1988) and familiar individuals are not chosen as mates (Blaustein and Waldman 1992, Waldman et al. 1992). Whether beewolf females meet their brothers in their maternal nest to accomplish learning of the family odor is not clear (in *P. banabacoa,* males and females stay in their mother's nest for some time, Genaro and Sanchez 1992**)**. Alternatively, there could be a correlation between the pheromone composition of brothers and the cuticular hydrocarbons of sisters. Females could then assess relatedness to a potential mate by comparing their own phenotype to that of the potential mate, so called phenotype matching (Dewsbury 1988, Pusey and Wolf 1996, Waldman et al. 1988), and discriminate against those males whose pheromone composition is closely correlated to their own chemical profile.

In species where an association between relatedness and spatial occurrence or phenotype matching is not possible, other means have to evolve to avoid inbreeding (Pusey and Wolf 1996, Simmons 1989). The major histocompatibility complex (MHC) has been shown to provide such a mechanism for individual and kin recognition in vertebrates. There is evidence for mate choice based on MHC compatibility for a variety of species (for reviews see: Penn 2002, Penn and Potts 1999, Tregenza and Wedell 2000), including mice (Eklund 1998, Potts et al. 1991), rats (Brown et al. 1987, Singh et al. 1987), fish (Landry et al. 2001), and humans (e.g. Ober et al. 1997, Wedekind et al. 1995, Wedekind and Füri 1997). A preference for odors that indicate dissimilar MHC alleles might increase fitness by providing progeny with a higher variability at the MHC loci and a more competent immune system (Knapp et al. 1996, Ober et al. 1997, Wittzell et al. 1999). In our study species, a similar genetic mechanism of kin recognition seems unlikely but can not be excluded based on current knowledge.

A crucial problem with the good genes models is how sufficient variability of the sexually selected traits is maintained despite strong directional selection. One possible solution is the hypothesis that coevolving parasites continuously challenge their hosts and select for different optimal genotypes over time (Hamilton and Zuk 1982). Under the complementary choice hypothesis adaptive genetic variation is maintained, because of the idiosyncrasy of genetic effects, i.e. male genes that are a good match for one female may not be as good for another (Colegrave et al. 2002, Garner and Schmidt 2003). Consequently, there is no persistent directional selection caused by directional female mate preferences (Møller and Alatalo 1999). In aculeate Hymenoptera, mate choice to avoid inbreeding and diploid males would inevitably maintain diversity at the sex determination locus. Thus, inbreeding avoidance in aculeate Hymenoptera might provide a promising model system to investigate the evolution of mate choice for complementary genotypes. Our results suggest that the sex pheromone of male European beewolves exhibits family specific variation in such a way that inbreeding avoidance due to complementary female choice should be possible.

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5.7 Appendix

Substance	**Mean [µg] ± sd**	**h² ± SE**	**p**
(S)-2,3-dihydrofarnesoic acid	11.02 ± 10.84	0.44 ± 0.58	0.053
(Z)-10-nonadecen-2-one + 1-Octadecanol	50.8 5±19.85	0.89 ± 0.67	0.0029
(Z)-11-eicosen-1-ol	243.34 ± 71.56	-0.13 ± 0.16	0.62
Tricosane	12.59 ± 3.79	0.30 ± 0.51	0.11
(Z)-9-pentacosene	35.48 ±12.30	0.28 ± 0.50	0.12
Pentacosane	5.92 ± 2.06	0.071 ± 0.35	0.29
(Z)-9-heptacosene	2.42 ± 3.88	0.89 ± 0.67	0.0028
Heptacosane	3.64 ± 1.49	0.18 ± 0.43	0.19

Table 3: Narrow-sense heritability (and standard error) estimates of individual pheromone components for male *P. triangulum,* analysis group A, calculated by full-sib analysis (General Linear Model). Number of families = 3, total number of individuals = 26.

Substance	**Mean [µg] ± sd**	**h² ± SE**	**p**
(S)-2,3-dihydrofarnesoic acid	21.65±22.09	0.72±0.52	0.0047
(Z)-10-nonadecen-2-one + 1-Octadecanol	32.90±19.95	0.192±0.372	0.17
(Z)-11-eicosen-1-ol	162.13±76.18	0.612±0.51	0.011
Tricosane	8.20±4.20	0.54±0.49	0.020
(Z)-9-pentacosene	23.04±17.75	1.07±0.50	0.000097
Pentacosane	2.40±1.53	0.70±0.52	0.0055
(Z)-9-heptacosene	1.15±2.11	0.25±0.40	0.13
Heptacosane	0.92±0.66	0.18±0.37	0.19

Table 4: Narrow-sense heritability (and standard error) estimates of individual pheromone components for male *P. triangulum*, analysis group B, calculated by full-sib analysis (General Linear Model). Number of families = 5, total number of individuals = 34.

Substance	χ^2	**degrees of freedom**	**p**
(S)-2,3-dihydrofarnesoic acid	16.6	4	< 0.01
(Z)-10-nonadecen-2-one + 1-Octadecanol	15.23	4	< 0.01
(Z)-11-eicosen-1-ol	9.97	4	< 0.05
Tricosane	12.24	4	< 0.05
(Z)-9-pentacosene	22.7	4	< 0.001
Pentacosane	12.88	4	< 0.05
(Z)-9-heptacosene	15.8	4	< 0.05
Heptacosane	6.6	4	n.s.

Table 5: Combined probabilities of the narrow-sense heritability estimates of pheromone components for male *P. triangulum*, analysis groups A and B as shown in Tables 3 and 4 (Sokal and Rohlf 1981). Number of families = 8, total number of individuals = 60.

6 A cuckoo in wolves' clothing? Chemical mimicry in a specialised cuckoo wasp of the European beewolf (Hymenoptera, Chrysididae and Crabronidae)

6.1 Abstract

Host-parasite interactions are among the most important biotic relationships. Host species should evolve mechanisms to detect their enemies and employ counterstrategies. Parasites, in turn, should evade detection to maximise their success. Females of the European beewolf (Philanthus triangulum, Hymenoptera, Crabronidae) hunt exclusively honeybee workers as food for their progeny. The brood cells containing the paralysed bees are the target of a highly specialised cuckoo wasp (Hedychrum rutilans, Hymenoptera, Chrysididae). Female cuckoo wasps enter beewolf nests to oviposit on paralysed bees that are temporarily couched in the nest burrow. The cuckoo wasp larva kills the beewolf larva and feeds on it and the bees. H. rutilans can be a major cause of immature mortality in P. triangulum. Observations suggest that beewolves attack the cuckoo wasps in the vicinity of their nests but do not recognise these parasitoids when they encounter them in the nest. Since insects heavily rely on chemical senses we hypothesised that the failure to detect this principal enemy is the result of chemical cloaking. Cuckoo wasps might either mimic their beewolf host or the paralysed honeybees. Cuticular hydrocarbons of honeybee workers, male and female beewolves, and cuckoo wasps of populations from Würzburg were analysed by GC-MS. There was little congruence between cuckoo wasps and honeybees. However, there was a considerable similarity between beewolf females and cuckoo wasps that was even larger than between beewolf females and their conspecific males. The occurrence of isomeric forms of certain compounds on the cuticles of the cuckoo wasps but their absence on beewolf females suggests that cuckoo wasps synthesise the cuticular compounds rather than sequester them from their host. Thus, this study provides evidence that a

specialised cuckoo wasp exhibits chemical mimicry of the odour of its solitary wasp host.

6.2 Introduction

The interaction between hosts and parasites or parasitoids is one of the most important forces driving evolutionary and ecological processes (e.g., Godfray 1994). In order to reduce the impact of parasitoids, host species should evolve mechanisms to detect their enemies and employ adequate counterstrategies (e.g. Tengö and Bergström 1977, Rosenheim 1988, Quicke 1997, Strohm et al. 2001). Parasitoids, in turn, should evolve mechanisms that reduce the probability of detection by their hosts to avoid such countermeasures. This sets the stage for an arms race between hosts and parasites. The resulting adaptations and counter adaptations should be most obvious if the parasitoid is highly specialised and has a large impact on host fitness (Tengö and Bergström 1977, Sick et al. 1994, Spencer 1998).

Progeny of brood caring aculeate Hymenoptera are particularly susceptible to parasitism. Bees and wasps provision brood cells with large amounts of valuable nutrients that serve as the only provisions for growth and development of their progeny. These valuable resources attract a variety of brood parasites, either cleptoparasites that reduce the amount of resources available to the host's progeny or parasitoids that obligatorily kill the host larvae. Mostly, females of these parasitic species have to enter the nest or the brood cell to deposit eggs or larvae. Thus, female brood parasites might be encountered in the nest by their host and might be driven away, injured or even killed (E. Strohm, unpubl. observations). Hosts might also abandon their nests and remove or destroy eggs of brood parasites (Rosenheim 1988, Kimsey and Bohart 1990). Since insects heavily rely on their chemical senses for any kind of recognition or localisation process, concealment of a brood parasite probably means chemical camouflage (compounds acquired from the host or the host's nest) or chemical mimicry (compounds synthesised by the mimic, definitions sensu Dettner and Liepert 1994). In this study, we investigated the interaction between a hunting wasp, the European beewolf, Philanthus triangulum (Hymenoptera, Crabronidae) and the highly specialised brood parasitoid Hedychrum rutilans (Hymenoptera, Chrysididae). We asked whether the brood parasitoid is concealed from the digger wasp and whether chemical camouflage or mimicry is involved.

Females of the European beewolf hunt exclusively honeybee workers (Apis mellifera, Hymenoptera, Apidae) as food for their progeny. A beewolf female searches for bees on flowers, paralyses the prey by stinging and brings it to a nest in flight. Several bees are temporarily couched in the main burrow (see Strohm and Linsenmair 1994/95 for details on nest architecture). Eventually, the female closes the nest entrance, excavates a side burrow and a terminal brood cell, brings in one to five paralysed bees, and oviposits on one of the bees (Strohm and Linsenmair 1999). Thereupon, she carefully closes the side burrow and subsequently has no contact to her progeny. Larvae hatch two to three days later, feed on the bees for another six to eight days, spin into a cocoon, and either develop into an adult after about four weeks or enter diapause.

The cuckoo wasp, H. rutilans, is a specialised brood parasitoid of the genus Philanthus (Kunz 1994, Kimsey and Bohart 1990). However, since in Central Europe only one member of the genus, P. triangulum, is fairly abundant, H. rutilans is effectively monospecific in this region. This considerable degree of specialisation is expressed by the unique oviposition strategy of H. rutilans. Most chrysidid wasps oviposit into the brood cell of their hosts at a defined stage of the provisioning cycle (Kunz 1994). In beewolves, however, the brood cell is excavated after the female has brought the bees to the nest and the entrance has been carefully closed. Thus, the female is present until the brood cell is finally closed. This leaves little opportunity for a cuckoo wasp to deposit an egg in the brood cell. As a consequence, H. rutilans females pursue two alternative strategies. Either they pounce and oviposit on a paralysed bee when the female alights with its prey and enters the burrow (Veenendaal 1987, E. Strohm unpubl. obs) or they enter the burrow and oviposit on the paralysed bees that are temporarily couched there (Kunz 1994). Thus, H. rutilans use the paralysed bees as a Trojan horse to bring the egg into the brood cell. In contrast to the immobile first larval stages of P. triangulum, the larva of H. rutilans is mobile. It climbs onto the beewolf larva, kills it and feeds on the host larva and the bees. Thus, infestation by H. rutilans, inevitably leads to a fitness reduction of the host female (therefore we consider H. rutilans a parasitoid although it might be classified as a cleptoparasite since it consumes not only the host larva but also provisions). The rate of parasitism varies between 3% and more than 30% (Strohm and Linsenmair 2000, Simon-Thomas and Simon-Thomas 1972, E. Strohm, unpubl. data). H. rutilans might even drive local aggregations of P. triangulum to extinction (Simon-Thomas and Simon-Thomas 1972).

In both situations, when H. rutilans oviposits on a bee that is carried into the nest and when entering the nest to oviposit on the couched bees, detection by the beewolf female might decrease the cuckoo wasp's success. First, when encountered in the nest burrow cuckoo wasps might be carried to the nest entrance by beewolf females and thrown out (Olberg 1953). Mostly, cuckoo wasps are not harmed by this due to the solidity and strong sculpturing of their cuticle and their ability to adopt a rolled-up defensive posture that protects the most vulnerable parts of the body (legs, mouthparts, antennae; Kunz 1994, Gauld and Bolton 1996). Second, beewolves might remove bees from the nest that have possibly been parasitized. Simon-Thomas and Simon-Thomas (1972) reported that beewolf females sometimes remove bees from the nest and that a considerable proportion of these bees are parasitized. Thus, a cuckoo wasp should avoid detection to minimise wastage of time and investment. This means that cuckoo wasp females should not be recognised when they are encountered by a host female in the nest. It might be even more important for cuckoo wasps not to leave any detectable signs of their presence when they had entered the nest and oviposited on a paralysed honeybee.

There are two evolutionary options for H. rutilans females to avoid olfactory detection by beewolves. First, cuckoo wasps could mimic the odour of honeybees that are temporarily couched in the main burrow. In this case, the cuckoo wasps might be able to sequester the bees’ odour from the paralysed prey that is temporarily stored in the main burrow. This might require prolonged stays in the nest and would represent chemical camouflage. Second, H. rutilans females might mimic their beewolf host. This would probably mean that they have to synthesise the relevant chemicals since it is unlikely that brood parasites of solitary Hymenoptera have access to sufficient amounts of host derived chemicals to warrant an efficient cloaking. Thus, they would have to evolve chemical mimicry. To assess both alternatives we analysed the composition of cuticular compounds of beewolf females, cuckoo wasps, and honeybees. Furthermore, we included beewolf males as the *a priori* most similar group to beewolf females and, thus, a crucial comparison for the chemical mimicry hypothesis. A reasonable null hypothesis for the resemblance among the species under study might be based on their phylogenetic relationship. Crabronids and apids are closely related and constitute the superfamily Apoidea, whereas chrysidids branch off very early (e.g. Gullan and Cranston 1994). Thus, the null hypothesis would predict that the cuticular profiles of beewolf females should be most similar to conspecific males, fairly similar to honeybees, and least similar to cuckoo wasps.

We investigated the following questions: Are cuckoo wasps detected and recognised by beewolf females outside and inside of the nest? Is the chemical composition of cuckoo wasps similar to their host or to their host's prey?

6.2 Methods

6.2.1 Behavioural observations

We observed interactions between cuckoo wasps and beewolf females in the field in a beewolf nest aggregation on the Campus of the University of Würzburg, Germany. Over several years there were about 100 – 500 beewolf nests (easily detectable due to the characteristic nest mounds) and 50 – 500 *H. rutilans* females (E. Strohm, unpubl. data, determined by capture-mark-recapture methods, Mühlenberg (1993)). Behavioural interactions between beewolf females and cuckoo wasps at 24 focal nests (located on an area of about 10 x 5 m) were recorded for a total of 54 hours. We observed whether beewolf females showed any sign of disturbance or agonistic behaviour when a cuckoo wasp was present in the vicinity of their nests. We recorded the approach of a cuckoo wasp to a nest, the duration it stayed on or near the nest mound, whether it entered the nest, the duration of stay in the nest, and whether a beewolf female was present in the nest and how it responded to the presence of the cuckoo wasp.

The interaction between host and parasite inside the nest was investigated using observation cages in the laboratory (for details see Strohm and Linsenmair 1994/95). These cages allow observation of the behaviour of host and parasite in the main burrow. Beewolf females, either from the laboratory population or from the field, were kept individually in such cages and one cuckoo wasp that was caught in the field was introduced per cage. Honey was provided ad libitum for both species. Honeybees were also provided ad libitum. Since a pilot study revealed that the cuckoo wasps need a humid retreat, petri-dishes with a layer of moist sand and gravel were placed into the flight compartment of each cage and moistened daily. Observations of interaction in the nest burrow were carried out under dimmed red light that did not elicit any disturbance in either species.

6.2.2 Chemical analyses

We caught females of H. rutilans in the vicinity of beewolf nests at a field site on the campus of the University of Würzburg, Germany. Beewolf females were taken from the same field site or from a laboratory population that was bred from the same population. Honeybee foragers (Apis mellifera carnica) from the hives nearest to the field site (less than 100 m away) were caught when leaving the nest. All individuals were killed by chilling (1 h, –20 °C). Chemicals on their cuticles were extracted in 0.5 ml distilled n-Hexane (Fluka) for 10 min (we confirmed that we did not extract the contents of any glands by this extraction method).

Capillary gas chromatography-mass spectrometry (GC-MS)-analysis was performed with a Fisons Instruments (Fisons, Egelsbach, Germany) GC 8000 Series coupled to a Fisons Instruments MD800 quadrupol mass detector. We used a DB-5MS fused silica capillary column (30 m x 0.25 mm i.d.; df = 0.25 µm) (J & W, Folsom, CA, USA). The

GC was programmed from 60° C for 1 to 310° C for 10 min with a temperature increase of 5°/min, with 2 ml/min flow rate of helium gas. We chose a splitless injection mode (1µl) at an injector temperature of 250° C and a splitless period of 60 sec. The mass spectrometer was operated in EI mode at 70 eV. The software Xcalibur for windows was used for data acquisition.

The chemical structure of the components of the cuticular hydrocarbons was determined by comparing retention times and diagnostic ions of the mass spectra with purchased chemicals and the use of a commercial MS database (NIST 4.0). Methylalkanes were characterized using diagnostic ions and by determining Kovats indices according to the method of Carlson et al. (1998). The position of double bonds was determined by DMDS derivatisation (Dunkelblum et al. 1985). The configuration of double bonds was classified by HRGC-FTIR (Attygalle et al. 1994, Schmitt et al. 2003). Some components could not be identified and for some alkenes the position of the double bond and its configuration could not be determined due to their small amounts on the cuticles. However, neither of the unidentified components occurs on both beewolf females and cuckoo wasps. Thus, these compounds do not confound the similarity between these two groups that are most important to our question. The alkenes listed in one line in Table 1 as the same compound for beewolves, cuckoo wasps, and honeybees are most probably identical since their mass spectra and the retention times are identical. Thus, the comparison between beewolf females and cuckoo wasps is not confounded by the incomplete identification of the alkenes.

6.2.3 Data analysis

The results of the behavioural observations are given as the mean ± SD and/or the median. We analysed patterns of chemicals on the cuticle by multivariate methods. Since we were interested in the similarity between cuckoo wasps, beewolf females, beewolf males, and honeybee workers, we performed a hierarchical cluster analysis to assess the pattern of similarity without a priori grouping. Furthermore, we conducted a discriminant analysis to test how the groups are separated by discriminant functions.

Due to the large number of peaks relative to the sample size the discriminant analysis might lead to confounded results with regard to the hypothesis tested. Thus, we reduced the number of variables for the discriminant analysis using principal component analysis (7 variables were extracted that represented 88 % of the variance of the total sample).

Since relative peak areas of a sample are not statistically independent we transformed the data according to Aitchison (1986, see e.g. Nielsen et al. 1999). However, the original transformation procedure makes it necessary to exclude compounds that do not occur in all samples. Thus, peaks that are zero in some samples but are present in other samples would not have been considered. When analysing whether groups can be separated by their profiles such a procedure is conservative. However, for the aim of this study, the exclusion of peaks that are only present in some samples would have erroneously increased the similarity between the groups and, thus, confounded the result. Therefore, we modified the transformation to avoid undefined values for peaks with an area of zero (log((relative peak area/geometric mean peak area)+1)). The resulting variables were reasonably normally distributed. We used the squared Euclidean distance as a measure of distance for cluster analysis and within groups average linkage as the method for combining clusters (other methods of combining clusters yielded qualitatively identical results). Analyses were calculated using SPSS 10.0.

6.4 Results

6.4.1 Are cuckoo wasps detected by beewolf females?

H. rutilans were active throughout the flight period of beewolves (Strohm et al. 2001). Cuckoo wasps flew over the nesting site and selectively landed on the mounds of beewolf nests. During the 54 hours we recorded 1024 landings of H. rutilans on beewolf nest mounds. In 259 cases (25.3%), cuckoo wasps flew off after a stay of ≤ 4 s. In 765 cases (74.7%) they remained on or in the vicinity of the nest mound (less than 10 cm) for > 4 s, the duration of these stays was 74 ± 250 s (median = 11 s). During these stays the cuckoo wasps often moved on the nest mound, vigorously antennating the surface. During prolonged stays at the nest, cuckoo wasps often moved to shaded areas and sometimes even placed themselves under some nearby structures like leaves. In 37 of the 765 cases the nest entrances were open and cuckoo wasps entered the nest for 13 - 270 s (mean: 118 ± 133 s, median: 60 s). In the remaining 728 cases the entrance was closed. Nevertheless, in 41 of these cases cuckoo wasps tried to dig through the closure; in 29 cases they abandoned digging after some time. In the 12 remaining cases they dug through the nest closure and stayed in the nest for 14 to 1263 s (mean: 384 ± 421 s, median 213 s). Thus, stays in the nest are rare and, with one exception, the durations are rather short, not supporting the hypothesis that cuckoo wasps sequester chemicals from bees that are stored in the burrow.

During the observation time we observed 89 beewolf females returning with a paralysed honeybee and entering their nest. In 38 cases a cuckoo wasp was present in the vicinity of the nest. In four of these cases we observed attempts of cuckoo wasp females to attach to a honeybee that was carried by a beewolf female while it entered the nest with its prey. In all four cases, the parasitoid was detected and driven away. In another 11 cases (of the 38) cuckoo wasps were driven away by beewolf females returning to the nest. In one of these cases the beewolf female grasped the cuckoo wasp with her mandibles. Most probably beewolf females detected the cuckoo wasp visually. Sometimes (46 cases of 765 cases), cuckoo wasps were driven away from a nest mound by the approach of another cuckoo wasps. We never observed beewolf females to throw H. rutilans females out of their nest although at least in one case the cuckoo wasp was in the nest when a female returned with a bee.

In observation cages in the laboratory, H. rutilans females were observed to enter beewolf nests and oviposit on the couched bees. Although in five cases the beewolf female entered the nest while a cuckoo wasp was present and came close to (less than 2 cm, five occasions) or even passed (three occasions) the cuckoo wasp in the burrow, the host female did not show any signs of disturbance or detection of the brood parasitoid. Notably, the cuckoo wasp either ran to a distant part of the nest when a beewolf female approached or it remained motionless and seemed to crouch to the wall of the burrow until the female had passed. Since females responded aggressively towards cuckoo wasps outside the nest, the lack of response despite close contact in the nest suggests that H. rutilans females are not detected in the dark nest burrow. Thus, we hypothesised that cuckoo wasps are chemically cloaked.

6.3.2 Are cuckoo wasps chemically cloaked?

The GC-MS analyses revealed alkanes, alkenes, and mehtylalkanes as the predominant hydrocarbons in all species. We found 16 substances on the cuticles of H. rutilans (N = 13 individuals), beewolf females had 15 (N = 8), males had 18 (N = 8) and honeybees had 33 peaks (N = 8) (Table 1). H. rutilans females shared 13 compounds with beewolf females, 9 with beewolf males, and 12 with honeybees. The cuticles of honeybees contained a varying proportion of saturated to unsaturated hydrocarbons that changed with increasing chain length from predominately saturated C25 and C27 to predominately unsaturated C33:1. Male beewolves showed large proportions of C23, C25, Z-9-C25:1, C33, and C33:1. The profile of beewolf females is characterised by very high proportions of the unsaturated Z-9-C25:1 and Z-9-C27:1. Cuckoo wasps also show relatively large amounts of the unsaturated C25:1 and C27:1 but besides the Z-9 isomers they possess similar proportions of Z-7-C25:1 and Z-7-C27:1 (Table 1). Thus, cuckoo wasps have closely related isomers of the major components of their host on their cuticles.

A cluster analysis (Figure 1) clearly separated honeybees from cuckoo wasps and beewolves in the first bifurcation. Among beewolves and cuckoo wasps, however, the distinction was less pronounced. The second bifurcation separated a group consisting of four beewolf females and all beewolf males from a group consisting of the other four beewolf females and the cuckoo wasps. Thus, cuckoo wasps more closely resemble certain beewolf females. The third bifurcation placed one individual beewolf female in a unique branch. Only the fourth bifurcation separated all cuckoo wasps from the three

Compound	Retention time(min)	A. mellifera Worker	P. triangulum Male	P. triangulum Female	H. rutilans Female
C21	33.00	0.394	0	0	0
C22	34.90	0.126	0	0	0
(Z)-11-Eicosen-1-ol	36.00	0.465	0	0	0
(Z)-9-C23:1	36.20	2.783	0.245	0	0
(Z)-7-C23:1	36.35	0.298	0	0	0
C23	36.65	22.21	15.63	9.721	10.09
3-MeC23	37.90	0	0	0.344	1.098
C24:1	38.00	1.137	0.248	0	0
C24	38.45	0.502	0.728	0	0
(Z)-9-C25:1	39.65	5.252	32.74	36.01	11.01
(Z)-7-C25:1	39.80	0.230	0	0	10.62
C25	40.10	24.55	9.093	5.345	20.28
13-, 11-, 9-MeC25	40.70	0.042	0	0.004	1.948
7-MeC25	40.80	0.010	0	0.002	0.975
5-MeC25	40.90	0	0	0	0.489
C26:1	41.30	0	0.263	0.551	0.211
NI	41.60	0	0	0.272	0
C26	41.75	0.379	0	0	0
(Z)-9-C27:1	42.90	1.908	1.998	37.37	13.87
(Z)-7-C27:1	43.00	0	0	0.013	6.315
C27	43.30	12.78	4.882	2.258	10.59
13-, 11-, 9-MeC27	43.80	0.363	0	0	1.578
NI	44.80	0.152	0	0	0
NI	45.65	0.025	1.970	0.816	0
C29:1	45.90	0.338	0.043	1.514	0.571
C29:1	46.00	0.644	0	0	0
C29	46.20	4.471	3.313	3.586	8.361
13-, 11-, 9-MeC29	46.70	0.246	0	0	0
NI	47.20	0.311	0	0	0
NI	47.40	0.105	3.150	0	0
NI	47.80	0.449	0	0	0
C31:1	48.70	4.718	0.523	0	0
C31:1	48.80	0.628	0.464	0	0
C31	49.00	5.347	1.759	2.186	1.982
NI	50.10	0.227	0	0	0
C33:1	51.20	0.753	0	0	0
C33:1	51.50	7.774	11.02	0	0
C33	51.80	0.373	11.93	0	0

Table 1: Retention times and mean relative peak area (in percent, not transformed) of compounds on the cuticle of A. mellifera workers, P. triangulum males, P. triangulum females, and H. rutilans females. NI = not yet identified.

beewolf females of that cluster. According to this analysis, H. rutilans females are considerably more similar to beewolf females than to honeybees and are about as similar to beewolf females as beewolf males.

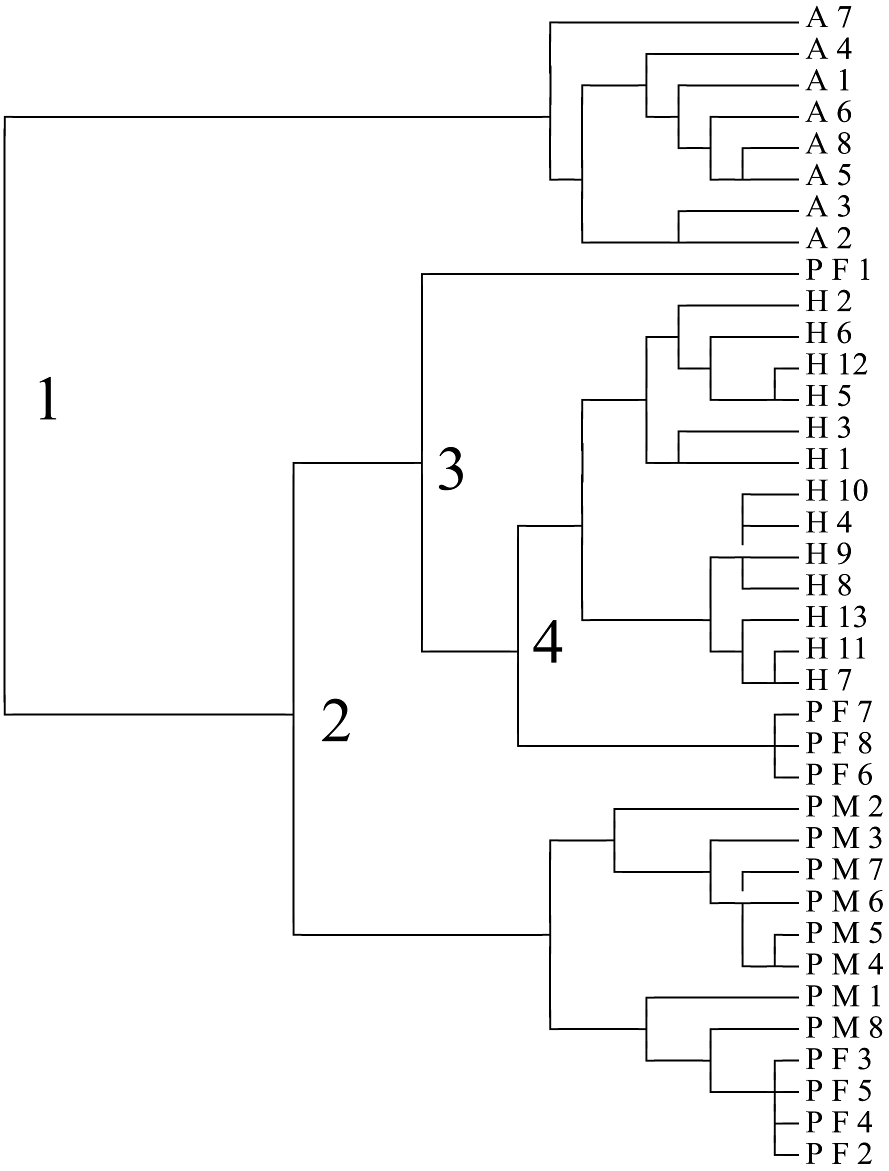

Figure 1: Dendrogram based on the cluster analysis of the cuticular compounds of individual P. triangulum females (= P F), P. triangulum males (= P M), H. rutilans females (= H), and A. mellifera workers (= A). Numbers after the species label indicate the different individuals. Numbers in the dendrogram indicate the first 4 bifurcations (see text).

The discriminant analysis, that followed the principal component analysis, yielded three discriminant functions that resulted in a complete separation of the four groups (Wilk's Lambda = 0.01, d.f. = 21, P < 0.001; Figure 2, Table 2). Discriminant function 1 represented 76.9 % of the variance and separated honeybees from the other three groups. Thus, by far the largest effect was between honeybees on the one hand and both sexes of beewolves and cuckoo wasps on the other hand. Discriminant function 2 represented 18.5 % of the variance and separated beewolf males from females and cuckoo wasps. Discriminant function 3, at last, separated beewolf females from cuckoo wasps and represented only 4.6 % of the variance.

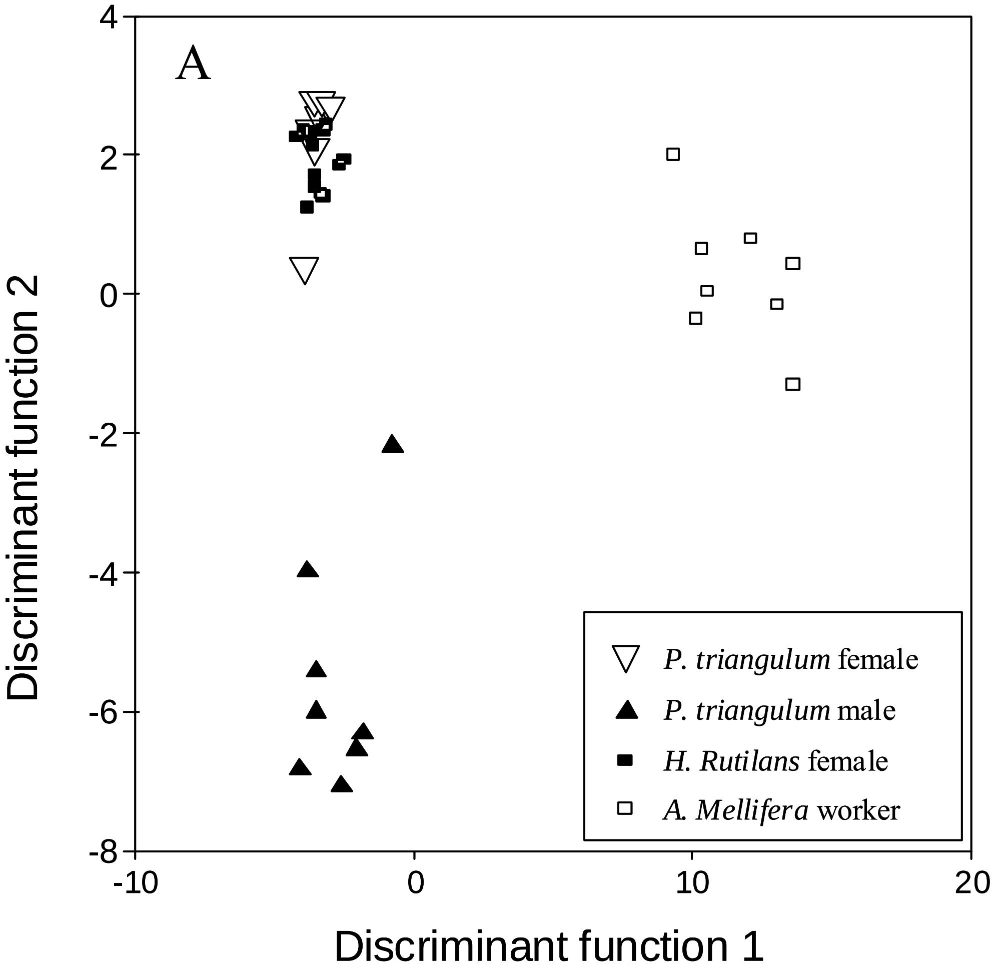

Figure 2A: Results of the discriminant analysis of the cuticular compounds of individual P. triangulum females, P. triangulum males, H. rutilans females, and A. mellifera workers. A: representation of the four groups for the first and second discriminant function.

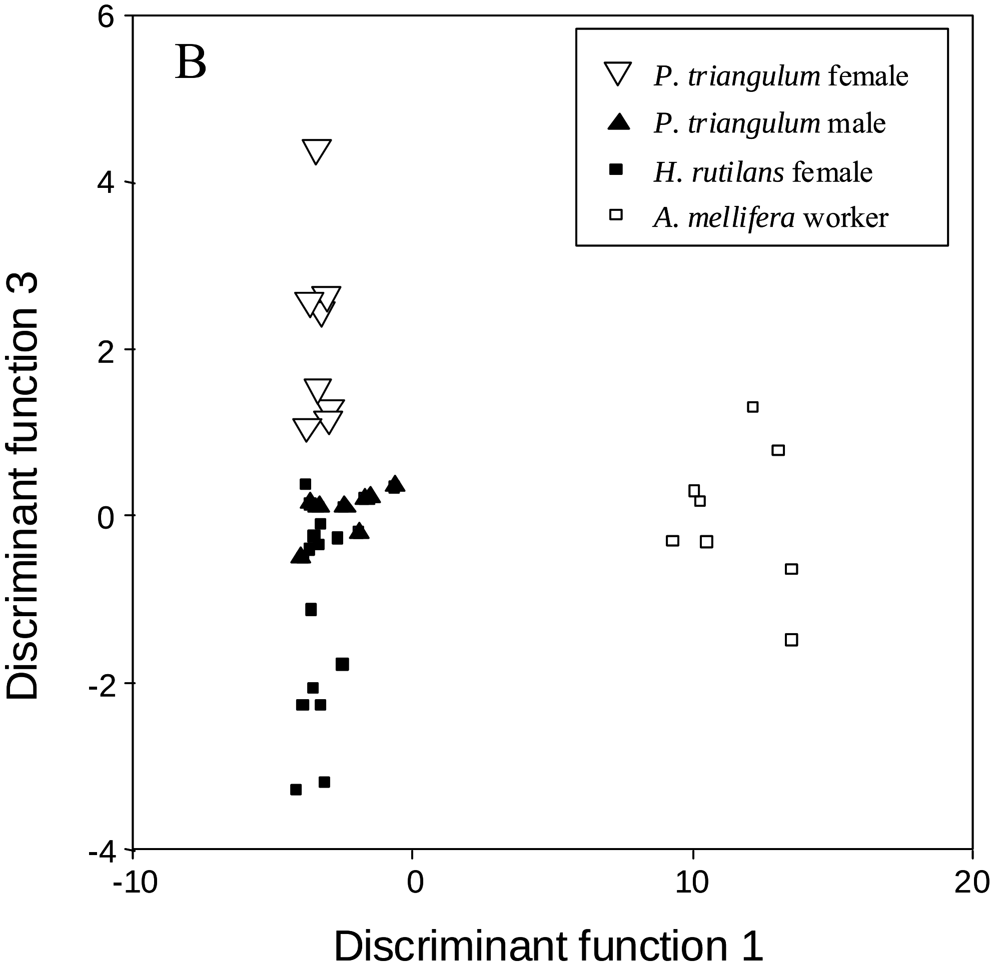

Figure 2: Results of the discriminant analysis of the cuticular compounds of individual P. triangulum females, P. triangulum males, H. rutilans females, and A. mellifera workers. A: representation of the four groups for the first and second discriminant function. B: representation of the four groups for the first and third discriminant function.

6.5 Discussion

Beewolf females attacked and evicted H. rutilans when they encountered them in front of their nest. This seems to be the rule for interactions between hosts and chrysidids although Linsenmaier (1997) reported that there are also cases where chrysidids do not elicit antagonistic behaviour by their hosts. Prolonged stays at hosts' nests as observed in H. rutilans, have also been reported for other chrysidids (Rosenheim 1987, Kunz 1994, Linsenmaier 1997). This behaviour is probably an adaptation that allows the chrysidids to adjust the timing of oviposition to the most suitable stage of the provisioning cycle. That H. rutilans females placed themselves under some cover (e. g. leaves) during prolonged stays might, besides the reduction of water loss, represent an attempt to hide themselves from the host females. Other chrysidid species seem also to hide near the entrance of a host nest and inspect the nest or brood cell after the host female has deposited provisions and departed for a new foraging flight (Linsenmaier 1997). This suggests that, similar to beewolves, most host species recognise cuckoo wasps visually outside the nest. Since chrysidids are all brightly coloured (see e.g. drawings in Linsenmaier (1997)) this is not surprising.

H. rutilans female versus:	**Wilk's Lambda**	**χ^2 (d.f. = 7)**	**P**
A. mellifera worker	0.016	64.4	< 0.001
P. triangulum male	0.035	51.8	< 0.001
P. triangulum female	0.242	21.9	0.003

Table 2: Results of pairwise discriminant analyses: H. rutilans is compared with honeybees, beewolf males as well as beewolf females. Given are Wilk's Lambda, χ^2 (d.f.) and P for the respective comparison.

However, H. rutilans females were not recognised by beewolf females in the nest although the nest owners passed the chrysidids several times. In contrast to Olberg (1953), we could not observe that chrysidids were thrown out of the nest by beewolf females neither in the study population in the field, nor in observation cages in the laboratory, nor during prolonged observations of beewolf nest aggregations as part of another study (Strohm et al. 2001). Possibly, the chrysidids that Olberg (1953) saw were not H. rutilans. Anyhow, our observations suggest that the H. rutilans females are not detected in the nests.

There are numerous reports of parasites of social species that gain access to their hosts' nests and protection from attacks mainly by chemical camouflage and more rarely chemical mimicry (Dettner and Liepert 1994, Stowe 1998, Lenoir et al. 2001, D'Ettorre et al. 2002, Moritz et al. 1991). Hydrocarbons are considered to represent the principal cues for nestmate recognition in social bees and wasps (Lenoir et al. 2001) and are most probably also involved in nest identification and species recognition in solitary species (Singer 1998). In our analysis, the GC-MS profiles of the cuticular hydrocarbons of H. rutilans and beewolf females show considerable similarity. At least, the null hypothesis based on the phylogenetic relationship of a closer resemblance between beewolves and honeybees than between beewolves and H. rutilans was clearly contradicted. The profiles of the chrysidids were as close as or even closer to beewolf females than the profiles of beewolf males. Thus, there is evidence that H. rutilans females are chemically cloaked and, thus, reduce the probability of detection in particular after a chrysidid had entered the burrow and oviposited.

A priori, chemical camouflage and mimicry seem unlikely to evolve in chrysidids that attack solitary hosts. Chemical camouflage, i.e. the acquisition of mimetic compounds from the host, might be difficult since the opportunity to sequester cloaking chemicals is limited compared with species that attack social hosts with large nests and a large number of colony members that are potential sources for the relevant compounds. Brood parasites of solitary brood caring Hymenoptera have rarely been studied (e.g. Hefetz et al. 1982). The only reported case of chemical camouflage in a brood parasite of a solitary species comes from Nomada bees. In some species of this genus, females have been reported to acquire mimetic odours by being perfumed by males during mating. Observations confirmed that females of these species do not elicit strong aggressive responses when encountered by host females of the genus Andrena (Tengö and Bergström 1977). In contrast, females of the parasitic bee genus Sphecodes, that parasitize halictid bees, seem not to resemble their hosts and are usually recognised and attacked by these (Sick et al. 1994). Chemical cloaking in chrysidid wasps has not yet been reported.

With chemical camouflage being not a feasible option for a brood parasite of solitary species, chemical cloaking might have evolved by synthesis of the compounds, i.e., chemical mimicry. For most chrysidids this is also unlikely since they attack a large number of different host species (e.g. Kunz 1994) with a varying composition of cuticular chemicals that is not compatible with an efficient chemical cloaking. However, our study species, H. rutilans, is de facto monospecific in the study area and is, thus, predestined to evolve chemical mimicry. The behavioural observations show that cuckoo wasps do not regularly stay in nests for long periods. This makes a sequestration of host chemicals that probably are only available in very small amounts at the walls of the burrow rather unlikely. The hypothesis that H. rutilans exhibits chemical mimicry is strongly supported by details of the composition of chemicals on the cuckoo wasp's cuticle. The occurrence of the Z-7 isomers of the major components of the beewolf cuticle, Z-9-C25:1 or Z-9-C27:1, in the brood parasitoid but the lack thereof in beewolf females contradicts an acquisition of the chemicals from their host. Thus, cuckoo wasps at least produce some of the compounds on their cuticle by themselves.

The probable chemical mimicry of H. rutilans females raises several interesting questions. First, beewolf females respond very aggressively towards conspecific females. Thus, the question is why cuckoo wasps that are obviously no perfect mimics do not elicit attacks. Second, the similarity in the composition of the cuticular hydrocarbons might be either the result of the same biochemical pathways in brood parasitoid and host (i.e., the pathways are homologous), or cuckoo wasps evolved alternative pathways (i.e., the pathways are the result of convergent evolution). At the moment we could only speculate about possible answers. Therefore, we postpone these aspects until we have data to test alternative explanations.

In conclusion, H. rutilans employ a combination of strategies to evade detection. They run away or remain motionless if encountered in the nest, and probably more important, they reduce the conspicuousness of scent marks left in the nest burrow or on the bee during oviposition since their cuticular compounds are reasonably similar to that of their host. This is to our knowledge the first case of chemical mimicry (sensu Dettner and Liepert 1994) in a parasitoid of a solitary wasp.

Acknowledgements

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7 Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect

7.1 Abstract

A hitherto unresolved problem in behavioral biology is how workers are prevented from reproducing in large insect societies with high relatedness. Signals of the queen are assumed to inform the nestmates about her presence in the colony which leads to indirect fitness benefits for workers. In the ant *Camponotus floridanus*, we found such a signal located on queen-laid eggs. In groups of workers regularly provided with either queen eggs, larvae and cocoons, with larvae and cocoons or with no brood, only in the groups with queen eggs workers did not lay eggs. Thus, the eggs seem to inform the workers about queen presence that let them refrain from reproducing. The signal on queen eggs is presumably the same that enables workers to distinguish between queen and worker-laid eggs. Despite their viability the latter are destroyed by workers when given a choice between both types. Queen and worker-laid eggs differ in their surface hydrocarbons in a similar way as fertile queens differ from workers in the composition of their cuticular hydrocarbons. When we transferred hydrocarbons from the queen cuticle to worker eggs the eggs were no longer destroyed, indicating that they now carry the signal. These hydrocarbons thus represent a queen signal that regulates worker reproduction in this species.

7.2 Introduction

The fundamental difference between solitary and highly social insects is reproductive division of labor between one or a few breeders and their non-breeding helpers (1-3). It is assumed that in large insect societies reproduction is regulated by pheromones (4).

One hypothesis suggests that these pheromones may be coercive tools of the breeder (the queen) to prevent its helpers (the workers) from reproducing against their own fitness interests (5, 6) as a form of parental manipulation (7). According to an alternative hypothesis, they may represent cooperative signals that inform workers how they can realize their fitness interests (8-10) in line with kin selection theory (11). In the presence of a fertile queen worker reproduction may impose costs on colony productivity which reduces the indirect fitness gains of workers (12-14). They should therefore either refrain from reproducing (self-policing) or control each other's reproduction (worker policing) (13).

So far, the presence of such a cooperative signal and its compounds has only been shown in the honeybee, *Apis mellifera*. Here, the queen mandibular pheromone with its main component 9 oxo-decenoic acid let workers refrain from reproducing (15-17). However, workers seem not always to respond to an artificial pheromone or to queen presence in *A. mellifera* (18, 19). In other species, there exists some evidence that such a signal also occurs (20, 21). Despite the existence of this signal it seems difficult to understand how it should be effective in a large colony, since not every colony member can regularly contact the queen to directly perceive her signal.

Thus, there must be alternative ways of indirect communication. One way has become manifest in the honeybee *A. mellifera* in which messenger bees distribute the queen mandibular pheromone throughout the colony (22, 23). A further possibility of indirect communication is the use of eggs as a vehicle to distribute a queen signal throughout the colony. This has been suggested for the ant *Myrmica rubra*, where queen produced egg clusters had some inhibitory effect on worker ovarian development (24) and for large, monogynous and polydomous colonies of *Aphaenogaster cockerelli* or *Oecophylla* weaver ant, in which the queen remains in one restricted nest zone but her eggs are distributed by workers all over the large nest area (25). However, no experimental proof exists so far for this hypothesis. We tested the presence of queen signals on queen eggs in the ant *Camponotus floridanus*.

In this species a single queen lays eggs while the majority of workers (presumably up to 10000 per colony) remain infertile. Even in subcolonies workers do not lay eggs although the queen is not present. However, brood items including eggs are usually carried into these subnests which suggests an indirect communication of a queen signal via eggs. In our experiments, we first demonstrated that the presence of queen eggs let workers refrain from reproducing. Then we showed that workers differentiate between queen- and worker-laid eggs. The pattern of discrimination corresponds to differences in the described composition of the egg surface hydrocarbons, which actually are qualitatively similar to the cuticular hydrocarbon profiles of the adults. Finally, a transfer of cuticular hydrocarbons of queens on worker eggs rescues the latter from being destroyed by workers, indicating that these hydrocarbons represent the hypothesized queen signal.

7.4 Methods

Animals. Queens of *Camponotus floridanus* (N = 75) were collected at the Florida Keys/USA after the mating flight in August 2001 and transferred to the laboratory. They were cultured at 25° Celsius (12h day, 12h night). Subsequently, the queens raised colonies with 1000 - 2000 individuals within the next year. Experimental worker groups were provided with honeywater and 1.5 cockroaches (*Nauphoetia* sp.) twice a week.

Egg inhibition experiment. The brood composition of queenless worker groups (N = 19; for each treatment 19 worker groups out of 19 colonies) was varied in three different ways. Group (a) received 250 workers without any brood, group (b) 200 workers with 50 larvae and 50 pupae and group (c) 200 workers with 35 ± 5 queen eggs, 35 larvae and 35 pupae. The groups were controlled for the presence of eggs twice a week. Whenever the number of eggs present in group c had dropped below six another 35± 5 queen eggs was added. Brood from parental colonies was regularly added to approximately maintain the brood composition (Group b: 50 larvae, group c: 35 ± 5 eggs). The groups were regularly controlled for the presence of eggs. The beginning of worker egg-laying is very conspicuous, since up to 700 eggs are produced within a week. Sporadic egg-laying by workers in group c cannot be excluded. However, we have no evidence that this occurred. First, the number of eggs never increased before the conspicuous occurrence of a lot of egg piles, our defined onset of worker egg-laying. Second, no males were produced in these groups, which would be expected if workers perceive the absence of the queen.

Discrimination of queen- and worker-laid eggs. In the first experiment freshly orphaned groups each containing 150 workers were provided with eggs of different origin: they received either 30 to 35 eggs from sister workers, 30 to 35 eggs from their own queen, or 30 to 35 eggs from a foreign queen. Paired worker groups were used, i.e. nine queen colonies were used from which three worker groups were isolated each time. During the next five, days the surviving eggs were counted daily for five days. In the second experiment, four worker groups were isolated from queen colonies. In this case they received either 30 to 35 eggs from their mother queen or from sister workers (N = 9 queenright colonies for each treatment), or 30 to 35 eggs from sister workers treated either with the cuticular hydrocarbons of foreign queens (N = 9 queenright colonies) or with cuticular hydrocarbons from sister workers (N = 5). The sample size of the last group is smaller due to an insufficient number of eggs available at that time. Remaining eggs were counted 1h, 2h and 24 h after the transfer.

Extraction and transfer of compounds. Single queens or two workers, respectively, were extracted 15 min in 1 ml hexane for each experiment. The extracts were fractionated on conditioned SiOH columns (Macherey & Nagel, Chromabond 500mg, glass) with 4 ml hexane and the non-polar hydrocarbon fraction transferred onto clean glass slides and the solvent evaporated. Thirty worker eggs were then swiftly rolled on the extract for 5 min. A solid phase microextraction fiber (SUPELCO, fiber coated with a 7-µm polydimethylsiloxane film) was used to roll the eggs. This allowed to simultaneously sample the hydrocarbon profiles of the manipulated eggs. The extracted profiles were directly injected into the gas chromatograph. Programming of the gas chromatograph was the same as for the cuticular extraction (see below). The hexane used had been distilled to the highest possible purity.

Chemical Analysis. Cuticular hydrocarbons from queens and workers and from eggs were extracted with solid phase microextraction (see above). The fiber was swiftly rubbed on the tergites of queens and workers for 3 minutes and on eggs for 2 minutes. Then the fiber was directly injected into the injection port of a ThermoQuest Trace GC with a split/splitless injector. We used a non-polar capillary column (DB 1, J&W Scientific, Folsom, CA, 20 m x 0.18 mm, 0.18 µm film thickness) with H_2 as carrier gas. The temperature was kept at 60° for two minutes with the split closed for the same time. Then temperature was raised at 60° C/min to 200° C. Temperature subsequently increased at 4° C/min to 320° and then held constant. The injector port was kept at 260° C and the FID at 340° C. Peak areas were computed with Chrom-Card 1.19 (CE Instruments).

One part of the GC/MS analysis was carried out with a Hewlett Packard 5890 GC directly coupled to a 5970B mass selective detector (quadrupole mass spectrometer with 70eV electron impact ionisation). The system was controlled by a Hewlett Packard series 300 computer with HP 5972/5971 Chem station. Chromatography was performed using a non-polar capillary column (Restek, RTX-5, 15m x 0.25mm, 0.25µm thickness), using Helium as the carrier gas at 1µl/min. Samples were injected in splitless mode, the split valve being closed before the sample was injected, and reopened 45 seconds later. The solvent delay was set at 3 minutes and the injector port at 250°C. The oven temperature was programmed to increase from 50°C (3 minutes) at 5°C/min to a final temperature of 300°C (10 minutes). Structures were determined by Equivalent Chain Length and the use of standard MS databases – NIST/EPA/NIH mass spectral library and J.Wiley and Sons.

The other part of the GC/MS analysis was performed with a Fisons Instruments GC 8000 Series gas chromatograph (Fisons, Egelsbach, Germany) coupled to a Fisons Instruments MD 800 quadrupol mass detector. The GC was equipped either with a J & W DB-5 fused silica capillary column (30 m x 0.25 mm ID; df = 0.25 µm; J & W, Folsom, CA, USA; temperature program: from 60°C to 310°C at 5°C/min and held for 10 min at 310°C) or with a J & W DB-1 fused silica capillary column (30 m x 0.25 mm ID; df =0.25 µm; J & W; temperature program: from 60°C to150°C at 10°C/min, from

150°C to 310°C at 1.5°C/min and held for 10 min at 310°C). Helium was used as carrier gas at a constant pressure of 90 kPa. Injection was carried out at 250°C in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV and a source temperature of 220°C. The software Xcalibur (ThermoFinnigan, Egelsbach, Germany) for windows was used for data acquisition. Methylalkanes were characterized by the use of standard MS databases, diagnostic ions and by determining Kovats indices by the method of Carlson et al. (26).

7.4 Results

When worker groups were isolated from the queen, some workers started laying eggs provided no brood or only larvae und pupae were present in the group (Fig. 1). In the groups that started egg-laying we followed the production of males. On average 64 days (± 13.5 days SD) after the onset of worker egg-laying, new males regularly emerged in 21 of the 23 colonies with worker egg-laying. We did not wait for male emergence in the other two colonies, since they started very late with egg-laying (at day 139 and 158). However, one colony with no queen brood provided produced larvae and the other with queen brood produced male cocoons. In contrast to these groups when the isolated workers were exposed to queen laid eggs, they refrained from reproduction. This is confirmed by the absence of male production in these groups. The refraining from egg-laying suggests that workers can identify the origin of the eggs, thus that these eggs carry a queen signal.

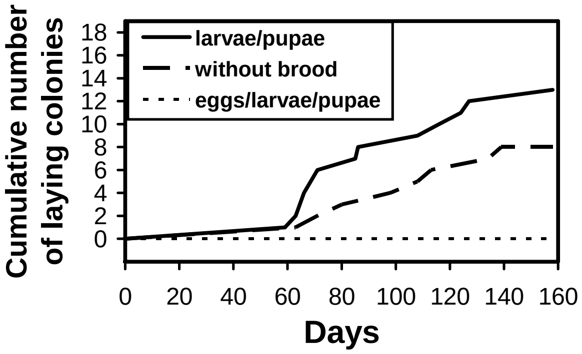

Figure 1: Inhibition of worker egg-laying by the presence of queen eggs. After 160 days of separation from the parental colonies the difference in worker egg-laying among the groups was significant (Overall comparison: Cochran Q test, N = 19, Q = 17.64, $p < 0.0001$; post hoc comparison one tailed Fisher's exact test: group b – group c, $p < 0.0001$; group a – group c, $p < 0.002$, group a – group b, not significant).

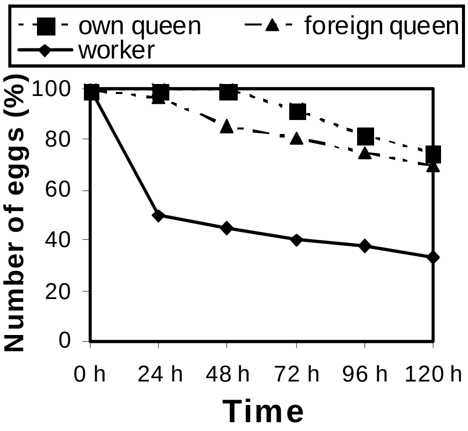

Figure 2: Discrimination of untreated queen and worker laid eggs. Only medians are presented. Already after 24 hours more than 62% of the worker eggs disappeared on average while the queen eggs remained almost untouched. The differences between the queen eggs and the worker eggs were statistically significant ($N_{groups} = 9$, Wilcoxon-Wilcox test for multiple comparisons, $p < 0.01$, own queen eggs versus worker eggs, $p < 0.05$, foreign queen eggs versus worker eggs, ns between queen eggs).

If there is a specific queen signal on these eggs, worker eggs should not elicit this response and workers should be able to discriminate between worker- and queen-laid eggs. When eggs from a queen or sister workers were given to freshly orphaned worker groups, the eggs from sisters were destroyed whereas eggs from their mother or from a foreign queen were tolerated (Fig. 2). Actually, it has been observed that workers always started destroying eggs immediately after egg transfer in groups with a final high loss of eggs. This indicates that queen eggs carry a specific signal that allows the workers to identify the origin of the eggs.

But what makes the eggs different? Chemical analysis revealed that queen eggs differ from worker eggs in the composition of their surface hydrocarbons (Fig. 3, 4). We found qualitative as well as quantitative differences (Fig. 4). The profiles of the egg surface compounds show qualitative similarities to the cuticular hydrocarbon profiles of either queens or workers (Fig. 4). Therefore, we tested whether the surface hydrocarbons of the queen eggs may represent the hypothesized queen signal. Due to the similarity of the cuticular hydrocarbon profiles of adults and the surface profiles of their eggs (Fig. 4) we simulated queen eggs by extracting and transferring hydrocarbon blends of the cuticle of foreign queens onto worker eggs. Successful manipulation was confirmed by gas chromatography of hydrocarbons extracted from the treated eggs (Fig. 4). Subsequently, the reaction of worker ants towards eggs carrying a transferred queen hydrocarbon profile was compared with that exhibited towards unmanipulated eggs from queen and workers and worker eggs carrying a transferred worker hydrocarbon profile. The result was unequivocal: significantly fewer worker eggs carrying the transferred queen hydrocarbon profile were destroyed than worker eggs although the manipulated eggs did not have the full protection of queen-laid eggs (Fig. 5).

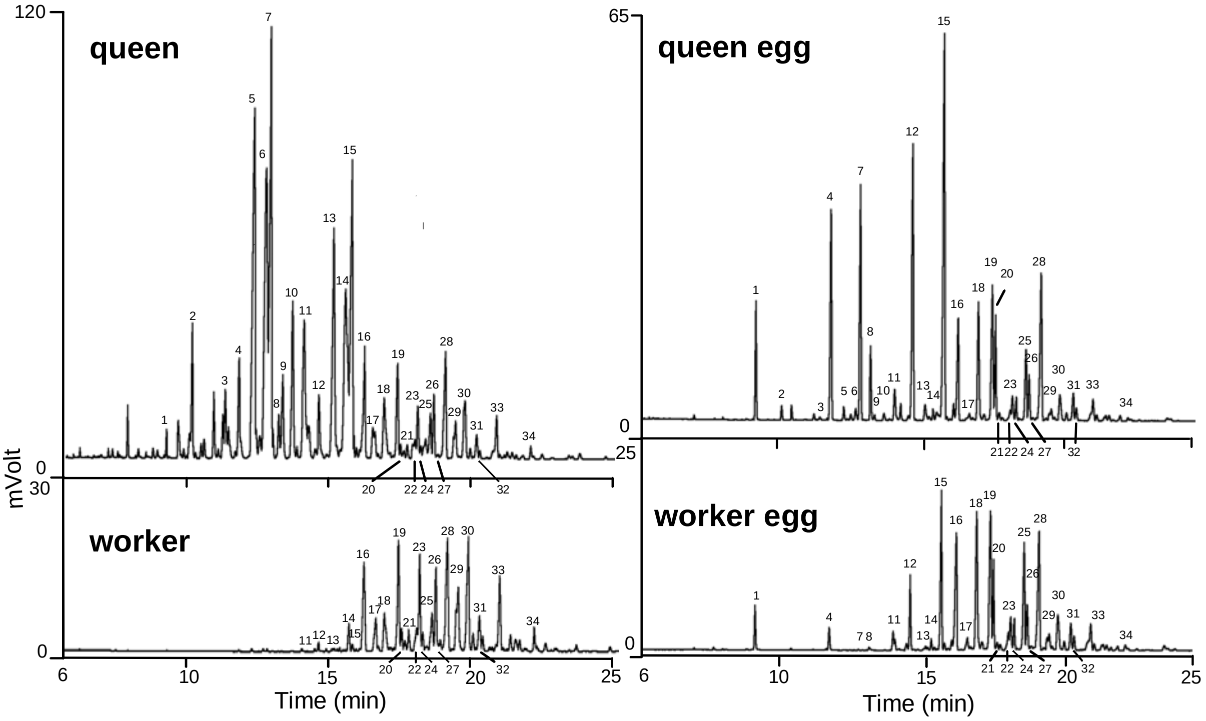

Figure 3: Chromatograms of the surface hydrocarbons of eggs and the cuticular hydrocarbons of fertile queens and workers. The compounds have been identified on the basis of retention times (in reference to GC/MS analysis) 1) n-pentacosane, 2) 3-methylpentacosane, 3) 10,14-dimethylhexacosane, 4) n-heptacosane, 5) 9-methyl-, 11-methyl-, and 13-methylheptacosane, 6) 11,15-dimethylheptacosane, 7) 3-methylheptacosane and 7,11-dimethylheptacosane, 8) n-octacosane, 9) 3,7-dimethyl-, 3,9-dimethyl-, 3,11-dimethyl-, and 3,13-dimethylheptacosane, 10) 10-methyl-, 12-methyl-, and 14-methyloctacosane, 11) 12,16-dimethyloctacosane, 12) n-nonacosane, 13) 9-methyl-, 11-methyl-, 13-methyl-, and 15-methylnonacosane, 14) 13,17-dimethyl-, 11,15-dimethyl-, and 9,13-dimethylnonacosane, 15) 3-methylnonacosane, 16) 3,7-dimethyl-, and 3,9-dimethylnonacosane, 17) 10-methyl-, 12-methyl-, and 14-methyltriacontane, 18) 4-methyltriacontane and 12,16-dimethyltriacontane, 19) 4,8-dimethyl-, 4,10-dimethyl-, 4,12-dimethyl-, and 4,14-dimethyltriacontane 20) n-hentriacontane, 21) 4,8,12-trimethyltriacontane, 22) 11-methyl-, 13-methyl-, and 15-methylhentriacontane, 23) 7-methyl-, 9-methylhentriacontane and 13,17-dimethylhentriacontane, 24) 11,15-dimethylhentriacontane, 25) 3-methylhentriacontane, 26) 5,9-dimethyl-, 5,11-dimethyl-, and 5,13-dimethylhentriacontane, 27) 7,11,15-trimethylhentriacontane, 28) n-dotriacontane, 3,7-dimethyl-, and 3,9-dimethylhentriacontane, 29) 3,7,11-trimethylhentriacontane 30) 3,9,15,21-tetramethyl-, and 3,7,11,15-tetramethylhentriacontane, 31) 4,8-dimethyl-, 4,10-dimethyl-, 4,12-dimethyl-, and 4,14-dimethyldotriacontane, 32) n-tritriacontane 33) 4,8,12,16-tetramethyldotriacontane 34) 5,9,13,17-tetramethyltritriacontane.

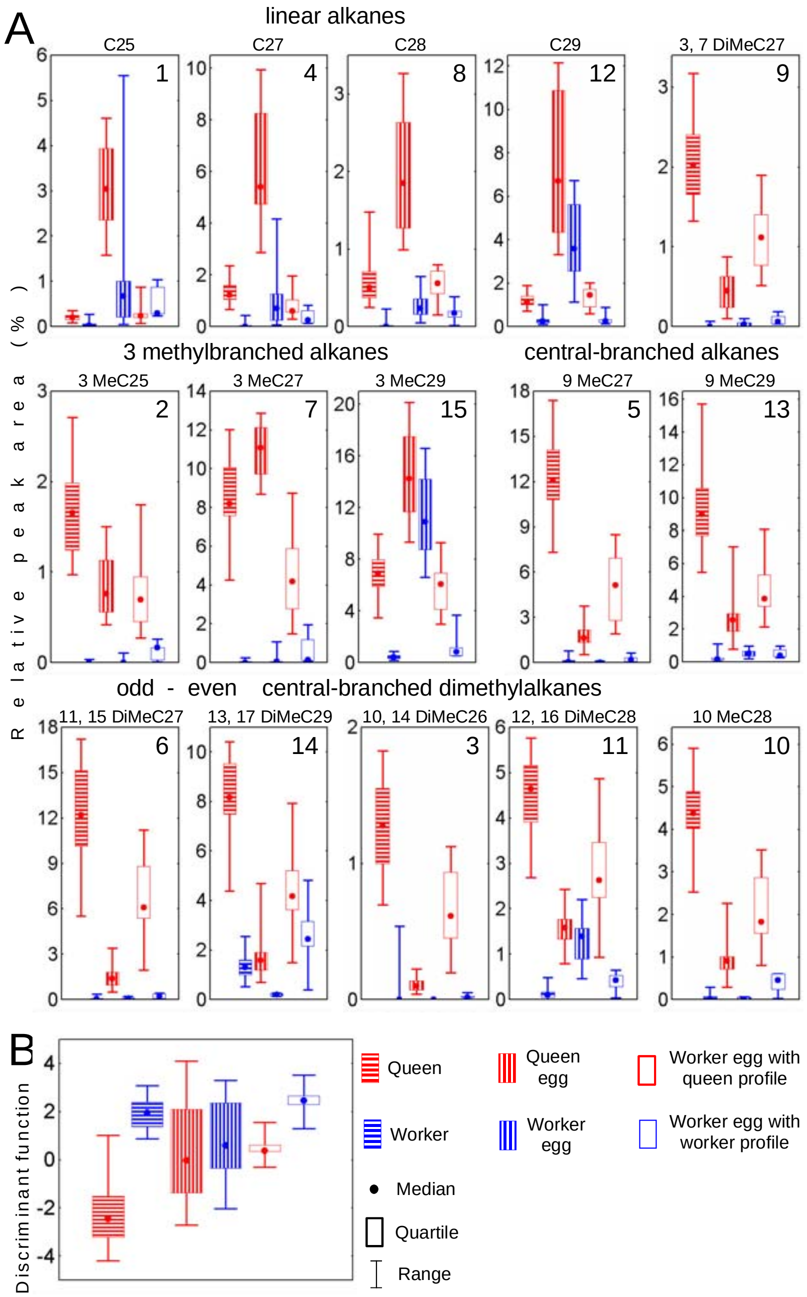

Figure 4: Differences in surface hydrocarbons between adult queens and workers, between queen and worker eggs and between worker eggs treated with either queen or worker extracts of cuticular hydrocarbons . N = 28 (queens), 33 (workers), 16 (queen eggs), 13 (worker eggs), 9 (worker eggs treated with queen extracts), 5 (worker eggs treated with worker extracts). Within a group all samples originated from different colonies to obtain independent data points. A) Major differences exist between queens and workers in 15 compounds. The differences in the medians of each compound between queens and workers and between queen eggs and worker eggs are significant (Wilcoxon test for paired samples, $p < 0.001$). The manipulation of the worker eggs either simulated queen origin or worker origin as before, since the direction of the differences in the medians between untreated eggs and between treated eggs were not different (Sign test, $p > 0.6$). Single variations are described by abbreviation of compound names. The numbers in the panels correspond to the compound names in figure 3, B) Differences in the profile of the remaining compounds of adult queens and workers were determined by a stepwise discriminant analysis. The resulting discriminant function was used to determine the similarity of the profiles of the untreated and treated eggs. Four compounds were excluded from the analysis. Compounds 20 and 34 were not normally distributed and compounds 16 and 17 did not show variance homogeneity according to Levene's test. In both cases the significance levels were corrected for multiple comparison according to Bonferroni. The stepwise procedure selected the compounds 31, 24, 23, and 27 (see compound names in Fig. 3). Only one discriminant function was extracted. The differences between the queens and workers are statistically significant (Wilk's lambda = 0.181, $p < 0.001$). The discriminant function correctly assigned queens and workers with the exception of three misclassifications of queens (leave-one-out criterion used). The plot of the egg hydrocarbon profiles employing this discriminant function shows, that the profiles of the selected compounds of the treated eggs are within the range of the natural profiles.

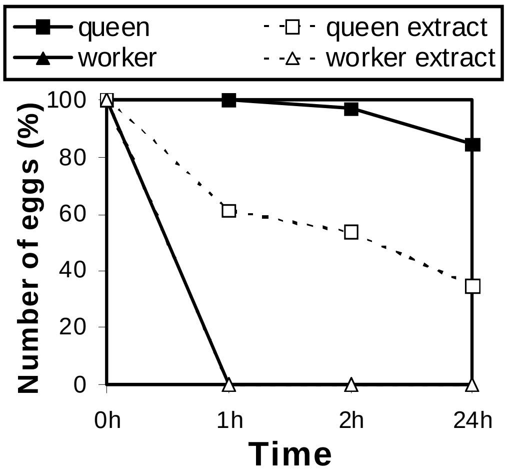

Figure 5: The survival of eggs treated with cuticular hydrocarbons in comparison to untreated eggs. The difference between the untreated eggs from queens and workers and the eggs treated with queen profile is statistically significant after 24h (Friedman's ANOVA, $N_{groups} = 9$, $p < 0.0005$). The important difference is between the worker eggs treated with queen cuticular hydrocarbons and the untreated worker eggs (Wilcoxon test for paired samples, $p < 0.02$). The sample size of the last group is smaller due to an insufficient number of eggs available at that time. However, the difference among all groups remains statistically significant with only the five samples including the control with manipulated worker eggs (Friedman's ANOVA, $p < 0.005$). The important differences between worker eggs treated with either queen extracts versus worker extracts are each significant (Wilcoxon test for paired samples, $p < 0.05$). The medians of eggs from workers and with worker profiles overlap.

7.5 Discussion

Our results show that queen eggs let workers refrain from reproducing in the ant *Camponotus floridanus*. This newly documented indirect way of queen signaling helps to understand the mechanisms of the regulation of reproduction in social insects, since the signaling way via eggs is of clearly cooperative character. Surface hydrocarbons of eggs seem to represent this signal, since workers use them to differentiate between queen- and worker-laid eggs as shown by our transfer experiment. These hydrocarbons reliably indicate the origin of the eggs, since they are closely connected to the differences in the cuticular hydrocarbon profiles between highly fertile queens and workers.

Egg inhibition. To our knowledge this study showed for the first time the inhibitory effect of queen eggs. Nevertheless, larvae have been shown to effect worker reproduction in at least two species. In the honeybee, *Apis mellifera*, larvae inhibit worker ovarian activation (19, 27-30). Larvae have also been found to affect worker reproduction in queenless worker groups in the ant *Pachycondyla apicalis* (31). However, larvae do generally not directly signal queen presence and therefore, this regulation mechanism clearly differs from our results.

Egg identification. In our study, workers destroyed worker-laid eggs but let queen-laid eggs alive. We showed that workers identify egg origin via the differences in their surface hydrocarbons. Our manipulation experiment excludes two alternative explanations for the loss of worker eggs. First, worker eggs may posses a lower viability which triggers worker egg destruction. Second, workers may identify the sex/ploidy of the eggs and destroy haploid, male-destined eggs preferentially. However, the transfer of cuticular hydrocarbons of the queen on worker eggs prevented their destruction. Therefore, workers primarily destroy eggs on the basis of their hydrocarbon profiles and not as a consequence of different viability or male-determination. Actually, in the groups that were followed up for male production many males were produced indicating their viability. Furthermore, there is no evidence so far that workers can recognize the sex of eggs (32).

Surface hydrocarbons of eggs and reproductive physiology. Our data are further supported from a study in the queenless ant *Dinoponera quadriceps* (33). Here, the amount of one compound of the cuticle of reproductive workers correlates with the amount of this substance on their eggs. While in *D. quadriceps* the difference relates to one compound, eggs of workers in *C. floridanus* differ from queen eggs in many compounds specific to the composition of the queen's cuticular hydrocarbons.

The close linkage between cuticular hydrocarbons and surface hydrocarbons of eggs is based on specific transport mechanisms in the hemolymph (34). Hydrocarbons are transported by lipoproteins to different tissues in the insect body including the ovaries and the cuticle. In the ovaries they are incorporated in developing oocytes (34). Differences between the profiles of the eggs in and the cuticle in *C. floridanus* may either be due to a different transport mechanism of hydrocarbons or to changes after oviposition.

In several ant species, the hydrocarbon profiles of adults correlate with the fertility of individuals which suggests that hydrocarbons represent a signal regulating reproduction (35-45). In fact, workers can identify gradual differences in the fertility of nestmates in some of these species (42, 46) as well as in others (47-49). In *Myrmecia gulosa*, workers can differentiate between the hydrocarbon profiles of reproductives and infertile workers (44). However, it has previously not been shown that cuticular hydrocarbons regulate worker reproduction.

Egg marking. Our transfer of queen cuticular hydrocarbons on worker eggs protected them from being destroyed, indicating that they represent a queen signal. This kind of destruction of worker eggs is actually a case of worker policing, i.e. the mutual control of the workers' reproduction (13, 32). Since *C. floridanus* is monogynous with a singly mated queen (50), workers should lay eggs even in the presence of their mother to maximize their inclusive fitness (13, 51). On the other hand, if worker reproduction reduces colony efficiency they should police each others reproduction despite their greater relatedness to their sons and nephews than to their brothers (13); this seems to be the case in *C. floridanus*.

Egg marking is known from several other species. In the honey bee *Apis mellifera*, worker policing of eggs occurs as well (52). Queen eggs differ from worker eggs by several compounds that are also present in the queen's Dufour's gland (53). Egg-laying workers mimic the queen specific profiles of the eggs (54-56). Although, there is experimental evidence, that Dufour's gland secrections let workers identify who laid the eggs (57), behavioral experiments failed so far to show which compounds are active (58-60). Egg marking also occurs in the fire ant *Solenopsis invicta*. Here, fertile queens apply Poison gland contents on their eggs (61). However, in this species workers do not lay eggs due to the lack of ovaries (4). Interestingly, the poison gland contents delay dealation of winged queens and consequently their ovarian activation in this species (62). In the queenless ant, *Dinoponera quadriceps*, the eggs of the reproductive workers are marked with a compound that is also found on their cuticle (33). Here, the marking is again not used for worker policing, but for queen policing, i.e. the dominant worker eats eggs from subordinates.

Regulation of reproduction. Our results in *C. floridanus* strongly suggest that components of the queens hydrocarbon profile serve as a signal that regulates reproduction in a dual way: i) it encourages workers to refrain from reproduction (self-policing (13)). (Fig. 1) and ii) it enables workers to discriminate between queen and worker-laid eggs and to destroy the latter if necessary (worker policing (13))(Fig 2). Although it is not known if it is a single compound that is the active signal, we do know that it is not nest-specific but common to all *C. floridanus* queens and can be detected by all *C. floridanus* workers and hence is acting as a true queen signal present on the surface of the queen and her eggs. This also suggests that the workers are particularly sensitive to the signal as they are able to detect it within a mixture that contains a large number of other like molecules. This could be achieved by a specific pheromone binding protein which selectively transports the signal molecules from the surface of the antennae to the receptors on sensory neurons. Krieger and Ross (63) have recently reported such a pheromone binding protein in the fire ant S*olenopsis invicta* which in this case allows workers to distinguish between queens with different genotypes. On the other hand, the differences between queens and workers and their eggs in *C. floridanus* are largely linked to compound classes that are structurally different (Fig. 3, 4a). Therefore, high receptor specificity would not be required to detect these differences.

In *C. floridanus*, hydrocarbons are reliable indicators of the presence of the queen and presumably also of her fertility due to the close linkage between hydrocarbon production and physiological processes. These processes are very basal and widespread as indicated by the close correlation of variations in the cuticular hydrocarbon profile and reproductive activity in many ant species (35, 37, 41, 42). Besides their function in protecting eggs and cuticle from desiccation (64-66) and contributing to nestmate recognition (67-69), hydrocarbon profiles additionally represent a queen signal that regulates reproduction in *C. floridanus* and maybe in many other social insects as well. Via the hydrocarbon profiles, workers could perceive the signal either directly from the queen or indirectly via her eggs. These two ways of signaling efficiently provide the workers with the information they rely on for their reproductive decisions.

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8 A chemical level in the coevolutionary arms race between an ant social parasite and its hosts

8.1 Abstract

Here we investigate the coevolutionary interactions between the slavemaking ant *Protomognathus americanus* and its *Leptothorax* hosts on a chemical level. We show that, although this social parasite is principally well-adapted to its hosts' cuticular hydrocarbon profile, there are pronounced differences in the fine-tuning of this adaptation. Between populations, chemical adaptation varies with host community composition, since the parasite faces a trade-off when confronted with more than one host species. In addition to adaptation of its own chemical signature, the slavemaker causes a reciprocal adjustment in its slaves' cuticular profile, the degree of which depends on the slave species. On the host side, successful parasite defence requires efficient enemy recognition, and in behavioural aggression trials, host colonies could indeed discriminate between invading slaves, which commonly accompany slavemakers on raids, and free-living conspecifics. Furthermore, hosts shifted their acceptance threshold over the seasons, presumably to reduce the costs of defence.

8.2 Introduction

Parasites exploit all levels of biological organisation, from genomes and cells to individual organisms and entire societies, and to this end they have evolved complex morphological, physiological, behavioural and chemical adaptations. The latter are especially important for parasites of social insects that have to break into the fortress of a colony that is normally closed to all but nestmate individuals (Wilson, 1971). This closure of the society, necessary to ensure that workers do not waste resources on non-kin, is maintained via a nestmate recognition system based on chemical cues on the cuticle (Howard, 1993; Lorenzi *et al.*, 1996). Among the cuticular lipids, hydrocarbons are thought to play the crucial role in nestmate recognition (Lahav *et al.*, 1999). In most social insect species, colony members share a common hydrocarbon signature ('gestalt odour', Crozier & Dix, 1979) that is probably maintained by storage of chemical compounds in the postpharyngeal gland and trophallaxis, allogrooming, and physical contact between nestmates (Soroker *et al.*, 1995; Lenoir *et al.*, 1999). Colony members learn the recognition cues shortly after eclosion, and individuals that do not match the internal template are usually rejected.

However, there are numerous examples of parasites that can overcome the nestmate recognition system and successfully integrate into social insect colonies (Kistner, 1979). This social integration is achieved by chemical insignificance (D'Ettorre & Errard, 1998), active biosynthesis of the host's chemical signature ('chemical mimicry' sensu Howard (1993), e.g. Howard *et al.*, 1990), or passive acquisition of recognition compounds ('chemical camouflage', e.g. Allan *et al.*, 2002). Since in this study we will not investigate the proximate mechanisms underlying chemical resemblance, we adopt the operational definition of (Dettner & Liepert, 1994) that describes chemical resemblance as mimicry irrespective of the origin of the chemical signal.

The above strategies are commonly used by social parasites, social Hymenoptera which, in an analogous manner to avian brood parasites, rely on other social insect species to rear their brood (Davies *et al.*, 1989). Chemical adaptations are especially critical for inquiline ant parasites, whose queens invade host colonies by sneaking and have to gain acceptance by the resident host workers to produce their own, exclusively sexual, offspring (Franks *et al.*, 1990). Another type of social parasite, slavemaking ants, produce workers specialized for fighting, which regularly conduct slave raids on neighbouring host colonies and steal their brood to replenish the labour force (D'Ettorre & Heinze, 2001). In some slavemaker species, e.g. of the genus *Polyergus*, queens usurp host colonies by sneaking in an 'inquiline-like' fashion and therefore also rely on elaborate chemical strategies to evade host aggression (Topoff & Zimmerli, 1993; D'Ettorre & Errard, 1998; Johnson *et al.*, 2001). Not surprisingly, workers of these species also show cuticular hydrocarbon patterns matching those of their hosts (Bonavita-Cougourdan *et al.*, 1997; D'Ettorre *et al.*, 2002). Other slavemaking species, in contrast, rely on overt aggression not only during slave raids, but also during host colony usurpation. Queens of these species invade a host colony, kill or drive away all adult individuals, and appropriate only the brood (Wesson, 1939; Buschinger, 1974). At first glance, the aggressive strategy of these slavemakers may seem to eliminate the need for mimicking their hosts' colony odour. However, aggressive invasion of a host colony is a risky task: 'Fighter queens' can invade only small host colonies (Buschinger, 1974; Herbers & Foitzik, 2002), and chemical disguise could thus increase their chance of success. Moreover, since slavemakers completely depend on their slaves for the regular tasks of colony maintenance (Stuart & Alloway, 1985), they have an interest in establishing a homogenous colony odour in order to ensure efficient communication with their slaves and lower the level of aggression (Heinze *et al.*, 1994). These parasites would therefore also benefit from resemblance to their slaves' cuticular profile, which has indeed been demonstrated for the European slavemaker *Harpagoxenus sublaevis* (Kaib *et al.*, 1993).

The slavemaker *Protomognathus americanus,* a small myrmicine ant distributed throughout northeastern North America, enslaves the three closely related *Leptothorax* species (*Temnothorax* according to a recent classification by Bolton (2003)) *L. longispinosus*, *L. curvispinosus*, and *L. ambiguus*. Recent studies on this host-parasite system have shown that, through frequent and destructive slave raids, the impact of the slavemaker on its hosts can be severe enough to trigger an escalating coevolutionary arms race (Foitzik *et al.*, 2001; Foitzik & Herbers, 2001; Blatrix & Herbers, 2003). However, in accordance with the geographic mosaic of coevolution theory (Thompson, 1999), the coevolutionary trajectories differ between populations, resulting in a patchwork of coevolutionary 'hot spots' and 'cold spots'. The strength of reciprocal selection in local populations depends on parasite prevalence (Foitzik & Herbers, 2001; Herbers & Foitzik, 2002), but can also be strongly influenced by the composition of the host community (Thompson & Pellmyr, 1992; Benkman, 1999). In an earlier study, we show that the coevolutionary arms race between *P. americanus* and its hosts proceeds faster in a community in New York with *L. longispinosus* as the only host species, than at a site in Ohio where a second host, *L. curvispinosus,* is included in the interaction (Brandt & Foitzik, submitted). Staged slave raids in the laboratory revealed a high degree of specialization and very efficient behavioural strategies of both host and parasite in New York, whereas the presence of the second host in Ohio disrupts the arms race and leads to less advanced reciprocal adaptations.

Chemical mimicry has been shown to occur in many myrmecophilous parasites, but to our knowledge it has never been examined in a coevolutionary context. Here we investigate chemical mimicry as a crucial component of the coevolutionary arms race between *P. americanus* and its hosts. To assay the fine-tuning of the slavemakers' chemical adaptation, we compared the cuticular profiles of free-living and enslaved *L. longispinosus* and *L. curvispinosus* workers as well as *P. americanus* workers from New York and Ohio on the qualitative and quantitative level. Based on our previous results, we formulate the following predictions:

We expect the specialized *P. americanus* population in New York to be closely adapted to the species-specific profile of its *L. longispinosus* host, while in Ohio, the more generalistic slavemaker faces a trade-off between adaptations to the two host species (Prediction 1).

Matching the hosts' species-specific signature is probably important for slavemaker queens invading a host colony. In established slavemaker colonies, the parasite, for reasons outlined above, should benefit from establishing a homogenous chemical profile. Therefore,
in *P. americanus* nests, slavemakers should cause a reciprocal adjustment of their own and their slaves’ chemical signature (Prediction 2).

Because there is no selection on hosts to rebel against their parasite once they are enslaved (Gladstone, 1981), hosts have little possibility to resist chemical manipulation by the slavemaker. However, host behaviours that preclude being enslaved in the first place could be selectively favoured. Since the first step to successful colony defence is reliable enemy detection, hosts could adjust their recognition threshold (Reeve, 1989) as a counter-adaptation to better identify invading slavemakers or slaves, which often participate in raids. We investigated host recognition abilities in aggression trials in late spring and early autumn, in which we monitored the reaction of host colonies to free-living and enslaved intruders. The expectation was that hosts can discriminate between free-living and enslaved conspecifics, and, if a lowered acceptance threshold entails costs, we would expect strong reactions to enslaved intruders only during the raiding season from July to September (Prediction 3).

8.3 Methods

Study system, colony collection and ant maintenance

P. americanus and its hosts occur in deciduous forests in northeastern North America and nest in hollow acorns, nuts and rotting sticks on the forest floor. Slavemaker colonies are typically small, containing between two and eight slavemaker workers and 20-50 slaves.

We collected ant colonies at the Huyck Preserve, Rensselaerville, Albany County, New York ('NY site') and in Harpersfield, Ashtabula County, Ohio ('OH site'), in the summers of 2002 and 2003. Colonies were transported to the laboratory in their natural nesting sites; in Regensburg, specimen for chemical analysis were immediately frozen in glass vials and stored at –20°C. Host colonies for behavioural experiments were housed under standard conditions (Heinze & Ortius, 1991) in an incubator (25°C for 14 h light, 17°C for 10 h dark).

Gas chromatography

For chemical analysis of the host species, three nestmate workers were pooled, whereas the slightly larger slavemaker workers were analysed individually. A total of 102 samples in nine categories was analysed (NY: free-living *L. longispinosus* hosts, *L. long.* slaves and *P. americanus* from colonies with *L. long.* slaves, N = 12 each; OH: *L. long.* hosts, *L. long.* slaves and *P. am.* from nests with *L. long.*, N = 10 each, and *L. curvispinosus* hosts, *L. curv.* slaves, and *P. am.* from nests with *L. curv.*, N = 12 each).

Cuticular hydrocarbons were extracted in 20 µl of pentane for 20 minutes, and 1µl of the solution was injected into an Agilent Technologies 6890N gas chromatograph equipped with an Rtx-5 capillary column (30m × 0.25mm × 0.50µm, Restek, Bellefonte, USA). We used helium as carrier gas at 1ml/min, the injector in splitless mode (1 min), and the temperature program of 2 min at 100°C, to 180°C at 30°C/min, from 180°C to 280°C at 3.5°C/min, and then held constant at 280°C for 20 min. Flame ionisation detector temperature was set at 300°C.

GC-MS analysis was done on an Agilent 5973 inert Mass Selective Detector with the same GC machine, temperature program and parameters, but equipped with an RH-5ms+ fused silica capillary column (30m × 0.25mm × 0.25µm). Electron impact mass spectra were obtained with an ionisation voltage of 70 eV and a source temperature of 230°C. Agilent G1701DA MSD ChemStation was used for data acquisition. Compounds were characterized by use of standard MS databases, diagnostic ions and Kovats indices (Carlson *et al.*, 1998).

Aggression trials

Aggression trials were conducted in May 2003 (N = 10 colonies each of *L. longispinosus* NY and OH, N = 15 colonies of *L. curvispinosus*) and repeated in September (N = 12 colonies of each host). Colonies for the spring and autumn experiments were collected in the summers of 2002 and 2003, respectively and were kept in the laboratory at least 6 weeks prior to the experiments. Laboratory culture can influence aggression levels in our study species; aggression was shown to increase under laboratory conditions in *L. longispinosus* (Stuart & Herbers, 2000), while for *L. curvispinosus* it remained constant for between-population trials and slightly decreased in within-population experiments (Stuart, 1987).

Each host colony was confronted with a nestmate, a free-living and an enslaved conspecific from the same population in random order, with at least four hours in-between trials. Intruders were marked with Edding paint on the gaster 30 min prior to the experiment and then introduced to the host colony. The nest entrance was blocked with cotton wool, and host workers' reaction was monitored during the first 10 min. Scans were conducted every 15 s, and all behaviours towards the intruder were recorded. As a measure of aggression, we used the sum of biting and mandible opening, standardized by the number of antennal contacts with the focal individual.

Statistical analysis

GC analysis yielded 97 peaks, which we analysed in two ways. In the qualitative analysis, presence-absence data of all peaks showing qualitative differences between individuals were used in a multiple correspondence analysis (MCA). The extracted factors were then used as variables in a discriminant analysis (DA). This procedure excludes peaks present in all individuals, which were included in a separate quantitative analysis. The areas of these 17 major peaks, which amount for about 90% of the total peak area, were standardized to 100%. Because relative peak areas represent compositional data, they were transformed following Aitchison's formula (Aitchison, 1986): $Z_{ij} = \ln [Y_{ij}/g(Y_j)]$, where Z_{ij} is the standardized peak area i for individual j, Y_{ij} represents the peak area i for individual j, and $g(Y_j)$ is the geometric mean of the areas of all peaks for ant j.

Variables as well as discriminant scores were checked for normality (K-S test, $P > 0.15$ in all cases), and homogeneity of variances was tested with Levene's test. One peak (65, Figure 1) did not have homogeneous variances and was excluded from the analysis, which was therefore based on 16 peaks. The assumption of equality of the variance-covariance matrices was fulfilled in all cases (Box's M-tests: $P > 0.15$). If sample size requirements permitted, a stepwise DA was performed. When this procedure extracted too many describing variables, the number of variables was reduced using a principal components analysis (PCA), and only factors with eigenvalues > 1 were used in the subsequent DA.

If several tests had to be performed on the same dataset, p-values given are adjusted using sequential Bonferroni correction (Rice, 1989).

8.4 Results

General findings

P. americanus workers generally exhibit a cuticular signature very similar to that of their hosts. As an example, chemical profiles of free-living *L. longispinosus* (NY) and *L. curvispinosus* (OH) hosts as well as slavemakers from nests with *L. longispinosus* slaves (NY) are shown in Fig. 1. The main cuticular compounds were alkanes (C25 – C31) and methylated alkanes.

Prediction 1: Profiles of **P. americanus** *and its free-living hosts*

Although all slavemakers exhibited similar cuticular profiles to their hosts, there were significant differences between the two populations regarding the fine-tuning of adaptation to the hosts species' signature. In order to investigate the chemical distance of *P. americanus* from New York and Ohio to their respective free-living hosts (*L. long.* in NY and *L. curv.* and *L. long.* in OH), we conducted a DA over these five groups.

In the both the qualitative and the quantitative analysis, slavemakers were statistically separated from their host species (MCA/DA: Wilks' $\lambda = 0.104$, $F_{16,\ 183} = 12.6$, $P < 0.0001$; PCA/DA: Wilks' $\lambda = 0.157$, $F_{16,\ 183} = 9.56$, $P < 0.0001$). In accordance with our prediction, the squared Mahalanobis distance between *P. americanus* and its hosts was significantly smaller in New York than in the Ohio community (Table 1a, MCA/DA: Mann-Whitney U test, N = 44, 12, U = 99, $P < 0.001$; PCA/DA: U = 96, $P < 0.001$). Ohio slavemakers were separated from their two host species by equal distances (Table 1a, Fig. 2, MCA/DA: Wilcoxon Matched Pairs test, N = 22, Z = 1.09, $p = 0.28$;

PCA/DA: Z = 0.11, *P* > 0.9). The two statistical procedures differed in that the qualitative analysis did not separate the two *L. longispinosus* populations (*L. long.* NY vs. OH: $F_{4,\,60} = 0.89$, *P* > 0.4), whereas the quantitative procedure did not identify the two slavemaker populations as discrete categories (*P. am.* NY vs. OH: $F_{4,\,60} = 1.24$, *P* > 0.3).

Prediction 2: Profiles of **P. americanus** *and their slaves*

Adjustment of the slavemakers' profile

In lack of an 'original' profile for *P. americanus*, which always occur in mixed nests, we tested whether slavemakers adjust their profile to their slaves by comparing the relative influence of the factors 'population' and 'slave species' on the profiles of the three groups of slavemakers (*P. am.* from OH with *L. curv.* or *L. long.* slaves, and *P. am.* from NY with *L. long.* slaves).

The qualitative analysis segregated the three groups (Wilks' $\lambda = 0.393$, $F_{8,\,56} = 4.16$, *P* < 0.001). However, with this method, it was not possible to determine whether 'population' and 'slave species' explained more of the divergence between groups (Squared Mahalanobis distances for *P. am.* from OH with *L. curv.* - *L. long.* slaves: 4.15; *P. am.* with *L. long.* slaves from NY - OH: 4.73; N = 10, Z = 0.46, *P* > 0.6).

A stepwise DA on the quantitative data (extracted peaks: 4, 45, 72) statistically separated all three groups (Wilks' $\lambda = 0.248$, $F_{6,\,58} = 9.74$, *P* < 0.0001). However, slavemakers occurring in sympatry in the Ohio community but exploiting different slave species had more divergent profiles (Squared Mahalanobis distance: 11.28) than the two groups from New York and Ohio with *L. longispinosus* slaves (distance: 6.97; N = 10, Z = 2.19, *P* < 0.03). Thus, although there are also population differences, the main factor influencing the quantitative adjustment of the cuticular profile of *P. americanus* is slave species.

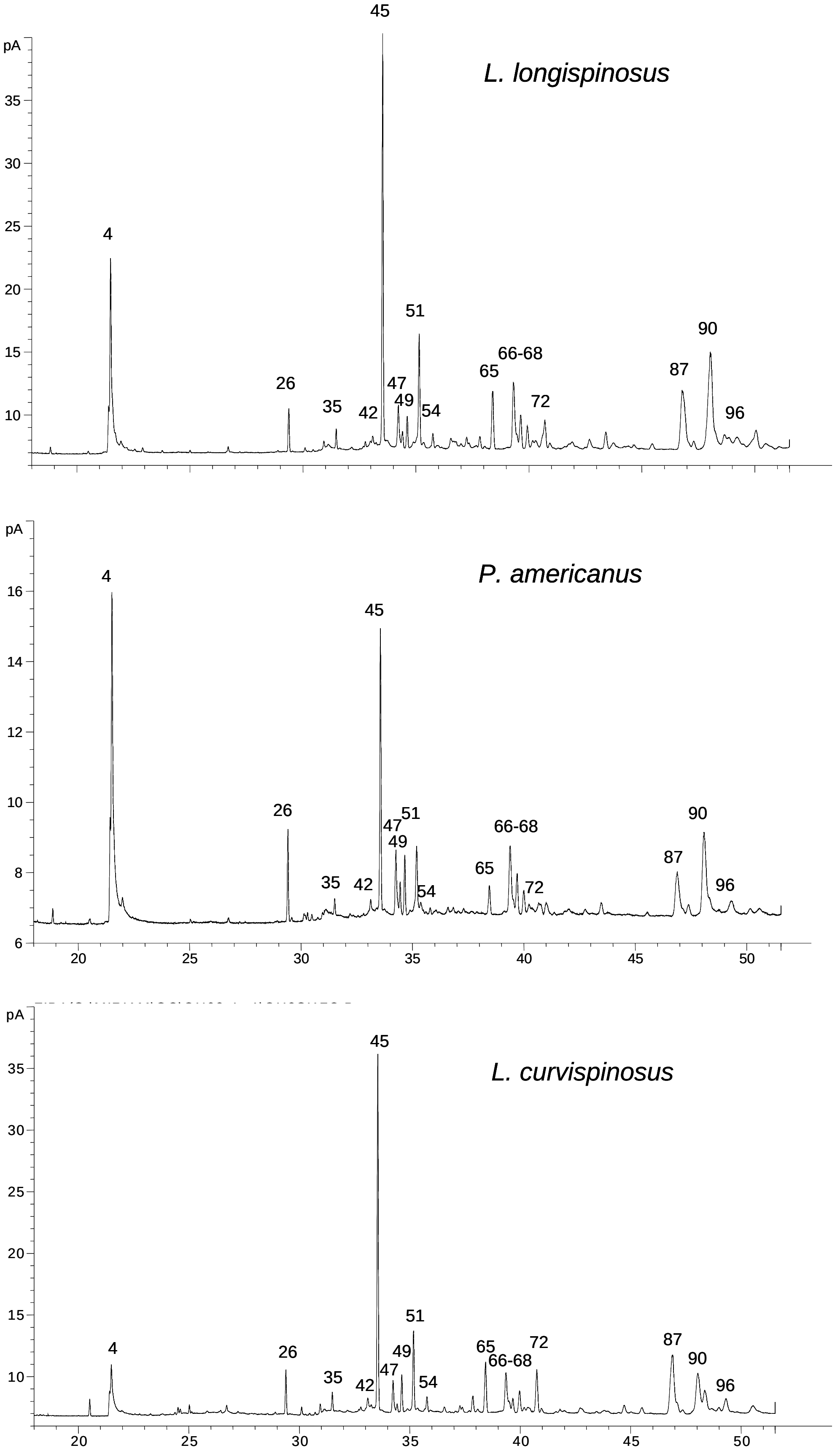


Figure 1: Gas chromatograms of free-living *L. longispinosus* hosts, *P. americanus* from a nest with *L. longispinosus* slaves (both from NY) and free-living *L. curvispinosus* hosts from Ohio. Peaks used in the quantitative analysis: (4) Octadecadienoic acid + Octadecenoic acid; (26) C25; (35) C26; (42) 3-meC_{26}; (45) C27; (47) 11- + 13-meC_{27}; (49) 5-meC_{27}; (51) 3-meC_{27}; (54) C28; (65) C29; (66) 9- + 11- + 13- + 15-meC_{29}; (67) 7-meC_{29}; (68) 5-meC_{29}; (72) 3-meC_{29}; (87) 9- + 11- + 13- + 15-meC_{31}; (90) 11,15- + 11,17- + 13,15- + 13,17-dimeC_{31}; (96) 11,15,19-trimeC_{31}.

		Qualitative analysis	*Quantitative analysis*
a)	*P. am.* NY vs. *L. long.* NY	7.5	4.3
	P. am. OH vs. *L. long.* OH	13.7	10.6
	P. am. OH vs. *L. curv.* OH	13.2	10.2
	P. am. NY vs. *P. am.* OH	2.3	0.7
	L. long. NY vs. *L. long.* OH	0.8	4.2
b)	***L. curv.* OH group**		
	free-living hosts – slaves	1.3	4.8
	P. am. – free-living hosts	15.9	25.5
	P. am. – slaves	12.4	23.4
	***L. long.* OH group**		
	free-living hosts – slaves	0.2	1.6
	P. am. – free-living hosts	6.5	2.3
	P. am. – slaves	6.1	5.1
	***L. long.* NY group**		
	free-living hosts – slaves	0.6	2.5
	P. am. – free-living hosts	12.5	2.5
	P. am. – slaves	12.5	4.9

Table 1: Squared Mahalanobis distances between a) *P. americanus* (*P.am.*) and its host species (*L. long.*, *L. curv.*) in New York and Ohio and between the two populations of the slavemaker and the host *L. longispinosus* (Prediction 1), and b) between free-living hosts, slaves and their slavemakers (Prediction 2).

Adjustment of the slaves' profile

In order to investigate the influence of the slavemaker on the cuticular profile of its slaves, a separate analysis was conducted for combinations of free living hosts, slaves, and *P. americanus* from nests with the respective slave species. Only one such combination exists for New York ('*L. long.* NY group'), while for the Ohio site, two groups of slavemakers were analysed separately depending on the slave species ('*L. long.* OH group' and '*L. curv.* group').

With the qualitative procedure, *P. americanus* were always separated from free-living hosts and slaves, suggesting that *P. americanus* retains some species-specific compounds (*L. curv.* group: Wilks' $\lambda = 0.208$, $F_{8, 60} = 8.94$, $P < 0.0001$; *L. long.* OH group: Wilks' $\lambda = 0.406$, $F_{8, 48} = 3.41$, $P < 0.005$; *L. long.* NY group: Wilks' $\lambda = 0.241$, $F_{8, 60} = 7.76$, $P < 0.0001$). However, in none of the comparisons, the DA distinguished free-living hosts from their enslaved conspecifics, hence the slavemaker does not alter its slaves' profile on a qualitative level (Table 1b, *L. curv.* group: $F_{4, 30} = 1.56$, $P > 0.2$; *L. long.* OH group: $F_{4, 24} = 0.18$, $P > 0.9$; *L. long.* NY group: $F_{4, 30} = 0.80$, $P > 0.5$).

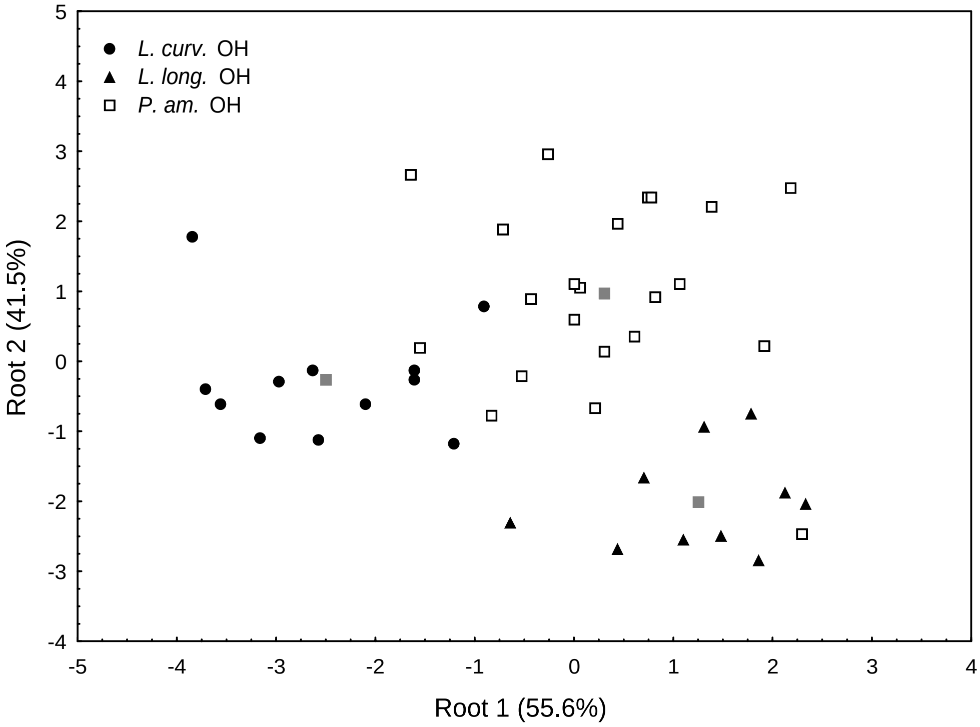
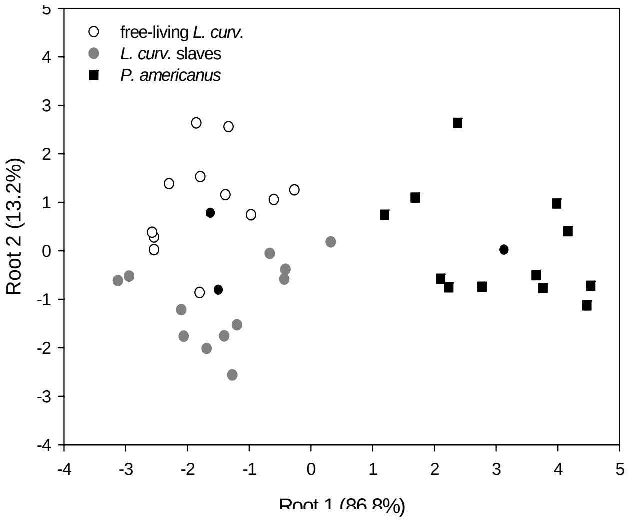


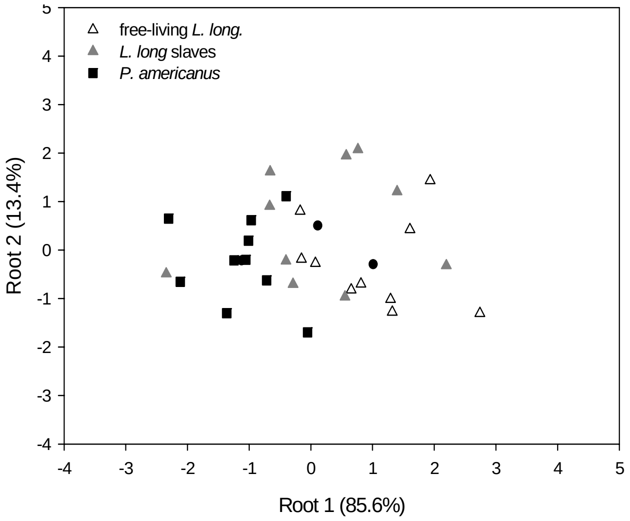
Figure 2: In the DA in the quantitative procedure, *P. americanus* from the Ohio community cluster between the two host species. Group centroids are shown as grey squares.

In the quantitative analysis, a stepwise DA extracted three peaks for the *L. curvispinosus* group (peaks 45, 26 and 47), two peaks for the *L. longispinosus* Ohio group (peaks 26 and 42), and two peaks for the *L. longispinosus* New York group (peaks 72 and 90).

a) *L. curv.* OH group



b) *L. long.* OH group



c) *L. long*. NY group

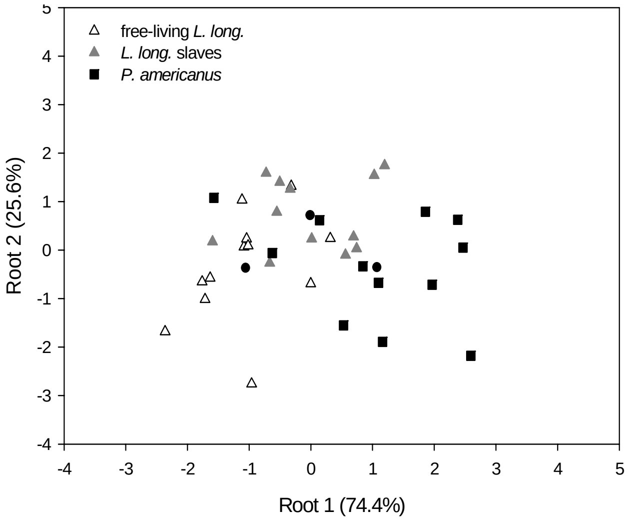


Figure 3: DA in the quantitative analysis of free-living hosts, slaves and *P. americanus* from nests with the respective slave species. a) *L. curv.* group, b) *L. long.* Ohio group, and c) *L. long.* New York group. Lines denote squared Mahalanobis distances between group centroids (black circles).

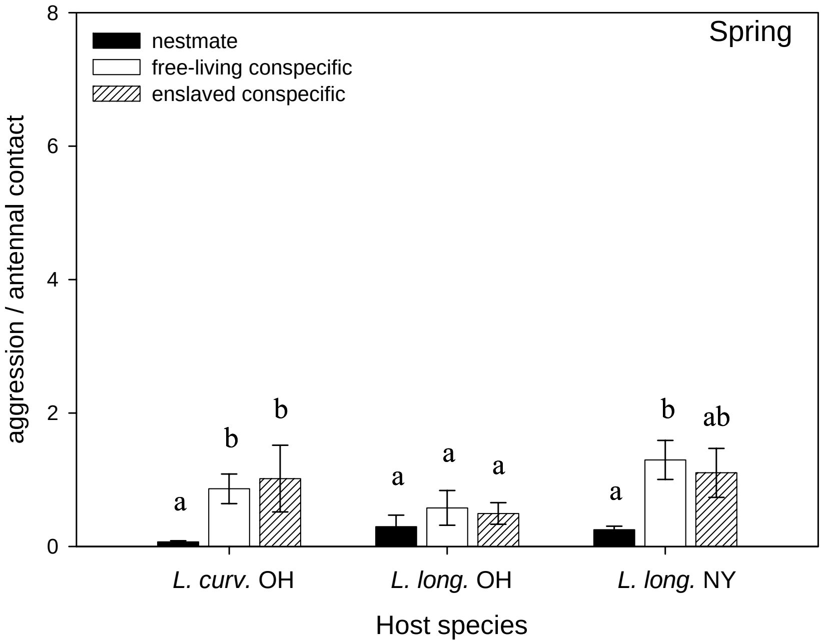
In contrast to the qualitative analysis, free-living hosts, slaves and slavemakers were now statistically separated in all three groups, indicating that *P. americanus* influences the relative amounts of the major peaks in the profile of both slave species. However, as shown in Fig. 3, there was less overlap in the *L. curvispinosus* group than in either of the comparisons including *L. longispinosus* hosts (*L. curv.* group: Wilks' $\lambda = 0.091$, $F_{6, 62} = 24.0$, $P < 0.0001$; *L. long.* OH group: Wilks' $\lambda = 0.473$, $F_{4, 52} = 5.90$, $P < 0.001$; *L. long.* NY group: Wilks' $\lambda = 0.427$, $F_{4, 64} = 8.47$, $P < 0.0001$).

Slavemakers adjusted the profile of their *L. longispinosus* slaves to their own, as *P. americanus* workers were separated from slaves by smaller Mahalanobis distances than from free-living hosts (Table 1b, *L. long.* OH group: $N = 10$, $Z = 2.50$, $P < 0.02$; *L. long.* NY group: $N = 12$, $Z = 2.20$, $P < 0.03$; *L. curv.* group: $N = 12$, $Z = 0.78$, $P > 0.4$). In these parasite nests, the slavemaker also induced a pronounced change in the slaves' chemical signature; thus *L. longispinosus* slaves were separated by equal distances from free-living conspecifics and their slavemakers (*L. long.* OH group: $N = 10$, $Z = 0.36$, $P > 0.7$; *L. long.* NY group: $N = 12$, $Z = 0.31$, $P > 0.7$). In contrast, *P. americanus* did not significantly alter the profile of *L. curvispinosus* slaves, which were consequently closer to free-living hosts than to their slavemaker ($N = 12$, $Z = 3.06$, $P < 0.005$).

Prediction 3: Discrimination ability of host colonies

In the behavioural experiments in spring (Fig. 4a), all host colonies were more aggressive towards alien conspecifics than towards nestmates, although this was only a trend in the *L. longispinosus* from Ohio (Friedman ANOVA, *L. long.* NY: $N = 10$, $\chi^2_F = 9.80$, $P < 0.01$; *L. curv.*: $N = 15$, $\chi^2_F = 15.97$, $P < 0.0005$; *L. long.* OH: $N = 10$, $\chi^2_F = 5.74$, $P = 0.057$). None of the hosts differentiated between free-living and enslaved conspecifics (*L. long.* NY: $Z = 0.97$, $P > 0.3$; *L. curv.*: $Z = 0.17$, $P > 0.8$).

a)



b)

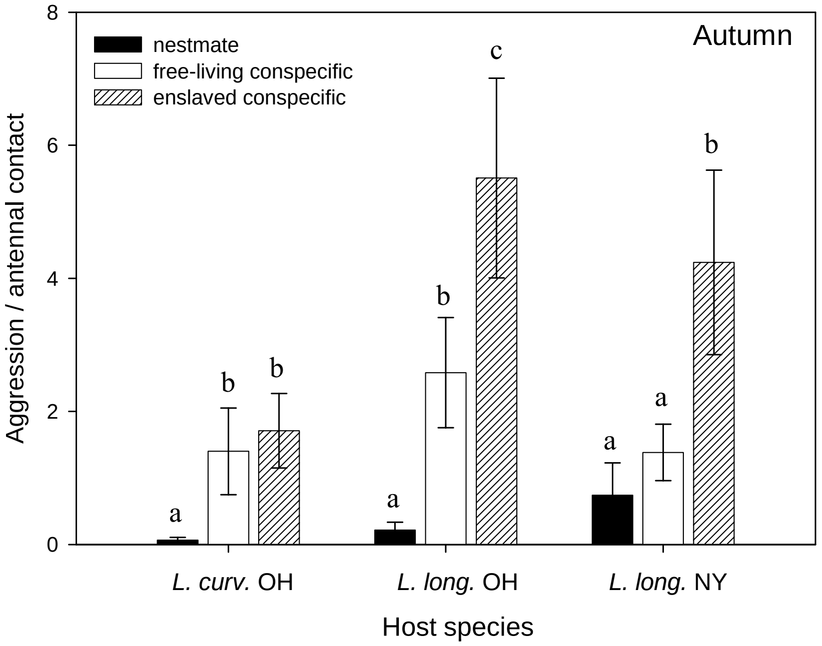


Figure 4: Reaction of host colonies towards introduced nestmates (control), free-living and enslaved conspecifics. Given is the number of aggressive acts, standardized by the number of antennal contacts with the focal individual (mean ± SE). a) experiments conducted in May, b) trials repeated in September.

In the trials in early autumn (Fig. 4b), *L. curvispinosus* showed nestmate recognition, but did not discriminate between free-living and enslaved intruders (N = 12, χ^2_F = 11.53, $P < 0.005$; nestmate - free-living: Z = 2.93, $P < 0.05$; nestmate - slave: Z = 2.67, $P < 0.05$; free-living - slave: Z = 0.39, $P > 0.6$). In contrast, *L. longispinosus* colonies from Ohio not only differentiated between nestmates and free-living intruders, but reacted even more aggressively towards enslaved aliens (N = 12, χ^2_F = 22.17, $P < 0.00005$; nestmate - free-living: Z = 3.06, $P < 0.05$; free-living - slave: Z = 2.35, $P < 0.05$). *L. longispinosus* from New York showed a similar reaction pattern, with slaves being more heavily attacked than aliens from free colonies (N = 12, χ^2_F = 15.16, $P < 0.001$; free-living - slave: Z = 2.35, $P < 0.05$).

In a direct comparison between seasons, *L. longispinosus* from Ohio were more aggressive towards free-living and enslaved intruders in autumn (free-living: N =10, 12, U = 24, ns after Bonferroni correction; slaves: U = 6, $P < 0.005$). An increase in aggression towards slaves later in season also occurred in the New York *L. longispinosus*, albeit only as a trend (U =31, $P = 0.056$).

8.5 Discussion

In this study, we investigated the coevolutionary arms race between *P. americanus* and its hosts on a chemical level. We show that the slavemaker exhibits chemical mimicry of its hosts' cuticular profile and that hosts, in turn, have developed specific recognition abilities and seasonally adjust their acceptance threshold.

Like other myrmecophilous parasites (e.g. Vander Meer *et al.*, 1989; Bonavita-Cougourdan *et al.*, 1997; Allan *et al.*, 2002), *P. americanus* exhibits chemical mimicry of its host species' cuticular profile (Prediction 1). However, since this species employs aggressive strategies during both colony usurpation and slave raids, why should concomitant chemical adaptations be necessary? Slavemaker queens, which have to take on an entire host colony by themselves, are often killed during invasion attempts and can successfully usurp only small colonies (Buschinger, 1974; Herbers & Foitzik, 2002); thus they would benefit from traits that help to evade attacks by host workers. Since it has been shown that *Leptothorax* ants react more aggressively towards heterospecifics than conspecifics (Stuart, 1991; Heinze *et al.*, 1996), mimicry of the host species' profile could thus offer *P. americanus* queens some protection. Chemical mimicry could also increase the success of slave raids. Before an attack is launched, a slavemaker scout has to investigate target host colonies; these scouts face the same situation as founding slavemaker queens. Although in later stages of a raid, slavemaker workers can recruit nestmates for aid, usually between 10-30 % of *P. americanus* workers are killed during raids (Foitzik *et al.*, 2001; Brandt & Foitzik, submitted). Considering that colonies of this species typically contain only few slavemaker workers, it should be selectively advantageous to decrease this death toll.

Although *P. americanus* from both studied communities have profiles closely resembling those of their hosts, we found substantial differences in the fine-tuning of this adaptation. In the New York community with only a single host species, slavemakers have chemical profiles very close to those of their *L. longispinosus* hosts. At the site in Ohio, where two host species co-occur, the slavemaker clusters in between these two hosts. As generalist species often face a trade-off between specific adaptations to different hosts (Futuyma & Moreno, 1988; Ward, 1992), the necessity to use two host species appears to impede closer adaptation to either one of them. *P. americanus* from Ohio were less proficient at obtaining new slaves and suffered higher losses during staged laboratory raids (Brandt & Foitzik, submitted). This may in part be explained by the present finding that the Ohio parasites do not match their hosts' profile as closely as their counterparts from New York: If host workers more easily recognize intruding slavemakers, they have a better chance of successful colony defence.

Apart from chemical mimicry of its hosts' species-specific signature as an adaptation for colony founding and slave raids, slavemakers should furthermore be selected to ensure smooth functioning of their colonies (Prediction 2). Indeed, we found a high degree of reciprocal adjustment of the profiles of slaves and their parasites, which, contrary to the proposition that *P. americanus* marks its slaves with a particular chemical substance (Alloway & Keough, 1990), was due not to qualitative differences, but to changes in the relative amounts of cuticular substances. A close chemical resemblance does not inevitably arise as a consequence of physical contact within the nest: For example, in mixed colonies of the temporary parasite *Lasius* sp. and its host *L. fuliginosus*, the two species did not exchange any chemical compounds despite frequent physical contact and trophallaxis (Liu *et al.*, 2000). The high degree of congruency in *P. americanus* colonies is thus likely to be the result of selection for a homogenous colony odour. It has been observed that artificially mixed colonies of *L. longispinosus* were less productive than homogeneous ones (Trampus, 2001). Less effective co-operation of slaves in heterogeneous colonies should therefore lead to selection on the parasite to reduce the degree of disparity. This could also be necessary to avoid intra-nest aggression. In colonies of the slavemaker *Harpagoxenus sublaevis*, hostile behaviours were more common among heterospecific than conspecific pairs of slaves (Heinze *et al.*, 1994). Hence, slaves in these systems retain the ability to recognize allospecific nestmates, which may explain slave aggression against adult and eclosing *P. americanus* workers (Alloway & Del Rio Pesado, 1983). This intracolonial aggression imposes a fitness cost on the slavemaker and probably impairs efficient functioning of the colony. A more homogenous odour may also facilitate communication between slaves and slavemakers, although it is probably not a prerequisite, as communication is possible even between very distantly related ant species (e.g. Maschwitz *et al.*, 2004).

Although *P. americanus* should always be under selection for close chemical resemblance with its slaves, the parasite is more successful in achieving this aim with *L. longispinosus* slaves. In nests with *L. curvispinosus*, in contrast, slavemakers cause only a minor reciprocal adjustment of their own and their slaves' profile. This difference cannot be explained by a closer phylogenetic relationship between the slavemaker and its *L. longispinosus* hosts (Foitzik *et al.*, submitted). Although the mechanism underlying the chemical integration of *P. americanus* and its slaves is as yet unknown, the most commonly used strategy in myrmecophilous species appears to be acquisition of host cues by contact rather than active synthesis of compounds (Lenoir *et al.*, 2001). The closer resemblance to *L. longispinosus* slaves may thus arise because parasite workers more effectively communicate with this slave species and are better able to encourage allogrooming and solicit food.

Our results indicate that *P. americanus* has evolved chemical mimicry for efficient host exploitation. Are there ways for hosts to counter this adaptation (Prediction 3)? In established slavemaker colonies, there is a profound asymmetry in selection pressures on the antagonists. While slavemakers profit from adaptation to their slaves, there is no selection on host workers, once enslaved, to withstand this manipulation. Slaves are imprinted on the slavemaker colony and thus have no means to detect that they have been enslaved, and even without this constraint, spiteful behaviours would not be selected for because they do not translate into fitness benefits (Gladstone, 1981). Hosts of slavemaking ants thus face the same dilemma as hosts of avian brood parasites that are hampered by the apparent difficulty of developing chick discrimination (Lotem, 1993). In this situation, the only way for hosts to evolve resistance is to avoid being parasitised in the first place, and the evolution of host defences against slave raids has been demonstrated by assaying the overall success of raids (Foitzik *et al.*, 2001; Brandt & Foitzik, submitted). Parasite pressure definitely selects for effective fighting abilities in hosts, but these have to be coupled with reliable enemy recognition, which was examined in the present study. Earlier work on *L. longispinosus* yielded evidence that these hosts are able to specifically recognize slavemakers as enemies (Alloway, 1990), but the results of our aggression trials allow additional conclusions.

First, whereas *L. longispinosus* hosts were more aggressive against slaves than unenslaved aliens, *L. curvispinosus* colonies did not discriminate between enslaved and free-living intruders. This result is not accounted for by the chemical distances between slaves and free-living conspecifics - otherwise, *L. curvispinosus* should have been more discriminating. Rather, the differences can be explained with the strength of parasite pressure: Whereas only one out of 16 *L. curvispinosus* colonies in the Ohio community was parasitised, one out of 7 *L. longispinosus* colonies in Ohio and one out of 8 in New York contained slavemakers (Brandt & Foitzik, submitted). A more rigid nestmate recognition system may protect a host colony from a slavemaker attack, but is likely to entail costs due to rejection errors. Since defences are only favoured if the benefits that ensue in the event of a parasite attack exceed the costs of maintaining the adaptation in the absence of parasites (Lotem *et al.*, 1992; Kraaijeveld & Godfray, 1997), the low probability of a slavemaker attack on *L. curvispinosus* apparently does not warrant investment in enemy recognition.

One way for hosts to decrease the costs of parasite defences is to adopt a conditional strategy: Hosts of avian brood parasites have been shown to respond to the presence of adult parasites by higher rates of egg rejection (Davies & Brooke, 1988), and in reed warblers, a decline in rejection was documented accompanying a seasonal relapse in parasitism (Brooke *et al.*, 1998). Similarly, our aggression trials showed that *L. longispinosus* hosts appeared to adjust their response to intruding slaves from late spring to early autumn. Although *L. longispinosus* from Ohio also showed a more hostile reaction to free-living intruders in the autumn trials, this finding cannot be explained by a general rise in aggression levels, because responses in the control experiments remained unchanged. Differences in the duration of laboratory maintenance cannot account for the observed effect either, as aggression levels in *L. longispinosus* were shown to increase under laboratory conditions (Stuart & Herbers, 2000). Rather, over the year hosts appear to lower their conspecific acceptance threshold, which is defined as the maximum amount of dissimilarity between template and cue that is tolerated without rejection (Reeve, 1989). It would be maladaptive to react to an intruding slave in spring with panic and nest evacuation, whereas such a response could potentially avoid the fatal consequences of a slave raid later in the season. A flexible adjustment of the acceptance threshold thus incurs the benefit of parasite defence while keeping the costs at a minimum. Lowering of the recognition threshold during the raiding season of the Formicine slavemaker *Polyergus rufescens* was also observed in the host *Formica rufibarbis* (Brunner *et al.*, 2002). Other social insects modified their acceptance thresholds in response to the nutritional status of their colony as an adaptation to conspecific threats (Bell *et al.*, 1974; Downs & Ratnieks, 2000).

Coevolution as the essential mode of interaction between parasite and host has been demonstrated for slavemaking ants with behavioural, genetic and ecological studies. Here we report a strong chemical component of the arms race, with selection pressure on parasites to break the code of nestmate recognition, and hosts evolving flexible discrimination abilities in response.

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9 General Discussion

The previous studies contribute to the understanding of the evolution of chemical communication in insects. Most specific aspects have already been discussed in the chapter 2 – 8. Two general aspects will be discussed in this chapter. One aspect will illustrate the various options to achieve specificity of a pheromone blend. The second one will highlight the distribution of benefits between sender and receiver and the resulting differentiation between cooperative and non-cooperative communication.

9.1 Chemistry of Semiochemicals

Like other organic molecules, semiochemicals are based on a chain of carbon and attached hydrogen atoms. This rather simple structure might become more complex by adding side chains, predominantly methyl-groups, or by introducing double bonds. When additional atoms such as oxygen or nitrogen, or other functional groups are added to the chain or substituted for hydrogens or carbons, the chemical nature of the molecule changes (Howse et al. 1998; Wyatt 2003). The majority of functional groups in insect pheromones are esters, alcohols, carboxylic acids, lactones, aldehydes, ketones and hydrocarbons. Less abundant are epoxides, ketals, acetals, phenols, halogens and ethers (Inscoe 1982; Silverstein 1984).

Pheromones are detected by receptor-proteins, which recognise the signal molecules by its three dimensional shape (Buck & Axel 1991; Clyne et al. 1999; Vosshall et al. 1999). Due to the configuration of hydrocarbons, molecules with the same formula can be assorted in a number of different structures, or even more complex, molecules with the same structure can have different shapes in three dimensions. These different variations are called isomers (Wyatt 2003). In the previous studies both types of isomers were identified. The constitutional isomers, which have the atoms connected in different ways, like the position of double bonds, E, Z configuration, and stereoisomers, which have the same connectivity but differ in the arrangement of atoms in space, such as (S)-2,3-dihydrofarnesoic acid. It was enantiopure in the beewolf scent marking pheromone (Schmitt et al. 2003).

Hymenoptera use compounds both with simple structures and with fairly complex configurations as signals or cues as obvious from our results. Cuticular hydrocarbons compositions in the examined ant species are mainly hydrocarbons without functional groups, predominantly alkanes, branched alkanes, some alkenes and some free fatty acids (Chapter 7 & 8). The honeybee cuticular hydrocarbon profile differs in its composition. Branched alkanes are the exception and simple n-alkanes and alkenes are the major constituents (Chapter 2; Blomquist 1980; Francis et al. 1985, 1989; Carlson 1988; Ogden et al. 1998). Additionally esters can be found in a significant amount (Blomquist et al. 1980; Buckner 1993). However, pheromones that evolved for communication purposes often contain fairly complex constituents with functional groups as examined in the beewolf male scent marking pheromone (Chapter 4; Rocca et al. 1983; Glancey 1986; Slessor et al. 1988; Schmitt et al. 2003).

Sex pheromones and recognition cues are under selection pressures to be species, nestmate, or sex specific (Phelan 1992, 1997; Vander Meer 1998). In case of the sex pheromone of *Philanthus triangulum*, the specificity is gained by using a mixture of relatively unique molecules, like (Z)-11-eicosen-1-ol, (Z)-nonadecen-2-on and (S)-2,3-dihydrofarnesoic acid, which also occur in only one isomeric form (Chapter 4; Schmitt et al. 2003). A second and more common way of gaining specificity in pheromone signals is by using a unique blend of relatively simple compounds as a multi-component pheromone. This is realised in the cuticular hydrocarbon profile of social insects like honeybees or the studied ant species. The unique complexity of the blend is demonstrated by the capability of these social Hymenoptera to distinguish not only between nestmate and non-nestmate, but also between kin, reproductive and non-reproductive individuals (Howard 1993; Vander Meer et al. 1998). Honeybees realise the nestmate recognition by using quantitative differences between the constituents of their cuticular hydrocarbon profile (Getz 1991; Breed 1998). *Camponotus floridanus* queens add to their colony profile alkanes and branchend alkanes with shorter chain-length to inform about their presence and fertility (Chapter 2, 7 & 8; Endler et al. 2004).

9.2 Communication – who benefits?

The classical ethological view is that in the context of social interactions animals should be able to predict what other individuals are about to do. To describe this ability to forecast other animals' behaviour, Krebs & Dawkins (1984) suggested the term "mind-reading". Communication is what evolves if being "mind-read" is beneficial for the receiver and this may evolve by exaggerating the cues by the sender. This process of exaggeration is called ritualisation and produces what we recognise as signals. This signalling is also thought to be adaptive for the signaller because it gains a net benefit from the response of the receiver (Harper 1991; Johnston 1997). This early theory of the evolution of communication assumes benefits both, sender and receiver (Harper 1991; Dusenbery 1992).

However, due to the selfish interests, which naturally occur between two individuals, benefits might rarely be equally distributed between the parties. In terms of natural selection, the receiver exerts strong selective pressures on signal design. This means that natural selection favours signallers whose signals elicit favourable responses. On the other hand, signal design might exert reciprocal selective pressures on receiver behaviour. Thus, it favours receivers who can accurately deduce the condition and intentions of signallers from their displays (Johnston 1997; Bradbury & Vehrencamp 1998). These two sets of reciprocal selective pressures are caused by conflict of interests. Each party will be selected to increase its benefits regardless of the benefits or costs for the other side (unless the partners are related). This conflict of interests generates a continuum between the two extremes, first, the receiver acquires all the benefits (e. g. eavesdropping) or, second, the sender acquires all the benefits (e. g. deceit or mimicry) (Figure 1).

The distribution of the benefits between sender and receiver leads to either cooperative or non-cooperative communication. Cooperative communication occurs, if both parties, obtain at least more benefits than costs from the communication process. In an evolutionary process, both, sender and receiver, will maximise its benefit. If the sender and the receiver will gain more benefits than costs, a cooperative communication process ensues, which subsequently might tend to evolve to either side of the continuum. It is also conceivable that non-cooperative communication switches into a cooperative communication because high selective pressure favours individuals who break down deceit, mimicry or eavesdropping by other individuals (Figure 1).

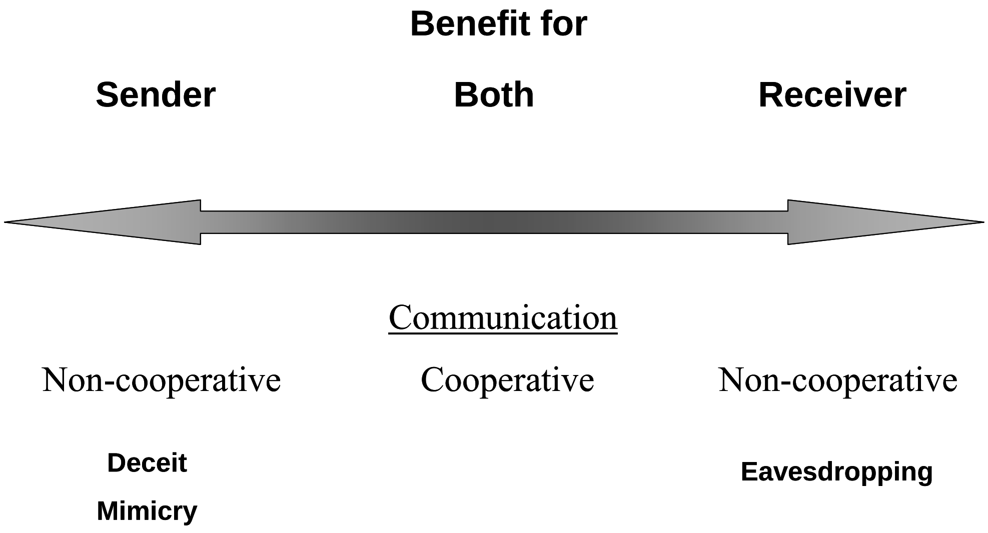

Figure 1: Distribution of benefits between sender and receiver and their effect on cooperative or non-cooperative communication

This thesis covers all these types of communication, from the mimicry of *H. rutilans* or of the slavemaker ant *P. americanus* to eavesdropping by *P. triangulum* females which uses the cuticular hydrocarbons emitted by honeybees as kairomones (Chapter 2, 6 and 8). It also shows that even an initial conflict of interests, for instance between the reproductives and non-reproductives in *C. floridanus*, and between the sexes of *P.*

triangulum, might end up in an evolutionary stable cooperative communication system that benefits both, sender and receiver (Chapter 2, 3, 4 and 7).

The non-cooperative communication is easily recognisable in the host-parasite interaction between the cockoo wasp and the beewolf female. The obvious disability to identify this parasitoid by the host provides evidence for chemical mimicry, which is already known from eusocial ants and bees, but for the first time shown in a solitary Hymenopteran species (Chapter 6, e.g. Dettner & Liepert 1994; Lenoir et al. 1997; D'Ettorre et al. 2002). The chemical analysis of the cuticular hydrocarbon composition shows a remarkable similarity between the host and the parasite. This is also shown in the social host-parasite system of *P. americanus* and its hosts (Chapter 8). In both cases the sender (parasite) bears all the benefits and the receiver (host) all the costs. This non-cooperative communication system is shifted to benefit the side of the sender.

Non-cooperative communication in the opposite direction was discovered in the exploitation of emitted cuticular hydrocarbons of the honeybee by one of its predators. This highly specialised beewolf females identify their prey by at least one very specific compound, (Z)-11-eicosen-1-ol. By using this long-chain alcohol as a kairomone, the receiver yields the benefits and the sender bears the costs. However, the occurrence of (Z)-11-eicosen-1-ol on the honeybee cuticle might serve a function in intra colonial social interactions and, thus, might provide a net benefit despite its detrimental effect as a kairomone (Chapter 2).

The evolution of the sex pheromone communication of the European beewolf was investigated with regard to the sensory exploitation hypotheses (Ryan 1990; Ryan & Rand 1990, 1993; see also Phelan 1992, 1997). There is strong evidence, that the males exploit the sensory ability of the females to detect (Z)-11-eicosen-1-ol in the context of prey identification. Thus, this long-chain alcohol became the main compound of the male pheromone (Chapter 4; Schmitt et al. 2003). Although there might exist a receiver preadaptation, which can be exploited by the sender, a cooperative communication system is likely to evolve. The selective pressure to increase their reproductive success acts on the females by enhancing their ability to be extremely sensitive for (Z)-11-

eicosen-1-ol. This might lead to a more precise identification of the prey by the monophagus beewolf females and therefore to a higher efficiency of hunting honeybees as provision for their progeny (Chapter 2 & 3). On the other hand, beewolf males increase their reproductive success by enhancing their efficiency to attract females for mating. The selection might favour individuals whose signal is most effective, that means using (Z)-11-eicosen-1-ol as an important compound of the sex pheromone might increase the number of matings. Even though there is a conflict of interests, males and females gain obviously more benefit than costs. A cooperative communication system has been evolved.

The cooperative communication between queens and workers in the ant *C. floridanus* evolved despite a conflict of interests with regard to reproduction (Hamilton 1964). One hypothesis suggests that a signal from the breeder may be a coercive means to refrain its workers from reproduction against their own fitness interests. If so, only the queen would benefit from its potential to manipulate the workers not to produce. This prediction is not supported by the experimental results of the investigated ant species (Alexander 1974; Hölldobler & Bartz 1985; Fletcher & Ross 1985). An alternative hypothesis assumes a cooperative signal that provides information on the condition of the queen (Seeley 1985; Keller & Nonacs 1993; West-Eberhard 2003). The interests of the breeder might be still different from the interests of the workers. However the workers gain benefits from an honest signal, which informs them of the queens' fertility and they respond by destroying worker-laid eggs if the queen is fertile or by developing ovaries to lay unfertilised eggs if the queen looses its fertility (Chapter 7; Endler et al. 2004). In this case a cooperative communication system also evolves since both, sender and receiver gain more benefits than costs.

The investigated species range from solitary to eusocial Hymenoptera and this thesis covers all types of communication, from cooperative to non-cooperative. It provides an insight into the diversity of chemical signals and their relevance with regard to the evolution of communication in a number different species.

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10 Summary

Insects exhibit complex systems of communication with chemical signalling being the most important mode. Although there are many studies on chemical communication in insects, the evolution of chemical signals is not well understood. Due to the conflict of interests between individuals, different selective pressures might act on sender and receiver. In this thesis I investigate different types of communication where either the sender, the receiver or both parties yield benefits. These studies were conducted with one digger wasp species, honeybees, one chrysidid wasp, and three ant species.

Senders might benefit by exploiting existing preferences of receivers. Such sensory exploitation might influence the evolution of male signals that are designed to attract females. The sex pheromone of male European beewolves *Philanthus triangulum* (Hymenoptera, Crabronidae) might have evolved according to the sensory exploitation hypothesis. A three-step scenario is supported by our studies. First, a major component of the honeybee alarm pheromone, (Z)-11-eicosen-1-ol, is also found on the cuticles and in the air surrounding foraging honeybees. Second, it could be shown, that (Z)-11-eicosen-1-ol plays a crucial role as kairomone for prey identification of honeybees by beewolf females. Third, a reanalysis of the beewolf male sex pheromone shows a remarkable similarity of compounds between the pheromone and the honeybee cuticle, besides the co-occurrence of (Z)-11-eisosen-ol. The majority of the cuticular hydrocarbons of honeybees occur also in the headspace of foraging workers. These results strongly support the hypothesis that beewolf males evolved a pheromone that exploits the females' pre-existing sensory sensitivity.

In addition, the male sex pheromone shows a significantly higher similarity among brothers than among non-related individuals, which might enable beewolf females to discriminate against brothers and avoid detrimental effects of breeding. Together with the studies on the possible sensory exploitation this result shows that both, male and female beewolves probably gain more benefits than costs from the pheromone

communication and, thus, the communication system as a whole can be regarded as cooperative.

To maintain the reproductive division of labour in eusocial colonies, queens have to signal their presence and fecundity. In the ant *Camponotus floridanus* (Hymenoptera, Formicidae) queens mark their own eggs with a distinctive pattern of cuticular hydrocarbons. Two different hypotheses have been developed. One suggests a form of worker manipulation by the queen. The alternative hypothesis assumes a cooperative signal that provides information on the condition of the queen. The results of our investigation clearly favour the latter hypothesis.

Chemical mimicry is a form of non-cooperative communication that benefits predominantly the sender. We provided conclusive evidence that the cockoo wasp, *Hedychrum rutilans* (Hymenoptera, Chrysididae), the primary brood parasitoid of *Philanthus triangulum*, evades recognition by beewolf females most probably by chemical mimicry of the odour of its host. Furthermore, the adaptation of the chemical signature in the social ant parasite *Protomognathus americanus* (Hymenoptera, Formicidae) to its *Leptothorax* (Hymenoptera, Formicidae) hosts was investigated. Although this parasite is principally adapted to its hosts' cuticular hydrocarbon profile, there are still pronounced differences between the profiles of parasites and hosts. This might be explained by the trade-off, which the parasites faces when confronted locally with two host species with different cuticular hydrocarbon profiles.

Non-cooperative communication in the sense that only receivers benefit was discovered in the exploitation of honeybees volatile cuticular hydrocarbons by beewolf females. By using emitted (Z)-11-eicosen-1-ol as a kairomone, the receiver, the beewolf female, yields the benefits and the sender, the honeybee prey, bears all the costs.

The results of these studies contribute to the understanding of the evolution of cooperative and non-cooperative communication with chemical signals taking into account differential benefits for sender and/or receiver.

11 Zusammenfassung

Insekten weisen ein komplexes System der Kommunikation auf, wobei chemische Signale die wichtigste Rolle spielen. Obwohl viele Studien über chemische Kommunikation an Insekten durchgeführt wurden, ist die Evolution von chemischen Signalen nicht gut verstanden. Aufgrund von Interessenkonflikten wirken unterschiedliche Selektionsdrücke auf Sender und Empfänger. In dieser Dissertation untersuchte ich verschiedene Typen von Kommunikation, bei denen entweder der Sender, der Empfänger oder beide von der Kommunikation profitieren. Als Modellorganismen wurden eine Grabwespenart (Crabronidae), Honigbienen (Apidae), eine Goldwespenart (Chrysididae) und drei Ameisenarten (Formicidae) studiert.

Sender können von der Ausnutzung existierender Präferenzen der Empfänger profitieren. Eine solche Ausnutzung kann die Evolution von Männchensignalen beeinflussen, die entwickelt wurden, um Weibchen anzulocken. Solch eine „sensory exploitation“ könnte die Evolution des Sexualpheromons von Männchen des Europäischen Bienenwolfs *Philanthus triangulum* (Hymenoptera, Crabronidae) beeinflußt haben. Unsere Studien unterstützen das folgende Drei-Stufen-Szenario: Erstens, eine Hauptkomponente aus dem Honigbienenalarmpheromon, das (Z)-11-Eicosen-1-ol, wurde auf der Kutikula und in der Umgebungsluft furagierender Honigbienen nachgewiesen. Zweitens konnte gezeigt werden, daß (Z)-11-Eicosen-1-ol eine wichtige Rolle als Kairomon bei der Identifizierung der Honigbiene als Beute durch Bienenwolfweibchen spielt. Schließlich zeigte eine detaillierte chemische Analyse des Bienenwolfmännchenpheromons, daß außer dem Auftreten von (Z)-11-Eicosen-1-ol weitere bemerkenswerte Übereinstimmungen zwischen dem Pheromon und der Honigbienenkutikula auftreten. Die meisten der kutikulären Substanzen der Honigbiene finden sich auch in der Umgebungsluft furagierender Honigbienen. Diese Ergebnisse bestätigen, daß bei der Evolution des Pheromons der Bienenwolfmännchen

bereits existierende sensorische Fähigkeiten der Weibchen eine wichtige Rolle spielten und somit die „sensory exploitation“ Hypothese unterstützt wird.

Das Sexualpheromon der Bienenwolfmännchen zeigt außerdem eine signifikant größere Ähnlichkeit zwischen Brüdern im Vergleich zu nicht verwandten Individuen. Dies könnte den Bienenwolfweibchen ermöglichen, bei der Paarung gegen Brüder zu diskriminieren und damit einen nachteiligen Effekt der Inzucht bei Nachkommen zu vermeiden. Dieses Ergebnis zeigt zusammen mit den Studien zur möglichen „sensory exploitation“, daß Männchen und Weibchen wahrscheinlich mehr Nutzen als Kosten aus diesem Kommunikationssystem erzielen und deshalb das System insgesamt als kooperativ betrachtet werden kann.

Um die reproduktive Arbeitsteilung in eusozialen Kolonien aufrecht zu erhalten, müssen Königinnen ihre Anwesendheit und Fekundität signalisieren. Bei der Ameisenart *Camponotus floridanus* (Hymenoptera, Formicidae) markieren die Königinnen ihre eigenen Eier mit einem unverwechselbaren kutikulären Kohlenwasserstoffmuster. Zwei unterschiedliche Hypothesen, die diese Form der Kommunikation erklären, wurden formuliert. Eine Hypothese schlägt eine Manipulation von Arbeiterinnen durch die Königin vor. Eine Alternativhypothese geht von einem kooperativen Signal aus, das Informationen über den Zustand der Königin übermittelt. Die Ergebnisse unserer Untersuchungen stützen eindeutig letztere Hypothese.

Chemische Mimikry ist eine Form von nicht-kooperativer Kommunikation, von der ausschließlich der Sender profitiert. Die Goldwespe, *Hedychrum rutilans* (Hymenoptera, Chrysididae), der wichtigste Brutparasitoid von *Philanthus triangulum*, entgeht der Entdeckung durch das Bienenwolfweibchen wahrscheinlich durch Imitierung des Geruchs seines Wirtes. Weiterhin wurde die Anpassung der chemischen Signatur des sozialen Ameisenparasiten *Protomognathus americanus* (Hymenoptera, Formicidae) an seine *Leptothorax* Wirtsarten untersucht. Obwohl dieser Parasit prinzipiell an das kutikuläre Kohlenwasserstoffprofil seines Wirtes angepaßt ist, gibt es trotzdem ausgeprägt Unterschiede zwischen den Profilen des Parasits und seines Wirtes.

Dies könnte durch einen „trade-off“ erklärt werden, dem die Parasiten ausgesetzt sind, wenn sie lokal mit zwei Wirtsarten mit unterschiedlichen kutikulären Kohlenwasserstoffprofilen konfrontiert werden.

Nicht-kooperative Kommunikation im Sinne, daß nur der Empfänger profitiert, wurde bei der Ausnutzung der flüchtigen kutikulären Kohlenwasserstoffen der Honigbiene durch seinen Prädator, das Bienenwolfweibchen, gezeigt. Durch die Nutzung von (Z)-11-Eicosen-1-ol als Kairomon profitiert nur der Empfänger, das Bienenwolfweibchen, wohingegen der Sender, die Honigbiene (Beute), alle Kosten trägt.

Die Ergebnisse dieser Studien tragen zu einem besseren Verständnis der Evolution von kooperativer und nicht-kooperativer Kommunikation mit chemischen Signalen unter Berücksichtigung des unterschiedlichen Nutzens für Sender und/oder Empfänger bei.

12 Curriculum Vitae

Geboren am 30.03.1964 in Würzburg

Hochschulausbildung

Seit Sept. 2000	Promotionsstudium an der Universität Würzburg mit dem Thema: „Communication in the Hymenoptera – Chemistry, Ecology and Evolution“ bei Prof. Dr. E. Strohm
Okt. 92 – Juli 94	Studium der Chemie an der Universität Würzburg
Okt. 94 – Sept. 2000	Studium der Biologie an der Universität Würzburg (Schwerpunkte im Hauptstudium: Tierökologie, Biochemie und Humangenetik)
Sept. 2000	Diplom Diplomarbeitsthema: „Chemische Ökologie und Einnischung bei westafrikanischen Scarabaeiden“ bei Prof. Dr. K. E. Linsenmair

Praxis und Auslandsaufenthalte

März 96 – April 96	Forschungspraktikum in der Elfenbeinküste, Westafrika (an der Forschungsstation der Universität Würzburg)

Mai 97 – Aug. 97	Forschungsaufenthalt für meine Diplomarbeit in der Elfenbeinküste (an der Forschungsstation der Universität Würzburg)
März 98 – April 98	Forschungsaufenthalt für meine Diplomarbeit in der Elfenbeinküste (an der Forschungsstation der Universität Würzburg)
Aug. 98 – Mai 99	Integriertes Auslandsstudium an der State University of New York at Albany, New York, USA Stipendium des Deutschen Akademischen Auslandsdienstes (DAAD) Eigenes Forschungsprojekt an In-vitro-evolution von Ribozymen bei Prof. Dr. Niles Lehman

Schulausbildung

1970 – 1976	Grund- und Hauptschule in Höchberg
1976 – 1980	Realschule in Höchberg Abschluss: Mittlere Reife
1989 – 1992	Westfalenkolleg in Paderborn Abschluss: Abitur

Berufsausbildung

1980 – 1983	Ausbildung zum Chemielaboranten im Raiffeisenkraftfutterwerk, Würzburg

Beruflicher Werdegang

1983 – 1984	Raiffeisenkraftfutterwerk in Würzburg
1984	Autol-Werke in Würzburg und Hannover
1985	Institut für medizinische Strahlenkunde der Universität Würzburg
1985 – 1989	Lehrstuhl für Lebensmittelchemie der Universität Würzburg

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Erklärung

gemäß § 4 Abs. 3 Ziff. 3, 5 und 8
der Promotionsordnung der Fakultät für Biologie der
Bayerischen Julius-Maximilians-Universität Würzburg

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation selbstständig angefertigt und keine anderen als die angegebenen Quellen oder Hilfsmittel verwendet habe.

Diese Dissertation wurde bisher weder vollständig noch teilweise einer anderen Hochschule mit dem Ziel, einen akademischen Grad zu erwerben, vorgelegt.

Ich erkläre weiterhin, daß ich außer meines Diploms in Biologie an der Universität Würzburg keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, den 15. September 2004