Evolution of the

Pheromone Communication System

in the European Beewolf *Philanthus triangulum* F.

(Hymenoptera: Crabronidae)

Dissertation zur Erlangung

des naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Gudrun Herzner

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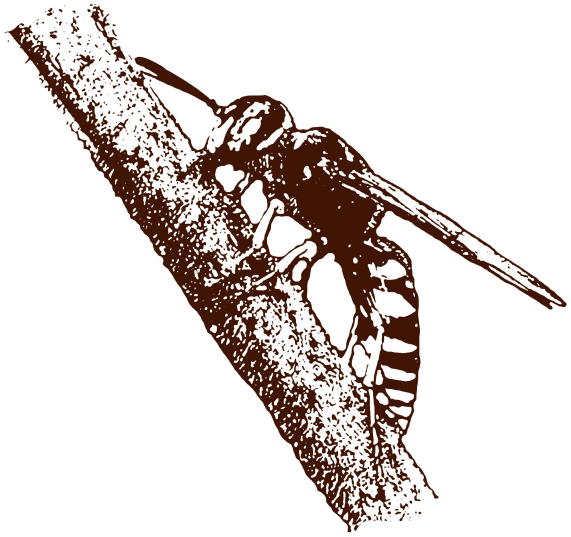

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Gutachter: Prof. Dr. K. Eduard Linsenmair

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CHAPTER 1

GENERAL INTRODUCTION

1.1 The asymmetry of sexual selection

The tremendous diversity of male secondary sexual characters and displays and the corresponding female preferences presents one of the most intriguing and controversial problems in evolutionary biology (Cronin 1991, Kirkpatrick and Ryan 1991, Maynard Smith 1991, Andersson 1994). Darwin (1871) proposed the theory of sexual selection to explain the evolution of spectacular and costly male advertisement signals, such as gaudy colouration and song, exorbitant courtship behaviours, as well as 'glands for emitting odours'. Put simply, he reasoned that these traits evolve because they enhance the mating success of their bearer. Darwin could not, however, explain, why sexual selection is usually more pronounced in males than in females, i.e. why males typically court and females choose.

This missing piece in Darwin's puzzle was later supplemented by a number of theoretical concepts. The bottom line of these concepts is that differences in the relative reproductive rates between males and females affect the strength of sexual selection. Females usually invest directly in offspring and can maximise their reproductive success by securing as many resources as possible to maximise the number and/or quality of their progeny (Trivers 1972). Males by contrast, generally invest no or few resources in offspring. They can increase their reproductive success by maximising the quantity of their mates (Bateman 1948, see also Andersson 1994). As a consequence, the operational sex ratio (Emlen and Oring 1977) becomes male-biased and females become a limiting resource for males. Females benefit from being choosy, i.e. maximising the quality of their mates to get the most out of their parental investment. Thus, mate competition is generally strongest in males who are under strong sexual selection to attract females most efficiently.

Though long dismissed, female choice is now widely accepted as the evolutionary cause of many ornate male secondary sexual characters, owing to numerous theoretical and empirical investigations (Cronin 1991, Andersson 1994). The evolutionary causes of female mate choice preferences themselves, however, are still vigorously debated (Kirkpatrick and Ryan 1991, Andersson 1994, Kokko et al. 2002, 2003). The many models explaining the origin and

maintenance of female preferences can roughly be divided into three broad categories: (1) direct benefits (Hoelzer 1989, Price et al. 1993), (2) indirect benefits (Fisher 1930, Trivers 1972, Zahavi 1975), and (3) receiver bias concepts (West-Eberhard 1984, Ryan et al. 1990, Christy 1995, Endler and Basolo 1998, Ryan 1998). In the following we will briefly outline these concepts and point at the differences and similarities between them.

1.2 The classical sexual selection models

The classical adaptive models of sexual selection include female choice for direct benefits, such as increased immediate survivorship and fecundity (Hoelzer 1989, Iwasa and Pomiankowski 1999), and choice for indirect benefits, like increased offspring quality and performance, which females acquire when mating with preferred males (Fisher 1930, Zahavi 1975, Lande 1981, reviewed by Kokko et al. 2002). The group of indirect benefit scenarios includes several more or less divergent variants called 'runaway sexual selection', 'sexy sons', 'good genes', 'indicator' or 'handicap' models, whose common thread is the genetic correlation and parallel co-evolution of male trait and female preference for that trait (Fisher 1930, Trivers 1972, Zahavi 1975, Lande 1981, Thornhill and Alcock 1983, Kirckpatrick and Ryan 1991, Colegrave et al. 2002, Kokko et al. 2002, 2003).

In Fisher's 'runaway' model (Fisher 1930), females who preferentially mate with males bearing certain attractive traits gain indirect benefits in the form of genes for attractiveness ('sexy sons') and viability. This non-random mating establishes a genetic association between the male trait and the female preference for that trait. When females exercise choice based on attractive male traits, not only will their sons inherit the trait, but their daughters will possess a similar preference for the trait. This positive feed-back loop leads to the so-called 'runaway' process of sexual selection.

The essence of the 'good genes' or genetic 'indicator' models is that mating preferences for male courtship traits evolve as adaptive mechanisms for assessing the genetic quality of potential mates (e.g. Hamilton and Zuk 1982). The male trait is thought to function as a reliable indicator of male quality under natural selection. All these indirect benefit models are based on the assumption that males with the most elaborate or exaggerated signals have intrinsically superior genes that confer greater fitness on the females' offspring (including

mating success of sons) (Andersson 1994, Johnstone 1995, Wilkinson et al. 1998, Møller and Alatalo 1999, Tomkins and Simmons 1999, Hine et al. 2002).

Although Fisher already suggested that ornate male traits may not only be attractive but additionally indicate high heritable viability, his name only became associated with the 'sexy son' hypothesis. In recent years, several theoretical studies have pointed out that the traditional dichotomy of Fisher's model versus the 'good genes' process is an arbitrary one that should be replaced with a more general concept of indirect selection: the 'Fisher-Zahavi model' (Eshel et al. 2000, Kokko et al. 2002, 2003; see also Kirkpatrick and Ryan 1991). There is a two-part reason for this: First, attractive male sexual signals may also function as indicators of male (genetic) quality, since males almost always vary in their ability to produce these signals (Eshel et al. 2000, Kokko et al. 2002). Second, quoting Kirkpatrick and Ryan (1991), female preferences become necessarily correlated with male traits when both have a heritable genetic basis and, thus, runaway processes have to be considered as an integral part of all good genes scenarios. The key prediction that arises from the Fisher-Zahavi model is that females choose males with high reproductive values based on indicator traits (Kokko et al. 2002).

Mate choice based on acoustic and visual male traits and displays has received much attention (e.g. Ryan 1983, Burkhardt and de la Motte 1988, Andersson 1994, Alcock 2001, Møller and Alatalo 1999, Klappert and Reinhold 2003, Tallamy et al. 2003), but comparatively few studies have focussed on chemical signals as indicators of male quality (Eisner and Meinwald 1995, Moore 1997, Sappington and Taylor 1990 a, b, c, Van Dongen et al. 1998, Hine et al. 2002). This is surprising since chemicals are likely to be the oldest and most universal of all cues and sex pheromones are probably the most important form of sexual communication in the vast majority of species (Dusenbery 1992, Penn and Potts 1998).

1.3 Choice for genetic compatibility

A recent alternative explanation for the evolution of female mate preferences nested within the indirect benefit models is the choice based on genetic compatibility (Trivers 1972, Tregenza and Wedell 2000, Colegrave et al. 2002). In this scenario offspring quality depends on the epistatic interaction between the individual female and male genotypes. Genes that are

good for one female may not be good for the other. Consequently, there will be no overall optimal male for all females but each individual female has her own 'best' mate.

These genotype-dependent, idiosyncratic, mate preferences have recently received much attention (e.g. Zeh and Zeh 1997, Tregenza and Wedell 2000, Colegrave et al. 2002, Penn 2002); as yet, there is surprisingly little empirical evidence supporting this hypothesis, with the exception of a few studies on the major histocompatibility complex (MHC)-based mate choice in some vertebrates (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).

1.4 Mate choice as a problem of communication

Many of the processes responsible for the evolution of female preferences and exaggerated male secondary sexual characters are now being discussed and investigated within the context of signal-receiver evolution (Guilford and Dawkins 1991, Endler 1992, 1993, Phelan 1992, 1997, Ryan and Rand 1993, Arak and Enquist 1995, Endler and Basolo 1998, Ryan 1998, Bradbury and Vehrencamp 2000, Schul and Bush 2002). This accrued from the insight that the process of male courtship and female choice is primarily a problem of communication. Accordingly, communication behaviour critically influences the reproductive fitness of individuals.

In the framework of communication theory the above mentioned sexual conflict of interest between the sexes can be seen as a conflict of interests between signallers and receivers and may thus be a major determinant in shaping sexual communication systems. Phelan (1992, 1997) took this conflict into account when formulating the theory of 'asymmetric tracking'. This theory is based on the assumption that female reproductive success is limited by the availability of resources needed to produce offspring, whereas male reproductive success is limited by the number of mating partners. As a consequence, males are under strong selection to find or attract receptive females. If males are the receivers, they can be expected to evolve very sensitive perceptual systems to detect female signals or cues that indicate the location of females most effectively. If males are the senders, as is mostly the case, the signal used will be designed to 'aim at' the sensitivity of the female perceptual system, i.e. the male signal

will be tracking the female response in evolutionary time. This idea is the pivotal point of the so-called receiver bias models, which will be described in the next section.

1.5 Receiver bias models of sexual selection

It was long assumed that mutual adjustments of signal traits and signal preferences, i.e. a co-evolutionary process for the benefit of both, sender and receiver ('for the good of the species'), has led to the patterns of communication seen today. This view has, however, repeatedly been challenged since Dawkins and Krebs (1978) noted, that signals often evolve to manipulate receivers in the signallers' own favour and not for a mutual benefit of both parties.

This thought and the growing interest in signal design efficiency have given rise to a number of models featuring a different perspective of sexual communication systems: e.g. pre-existing preference, sensory exploitation, and sensory trap models (reviewed in Endler and Basolo 1998, Ryan 1998). These models, which can all be integrated into the more general concept of receiver biases, suggest that male courtship signals may evolve to maximally stimulate pre-existing female perceptive systems (Ryan et al. 1990, Basolo 1990a, b, 1995a, b, Endler 1992, Proctor 1992). In receiver bias scenarios, the male signal does not have to be associated with male viability or other aspects of quality. Female (receiver) biases for certain stimuli may either simply arise as epiphenomena of how sensory or cognitive systems work (sensory exploitation model, Ryan et al. 1990, Enquist and Arak 1993) or they evolve because they are adaptive in naturally selected contexts, like predator avoidance or foraging (sensory trap model, pre-existing preference, West-Eberhard 1984, Christy 1995). In any case, they may (incidentally) lead to female preferences that predate male signals and may have important implications for the evolution of novel male advertisement signals and the direction of sexual selection (Kokko et al. 2003).

One major distinction between the receiver bias and the indicator models is the chronological order of the origin and the causal relationship between the signal and the preference for the signal. In the indicator models the male signal is considered to be the causal agent in the evolution of the receiver preference, while in the receiver bias model the evolution of the preference precedes the evolution of the signal (e.g. Endler and Basolo 1998). Thus, one

decisive prediction to distinguish a receiver bias from an indirect benefits effect is that the female preference existed before the male signal evolved (Ryan 1990, Shaw 1995). The second, complementary approach is to investigate and reveal the reason for or base of the bias (Christy 1995).

All receiver bias models share the view that the evolution of male sexual signals is influenced by pre-existing characteristics of the females' sensory or neural systems. The sensory trap model explicitly asserts that the receiver response evolved in a context unrelated to sexual selection. Male signals are thought to mimic model stimuli that stimulate female biases that evolved under selection forces in natural selection contexts (or at least, this happened at an early stage of the evolution of the communication system) (Wickler 1965, West-Eberhard 1984, Christy 1995). If females use e.g. odours to locate their food or prey, male pheromones that are sufficiently similar to this odour to attract foraging females will be favoured by sexual selection (Christy 1995). The critical prediction of the sensory trap model is that the mimic stimulus (male signal) elicits the same female response as the model stimulus (e.g. prey cue) when contextually transposed (i.e. into the prey capture context, Christy 1995) and thus offers a feasible empirical test.

Evidence for receiver bias processes (including sensory exploitation and sensory traps) influencing the evolution of visual, acoustic and mechanical courtship signals has been steadily increasing during the last two decades (e.g. Ryan et al. 1990, Basolo 1990a, 1995a Proctor 1991, 1992, McClintock and Uetz 1996, Hebets and Uetz 2000, Greenfield and Weber 2000, Rodd et al. 2002, Stålhandske 2002, Madden and Tanner 2003, Christy et al. 2003a, b, Smith et al. 2004, MacLaren et al. 2004). As with indirect benefits models, surprisingly few studies have explored the role of receiver biases in shaping the composition of sex pheromones (West-Eberhard 1984, Christy 1995, Phelan 1992, 1997).

1.6 Signal design and signal content

When studying communication systems, two basic components of signals have to be considered: signal design or structure and signal content (which roughly correspond to the 'efficacy' and 'strategy' of a signal, sensu Guilford and Dawkins 1991, and is related to the 'pattern recognition program' and 'general assessment program', sensu Burley 1985; see also

Endler 1992, 1993). 'Signal design' is aimed at getting the attention of the female and may thus depend on characteristics of the female sensory system. Natural selection will favour signals that are easily transmitted through the environment, easily received or detected by females and that can unambiguously be discriminated from others (Endler 1992, 1993). 'Signal content' relates to the function of the signal, e.g. conveying male quality (Zahavi 1975, Hamilton and Zuk 1982, Maynard Smith 1994), and may depend on the genetic equipment as well as the phenotypic condition of the male generating the signal. Sexual selection will favour signals that elicit a response in the receiver (female) that maximises the mating success of the sender (male). Receiver bias models only make predictions about signal design and do not address signal content, whereas the indicator or good genes models are primarily concerned with signal content.

1.7 Outline of the thesis

Endler (1992, 1993) pointed out that although many studies of sexual selection have focussed on understanding signal content, fewer have addressed signal structure. In the current study we investigate both aspects, the design and possible information content, of the male pheromone in one model species, the European Beewolf *Philanthus triangulum* F. (Hymenoptera: Crabronidae). We explore different variables determining female preference and male pheromone evolution and suggest a selective regime that might have led to the pheromone composition and individual variation visible in this species today. The major part of the study addresses signal design and tests key predictions of the sensory trap hypothesis (chapters 3 to 7). Signal content and its implications for female choice are then investigated and discussed in chapter 8. Chapters 3-8 consist of publications or manuscripts (submitted or in preparation). Therefore, there is necessarily some redundancy among these chapters.

The European Beewolf constitutes an excellent model system to investigate the evolution of a pheromone communication system. A short overview of the behaviour of this digger wasp that is essential for the understanding of the following chapters is given in chapter 2. Female *P. triangulum* hunt and provision their progeny exclusively with honeybees, *Apis mellifera*. Males establish territories and scent mark them with a pheromone from special head glands to attract females. Notably, the major compound of the males' pheromone, (Z)-11-eicosen-1-ol, is one of the major compounds of the alarm pheromone of honeybees, the prey of the females

(Free et al. 1982, 1983, Pickett et al. 1982, Schmidt et al. 1990). The occurrence of this otherwise rare long chain alcohol in the odour bouquets of both, male beewolves and honeybees, the exclusive prey of the females, suggests that males may have adopted 'bee odour' substances into their pheromone to capitalize on a pre-existing female bias. Here we propose and test three main predictions of a sensory trap scenario for the evolution of the male pheromone (signal design) in European Beewolves.

First, honeybees smell of (Z)-11-eicosen-1-ol during foraging (and not only in alarm situations). A description of the cuticular hydrocarbon pattern and the odour bouquet (head space) of foraging honeybees, *Apis mellifera*, describing several new compounds, is given in chapter 3. Second, beewolf females use this characteristic odour, and (Z)-11-eicosen-1-ol in particular, as a cue (kairomone) for prey recognition and hence have evolved a high sensory sensitivity for it. The prey recognition mechanisms of female beewolves and the role of (Z)-11-eicosen-1-ol in prey hunting are illustrated in chapter 4. Since beewolf females use olfactory cues for prey location and identification, they can be expected to have evolved special sensory (olfactory) or perceptive structures and abilities. Chapter 5 offers an inventory of the flagellar sensilla of male and female beewolves and describes a sexual dimorphism that may reflect an adaptation for prey recognition in females. The third prediction holds that males have incorporated (Z)-11-eicosen-1-ol into their pheromone to catch females in a sensory trap. Chapter 6 comprises the re-identification of the male pheromone of *P. triangulum* revealing marked differences to the previously published pheromone composition. The focus of chapter 7 is the comparison of the honeybee odour and male pheromone based on our own results regarding their compositions and the actual test for a sensory trap process. It provides a thorough discussion of different selection pressures that might have influenced the female preference and the design of the male signal (the pheromone).

Chapter 8 deals with the information content of the male pheromone and proposes a genetic basis for the composition of the pheromone. In this chapter the fitness advantages that females might gain by exercising choice based on the signal's content are interpreted within the framework of the complementary female choice hypothesis. The general discussion in chapter 9 summarises the results and outlines a selective scenario and evolutionary pathway for the origin and maintenance of the female preference and the male pheromone in *P. triangulum*.

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CHAPTER 2

THE EUROPEAN BEEWOLF *PHILANTHUS TRIANGULUM* (HYMENOPTERA: CRABRONIDAE)

The European Beewolf *Philanthus triangulum* is a solitary, ground nesting wasp that belongs to the family Crabronidae (formerly Sphecidae; superfamily Apoidea). It has a wide geographical distribution ranging from Europe (from Scandinavia to Greece) to Africa, Kasachstan, Turkmenistan and Irane (Blösch 2000, Hansen 1997, Lomholdt 1975) and can prevail over a relatively wide range of climatic and microclimatic conditions. *P. triangulum* is a pioneer species, frequently colonizing new habitats in warm and sandy regions (e.g. Hirschfelder 1956). Overall population densities are therefore mostly low; under favorable climatic conditions (long dry summers), however, single nest aggregations can count several hundred individuals. In our study region the European Beewolf occurs from June to September with varying abundances and one or two generations per year.

2.1 Females

Female European beewolves construct subterranean nest burrows in sandy soil bearing little vegetation and often fully exposed to the sun (Fig. 2.1). Females are strictly monophagous and hunt exclusively workers of the western honeybee, *Apis mellifera*. They feed on floral nectar but primarily rely on the nectar content of the paralyzed honeybees' crops (Rathmayer 1962) for their own energy supply. They search for honeybees on flowers. When they encounter a prey, they pounce at it, paralyse it by stinging (Fig. 2.2), and bring it in flight to their nest (Fig. 2.3) as provision for their offspring (Strohm 1995).

Females excavate a main burrow and several side burrows more or less perpendicular to the main burrow (for a detailed description of nest architecture see Strohm and Linsenmair 1994/95). At the end of the side burrow they form a brood cell, provision it with one to five paralyzed bees, and oviposit on one of the bees (Fig. 2.4 a). They close the brood cell immediately after oviposition and provide no further care to their offspring. Due to the nest architecture each individual is separated from its siblings in the nest and the bees in one brood cell constitute the total quantity of food available to a single larva (Fig. 2.4 b). The larva

hatches 2-3 days after oviposition, feeds on the bees for another 5-8 days (Fig. 2.4 c), and spins into a cocoon (Fig. 2.4 d). It either completes development and metamorphosis and emerges the same year or it enters diapause and hibernates to emerge the next summer. In the laboratory the total development from the egg to eclosion requires about four to five weeks at 26-28°C. Prior to oviposition female beewolves spent a considerable amount of time extensively licking the entire surface of the honeybee prey. This treatment has been shown to delay the onset of fungus growth and Strohm and Linsenmair (2001) hypothesized that females apply fungicidal substances onto the prey's cuticle.

Figure 2.1: Beewolf female at her nest entrance.

In beewolves, adult body size critically depends on the amount of food available during larval development, i.e. the number of bees in the brood cell. Prey hunting has been shown to be costly (Strohm and Marliani 2002) and the 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). The location and identification of honeybees is thus the most important component of female reproduction. 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)

Figure 2.2: Beewolf female stinging and paralyzing a honeybee.

Figure 2.3: Beewolf female carrying the paralyzed honeybee prey to her nest.

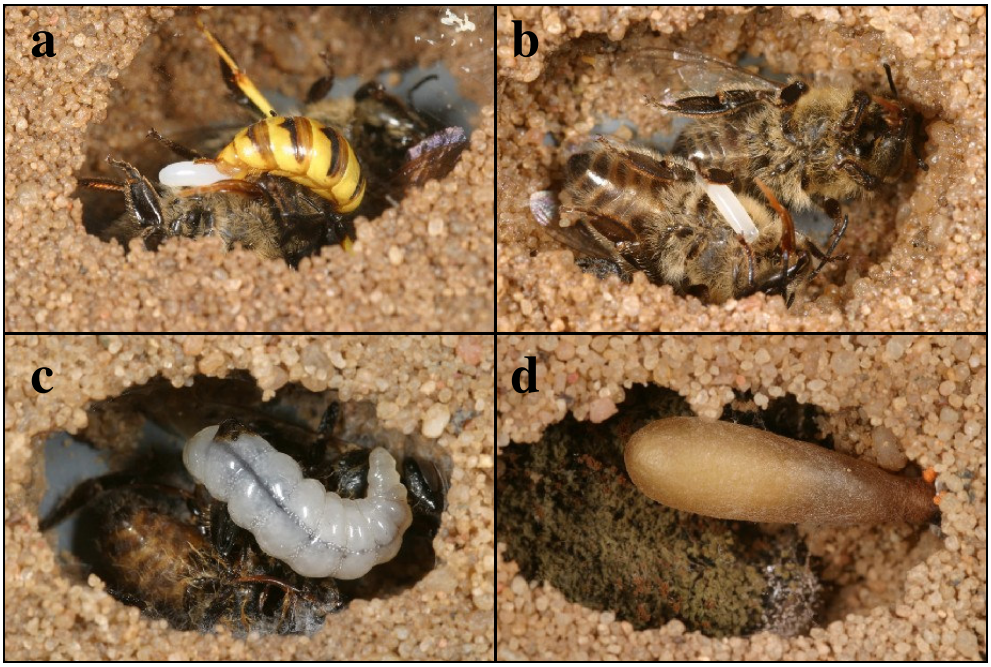

Figure 2.4: Inside a brood cell: **(a)** Female beewolf laying an egg on a paralyzed bee. **(b)** Two provisioned honeybees, one carrying a beewolf egg. **(c)** Beewolf larva feeding on its provisions. **(d)** Beewolf cocoon.

Figure 2.5: Beewolf male scent marking his territory. Note the clypeal brush with which the pheromone is applied to the vegetation.

2.2 Males

Males of the European Beewolf establish small territories (about 0.25-0.5 m^2) that do not contain any resources essential to the females. They scent mark these territories with a secretion from a cephalic gland, a behaviour which is believed to function to attract females for mating (Simon Thomas and Poorter 1972, Evans and O'Neill 1988, Strohm 1995, Strohm and Lechner 2000). Receptive females move toward territories from the downwind side. When they alight in the territories, males immediately approach them and the copulation ensues without any further courtship behavior (personal observation). Thus, the male pheromone most likely plays the predominant role for mate choice. Males usually form aggregations with several males establishing territories in close vicinity to female nests. These so-called leks allow females to compare and choose between males with presumably low costs. Females of the European Beewolf most probably mate only once a few days after emergence (Evans and O'Neill 1988) and the males' reproductive fitness depends on the number of matings they can achieve. The attraction of receptive females and the obtaining of matings are therefore the most important components of male reproduction. Males feed on flowers, in the study region mostly on thistle (*Cirsium arvense*), field eryngo (*Eryngium campestre*), and goldenrod (*Solidago canadensis*) (see also Olberg 1953). During the night they stay in small burrows in sandy soil.

Figure 2.6: Copulating pair of beewolves. Female on the left (Photo: Martin Kalthenpoth).

Figure 2.7: Male beewolf feeding on Field Eryngo (*Eryngium campestre*)**.**

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CHAPTER 3

VOLATILES OF FORAGING HONEYBEES *APIS MELLIFERA* L. (HYMENOPTERA: APIDAE) AND THEIR POSSIBLE ROLE AS SEMIOCHEMICALS AND KAIROMONES

Thomas Schmitt, Gudrun Herzner, Peter Schreier & Erhard Strohm

3.1 Summary

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.

3.2 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 by using chemical cues (Moore 1987, Moritz et al. 1991, Breed et al. 1992, Beekman et al. 2002). Guard bees antennate approaching bees for identification. It has therefore 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 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.

3.3 Material and Methods

3.3.1 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).

3.3.2 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 3.1). 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 metres from 10-15 beehives.

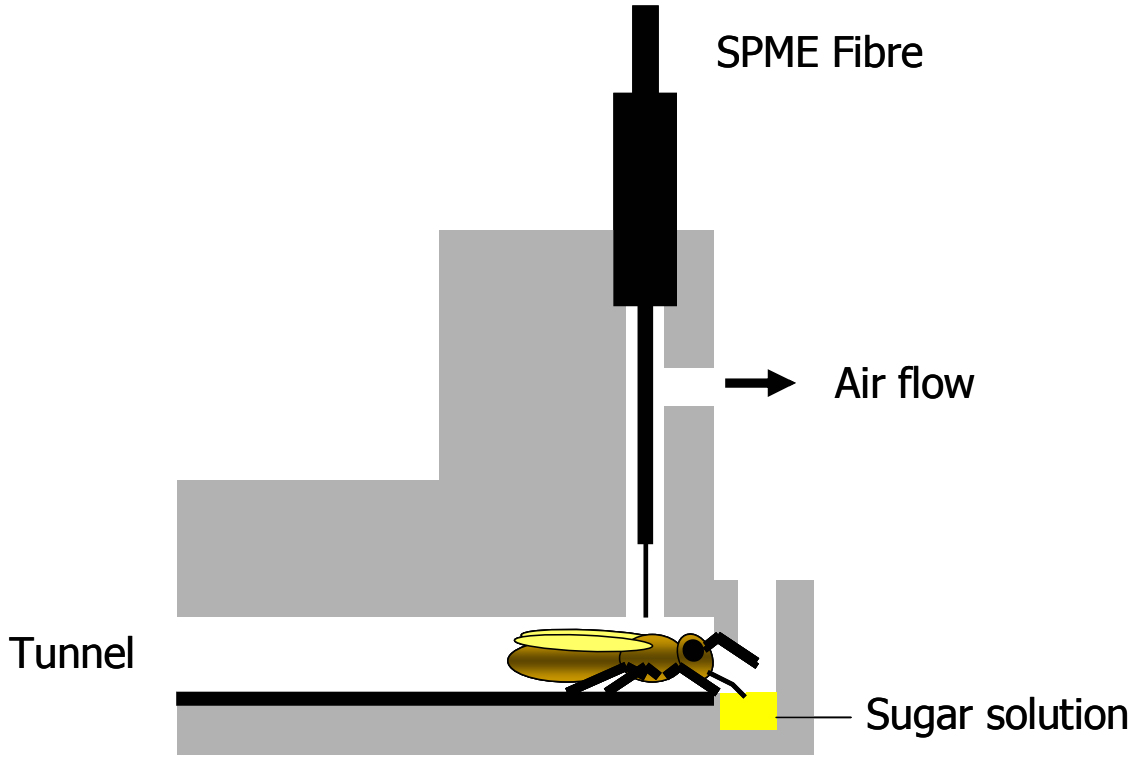

Figure 3.1: Schematic draft of the arena for the sampling of volatiles emitted from undisturbed foraging honeybee workers.

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 occured less than twice as much in the experimental arena than in the control arena to come from the surrounding air. These compounds were not included in the analysis.

3.4 Results

3.4.1 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 3.1). 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. Remarkably, we identified (Z)-11-eicosen-1-ol as a new minor component of the cuticular hydrocarbon composition of foraging honeybee workers.

3.4.2 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. Minor alkanes as well as alkenes and in one of four trials geraniol and farnesol also occurred in the air surrounding bees. However, the proportions of all components differed considerably from the cuticle extracts. Generally, highly volatile compounds were 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 3.1).

Table 3.1: 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.

	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	***	

3.5 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). In the present study we found traces and minor constituents with shorter chain lengths and characterised them as alkanes, alkenes, alcohols, and terpenes. As expected, the more volatile compounds were present in higher proportions in the headspace of foraging honeybees as compared to their cuticles. Long chain alkanes and alkenes up to C29 also emanated from the cuticle of honeybee workers in detectable amounts. This is surprising, since these substances have generally 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). 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. We found minor amounts of (Z)-11-eicosen-1-ol on the honeybee cuticles. This substance 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 most abundant hydrocarbons with functional groups in the headspace of honeybee workers are aldehydes from chain lengths 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 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 in addition to 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 but it possibly also occurs in the honeybees' Dufour's gland which seems to be slightly leaking (A. Hefetz, pers. comm.). We do not know whether the (Z)-11-eicosen-1-ol on the honeybees' cuticles does serve a pheromonal function in non-alarm situations. It may, however, provide 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 females of the European Beewolf, *Philanthus triangulum*, 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 for (Z)-11-eicosen-1-ol the significance of the 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.

3.6 References

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CHAPTER 4

PREY RECOGNITION BY FEMALE EUROPEAN BEEWOLVES AND ITS POTENTIAL FOR A SENSORY TRAP

Gudrun Herzner, Thomas Schmitt, K. Eduard Linsenmair & Erhard Strohm

4.1 Summary

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.

4.2 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 specialized 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.

4.3 Material and Methods

4.3.1 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.

4.3.2 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' response 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".

4.3.3 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 solution dummies 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.

4.3.4 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.

4.3.5 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).

4.3.6 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.

4.4 Results

4.4.1 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. 4.1). 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. 4.1). 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. 4.1). 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.

4.4.2 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 4.2 (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.

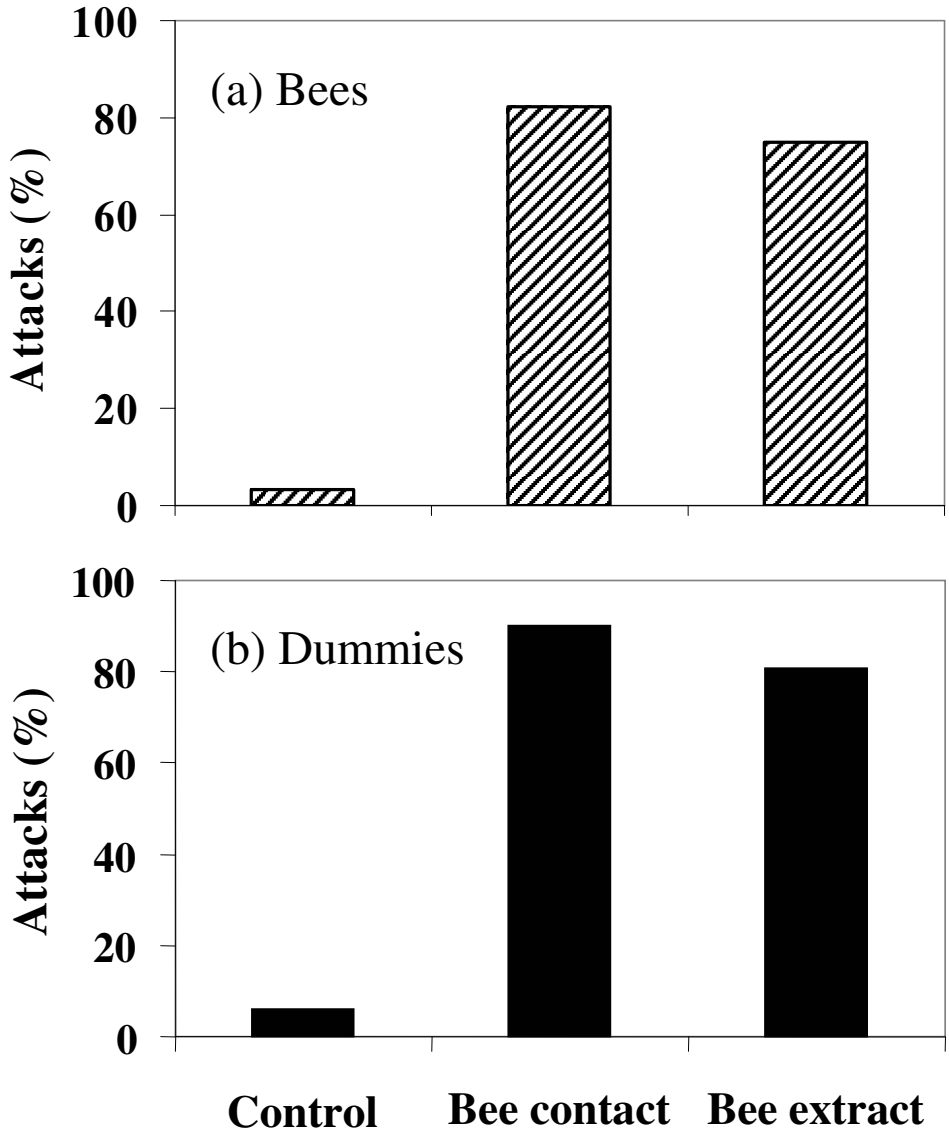

Figure 4.1: Proportion of attacks on differently treated prey objects. **(a)** Honeybees: Odourless control (n=29) bees were not attacked by beewolf females. Re-scented bees that had been in contact with live honeybees (bee contact, n=34) or were treated with cuticle extracts of honeybees (Bee extract, 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).

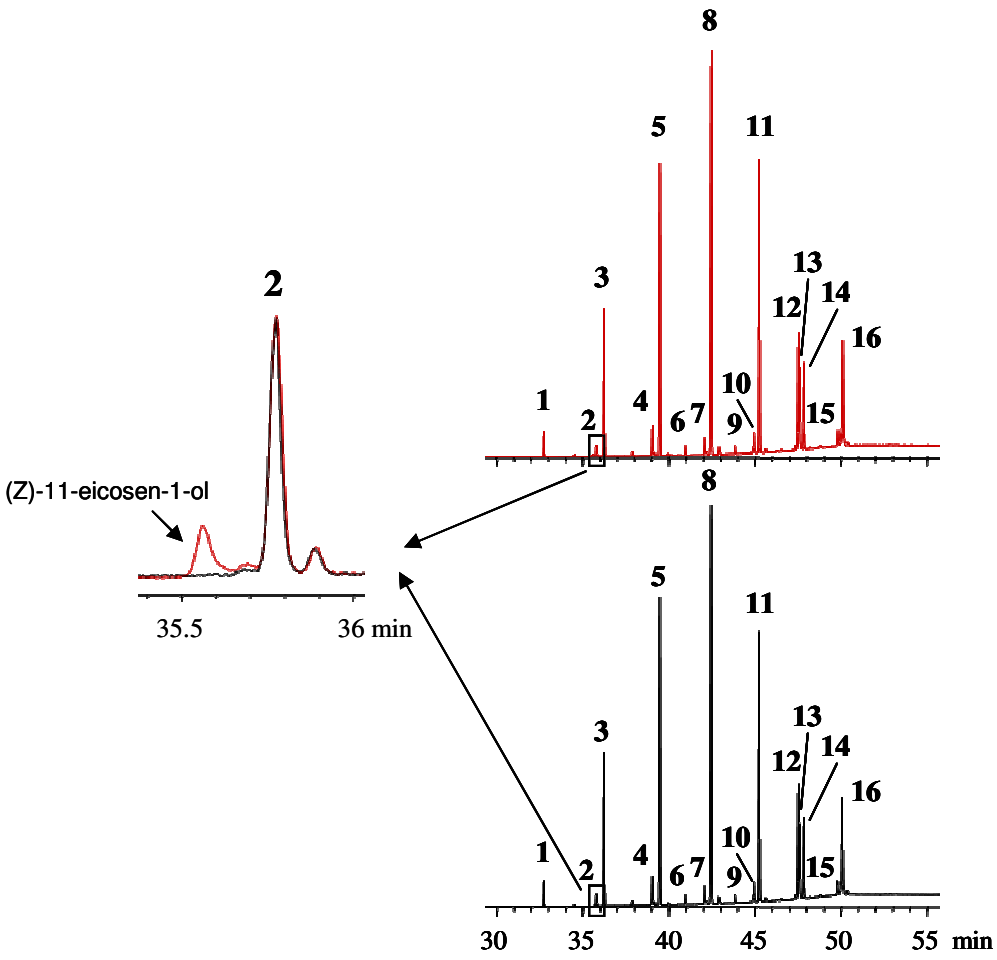

Figure 4.2: Comparison of the chromatograms of a cuticular extract of ten honeybees (red) and the hydrocarbon fraction of the same extract (black). The chart on the left shows a magnified section of an overlay of both chromatograms. Note that (Z)-11-eicosen-1-ol is only a minor peak of the chemical profile 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.)

The results of the second set of bioassays are illustrated in Figure 4.3. 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.

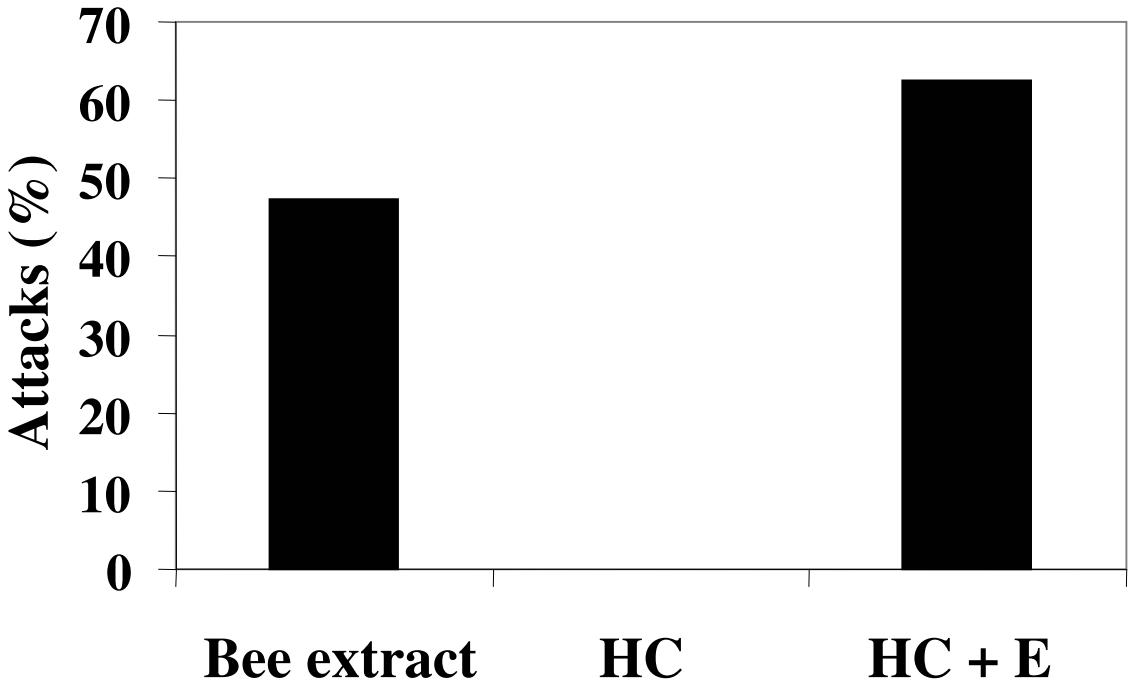

Figure 4.3: Proportion of attacks on honeybee dummies treated with differently processed honeybee extracts. In contrast to the natural honeybee extracts (Bee extract, n=19) the pure hydrocarbon extracts HC, (from which (Z)-11-eicosen-1-ol had been removed by chromatography; n=14) never elicited attacks on dummies (Fisher's exact test, p=0.002). After the re-addition of (Z)-11-eicosen-1-ol (HC+E), the hydrocarbon solution was significantly more attractive to beewolf females (n=8; Fisher's exact test, p=0.002).

4.5 Discussion

4.5.1 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*.

4.5.2 (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). Specialized 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).

4.5.3 (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 specialized – 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.

4.6 References

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CHAPTER 5

FLAGELLAR SENSILLA IN MALE AND FEMALE EUROPEAN BEEWOLVES, *PHILANTHUS TRIANGULUM* F. (HYMENOPTERA: CRABRONIDAE)

Gudrun Herzner, Thomas Schmitt, K. Eduard Linsenmair & Erhard Strohm

5.1 Summary

We investigated the morphology of the antennal sensilla of a sphecid wasp, the European beewolf *Philanthus triangulum*, to provide an inventory for the species and to compare the sensillar equipment between the sexes. The density of sensilla increased from the base to the tip of the antennae. We distinguished nine different types of sensilla. One type has not yet been described in Hymenoptera. The large s. basiconica occurred only on the antennae of female beewolves. We discuss the functional significance of the difference between the sexes and compare our results with data from other sphecids and the honeybee *Apis mellifera*.

5.2 Introduction

Insects heavily rely on the perception of chemical stimuli for foraging and intraspecific as well as interspecific communication (e.g. Cossé et al. 1995, Attygalle et al. 1996, Nishida et al. 1996, Yarden et al. 1996, Paulmier et al. 1999, Drijfhout and Groot 2001). The most important receptor organs in this case are antennal sensilla. Accordingly, insect antennae possess a considerable diversity and high density of sensilla with an olfactory or gustatory function (Inouchi et al. 1987, Isidoro et al. 1996, Kim and Leal 2000). The incidence, density, and distribution of different types of sensilla differ among species and, to a variable extent, between sexes within a species (Esslen and Kaissling 1976, Ågren 1978, Martini 1986a, Jourdan et al. 1995, van Baaren et al. 1999). These differences in sensillar equipment are

probably related to differences in ecology, mating system and other behavioral aspects of the species or the sexes (Wcislo 1995, Merivee et al. 1999).

In order to maximise their reproductive success, females should be selected to find food resources or oviposition sites (e.g. certain plant species) most effectively. By contrast, the primary interest of males is to locate females (Phelan 1992, 1997). Thus, the most relevant odors differ between males and females. As a consequence, the abundance and/or distribution of different types of sensilla on the antennae of males and females might differ. This difference is probably most pronounced in species where either males or females are highly specialized. In this study, we investigated the sensillar typology and distribution on the antennae of males and females of a sphecid wasp, the European beewolf *Philanthus triangulum*, using scanning electron microscopy (SEM) and light microscopy (LM). Since the sexes differ considerably in this species with regard to the chemical stimuli that are most important for reproduction, we expected differences in their sensory system.

For males of the European beewolf olfaction is primarily important in two (perhaps three) different contexts. First, they scent-mark territories to attract receptive females (Evans and O'Neill 1988, Strohm and Lechner 2000). Males regularly visit neighboring territories and hover downwind, possibly assessing the intensity of their neighboring male's odor (E. Strohm, unpublished observations). Males might use this information to regulate the frequency of their marking runs to match the intensity of their odor to neighboring territories. Thus, males probably perceive the sexual pheromone. Second, males feed on flowers, in the study region mostly on thistle (*Cirsium arvense*) and goldenrod (*Solidago canadensis*) (see also Olberg 1953). Thus, males have to perceive flower odors. During the night males stay in small burrows in sandy soil which they probably find by visual stimuli, but olfaction might also be involved (E. Strohm, unpublished observations). Probably none of the contexts mentioned above require extreme sensitivity on behalf of the males (as would be the case if males are attracted by female pheromones as in many moths, see e.g. Schenk 1903, Hansson 1995). There is no evidence for any aphrodisiacs emitted by females of the European beewolf.

Females rely on olfaction in mainly four different contexts. First, and probably most important, is the foraging for larval provisions. Female European beewolves are highly specialized in that they exclusively hunt workers of honeybees, *Apis mellifera*. Beewolf

females search for honeybees on flowers. They approach a potential prey and identify it while hovering downwind (Tinbergen 1935). Then the beewolf female pounces on the bee and paralyzes it by stinging. The paralyzed prey is then carried in flight to a nest that has been excavated in bare sandy soil. One to six paralyzed bees are provisioned in a brood cell and an egg is laid on one of the bees. The larva feeds on the bees, spins into a cocoon and hibernates and emerges next summer. The location and identification of honeybees is the most important component of female reproduction. Thus, females should have a suitable sensory equipment to detect their prey. Second, females have to locate a male territory for mating. Females most probably mate only once a few days after emergence (Evans and O'Neill 1988). Third, in particular when returning with prey females have to locate their nests reliably and quickly in order to minimize the probability of parasitism (Evans and O'Neill 1988, Strohm et al. 2001). The localization of the nest is mainly accomplished by visual stimuli (Tinbergen 1932, Tinbergen and Kruyt 1938). However, olfaction is probably involved in the identification of the nest (E. Strohm, unpublished observations). Finally, females also feed on floral nectar. However, they rely much less on nectar than males since for their own energy supply they primarily feed on the nectar content of the paralyzed honeybees' crops (Rathmayer 1962).

This study has two aims. First, we give an inventory of the sensilla on the antennae of male and female European beewolves. Second, we ask whether the differences between male and female behaviour might have favoured differences in the equipment with antennal sensilla. The higher complexity and importance of chemical stimuli that are relevant for females, leads to the expectation of more types of sensilla and possibly a higher density on female antennae. Sensilla types that are found on female but not on male antennae probably play a role in the location and/or identification of the honeybee prey.

5.3 Material and Methods

5.3.1 Specimens

Beewolf males and females were obtained from a laboratory population, reared at the Biocenter of the University of Würzburg, Germany, or taken from a field population nesting close to the Biocenter. In the laboratory, beewolves were kept in an environmental chamber at 26°C/22°C day/night with a 14h/10h light/dark cycle. Males could establish territories and

females were provided with honeybees *ad libitum*. Both sexes were fed with honey *ad libitum*.

5.3.2 Morphology

For scanning electron microscopy (SEM) of the outer cuticular structures entire heads with antennae attached (for purposes of orientation) and excised antennae were used. Excised heads and antennae of freshly freeze-killed females were immediately fixed in alcoholic Bouin (Romeis 1989) for 24 h and then washed in 80% ethanol. Male heads and antennae were taken from chilled individuals, fixed in 6.25 % glutar aldehyde and washed in phosphate buffer (pH 7.4). The antennae of both sexes were then dehydrated in a graded acetone series, critical point dried in CO_2 (Bal-Tec CPD030, Balzers, Liechtenstein) and glued (with conductive glue) onto the SEM supports. Finally the specimens were gold/palladium-coated (Balzers Union MED010 sputter-coating unit, Balzers, Liechtenstein) and viewed in a Zeiss DSM962 scanning electron microscope at an acceleration voltage of 10-15 kV. Micrographs were taken with a Contax camera fitted to the SEM. Beewolves hold their antennae slightly upwards (about 30°), slightly outwards (about 15°), and entirely straight during flight. Thus, for positional information we consider the ventral side of the antenna the side facing downwards and the dorsal side the side facing upwards. The measurements are based on antennal sensilla from three females and three males.

The anatomy of the cuticular structures was investigated using standard histological techniques and light microscopy (LM). Antennae were fixed in 0.1 M Cacodylate buffer containing 2.5 % glutar aldehyde, 2 % paraform aldehyde, 6 % saccharose, and 2 % DMSO, embedded in Epon and cut to 1 or 2 µm thick slices with an ultramicrotom (Reichert-Jung Ultracut) using a diamond knife. These semithin sections were mounted on glass slides, stained with methylene blue or AZAN (Romeis 1989) and observed with a light microscope (Leitz Laborlux S). Photographs were taken with a Nikon Coolpix 990 digital camera attached to the microscope.

For the inventory of the sensilla we primarily follow the sensillar classification of Schenk (1903), Esslen and Kaissling (1976), and Ågren (1977), based on morphological characters. This classification is, however, preliminary and should be replaced by that proposed by Altner

(1977), when the internal structures of the sensilla and structure-function relationships have been established.

5.4 Results

5.4.1 General morphology of the antennae

The antennae of *P. triangulum* are filiform. They are made up of a long scape at the proximal end, followed by a rounded pedicel, to which the flagellum is attached. Females have 10 flagellar annuli, males 11. Annuli are counted from proximal to distal. The first annulus is considerably longer than all the other annuli. Compared to honeybees, the flagellum is rather thick. The cuticle between the sensilla or setae is coarser on the ventral than on the dorsal side. On the ventral side of the last annulus there is a conspicuous kidney-shaped, smooth area that bears very few sensilla or setae (Fig. 5.1).

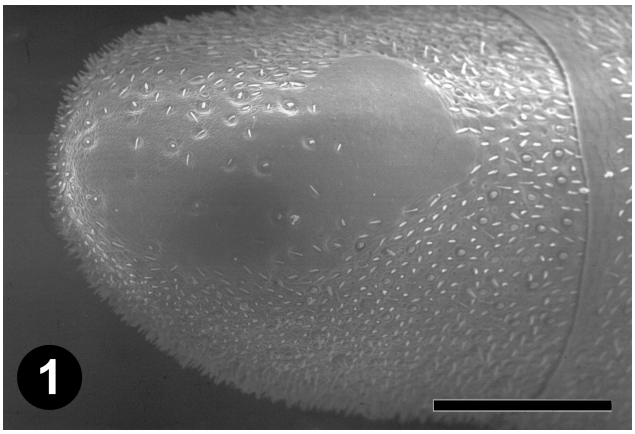

Figure 5.1: Female, 10th annulus, ventral side. Kidney-shaped area almost free of sensilla and setae. The surrounding area bears a high density of different sensillar types. Bar is 100 µm.

5.4.2 Sensilla and setae

We were able to classify nine different types of sensilla that are described in the following.

Sensilla placodea (Fig. 5.2). These so-called pore plates are oval shaped, about 7-8 µm long and 3-4 µm wide with their longitudinal axis parallel to the longitudinal axis of the antenna.

The distal end of the plate rises higher above the cuticle than the proximal end. The cuticular apparatus is encircled by a fissure and connected to the surrounding cuticle by a joint-like membrane. The plate of the sensillum bears many pores, which are arranged radially over the whole surface. The pore plates are the most numerous type of sensilla on the antennae of both males and females. They are found all around the annuli, although more rarely on the ventral side. In females they occur on annuli 1 to 10 dorsally, and 2 to 10 ventrally. Males have s. placodea on the annuli 2 to 11 on both sides of their antennae. The abundance of this sensillum is lowest on the first annulus and increases towards the tip of the antenna.

Sensilla basiconica (Fig. 5.3). The size of this large sensillum with its longitudinally sculptured shaft is about 10 x 5 µm. The shaft ends in a perforated dome constituting the blunt tip of the peg and has a apparently flexible socket that is about as wide as the length of the sensillum. The s. basiconica are restricted to females, where they can be found only dorsally or laterally on the inner side of annuli 3-10. They increase in number from the proximal to the distal end of the antenna.

Pit organs (Fig. 5.4a). They belong to the so-called "peg in pit" sensilla and are characterized by a round cuticular opening to the outside, in which no peg is visible. SEM investigations revealed that the two types cannot be easily distinguished by characters at the surface of the antenna. Although the cuticular openings of the s. ampullacea seem to be slightly smaller than the ones of the s. coeloconica, a clear differentiation of these two sensillar types was not possible. Therefore, we grouped them together as "pit organs" for the investigation of their distribution. Pit organs are found on the ventral side of the antennae in both sexes. Very few were found laterally on the outside of the flagellum. Male antennae bear pit organs on the annuli 3 to 11, female antennae on the annuli 2-10. The density of pit organs increases towards the tip of the antenna.

In the semi-thin sections we identified both s. coeloconica (Fig. 5.4b) and s. ampullacea (Fig. 5.4c) unambiguously. The s. ampullacea have a peg situated at the bottom of a 40-50 µm long and 6.7-8.5 µm wide ampulla or tube. The longitudinally furrowed peg has a length of 20-25 µm and a diameter of 2-2.7 µm. In contrast, the s. coeloconica are characterized by an only 17-20 µm deep and 8.4-10.2 µm wide cavity, two thirds of which are embedded within the

thick cuticle. They possess a much shorter central peg (8-10.7 µm) that is encircled by folds. The two sensillar types are often found intermingled with each other.

Sensilla trichodea A (Fig. 5.5). The peg of this sensillum is smooth and has a slightly tuberculoid base that is connected to the surrounding cuticle via a joint-like membrane. The s. trichodea A are found mainly on the dorsal side of male and female antennae. They also occur in fewer numbers laterally at the inside of the annuli, but are much shorter there (about 5 µm compared to 8.6-9.7 µm dorsally). In males the annuli 3-11, in females the annuli 2-10 are equipped with these sensilla, with the highest density at the apex of the antenna in both sexes.

Sensilla trichodea B (Fig. 5.6). The s. trichodea B have longitudinal furrows and a narrow socket. In most cases they are bent slightly downwards (towards the antennal surface). In the crater surrounding the sensillum two little holes can be seen (Fig. 6). These very thin and sharp-tipped sensilla are found on the annuli 1-10 on the ventral and on the annuli 2-10 on the dorsal side of female antennae. In males they occur on the annuli 2-11 dorsally and 3-11 ventrally. The most distal annuli bear more sensilla of this type than the proximal ones. Lengths measured were usually in the range of 8.8-9.3 µm at a width of only 0.9 µm.

Sensilla trichodea C/D (Fig. 5.7). The antennae of beewolf males and females bear a group of sensilla of variable size that very closely resemble the s. trichodea C/D described by Ågren (1989) for other sphecid wasps (see also Esslen and Kaissling 1976). Esslen and Kaissling (1976) differentiated the s. trichodea C and D in honeybees by, among other things, the sharpness of the tip. These two types are not differentiated here, since they were not separable by SEM, due to their uniformly rather blunt tips in *P. triangulum*. S. trichodea C/D are characterized by longitudinal furrows and a wide, possibly flexible socket. There are indications of an apical pore. They occur in both sexes, in females on all annuli dorsally and ventrally, in males on all annuli dorsally and on the ventral side from annulus 2-11. In females very few s. trichodea C/D can also be found on the scape and pedicel. The sensillum length is very variable and ranges from 8 to almost 16 µm, at a relatively constant width of 1.8-2.2 µm. While the shorter sensilla are mainly found close to the ventral or dorsal midline, there is a row of very long sensilla laterally. S. trichodea C/D are sometimes slightly curved upwards from the antennal surface.

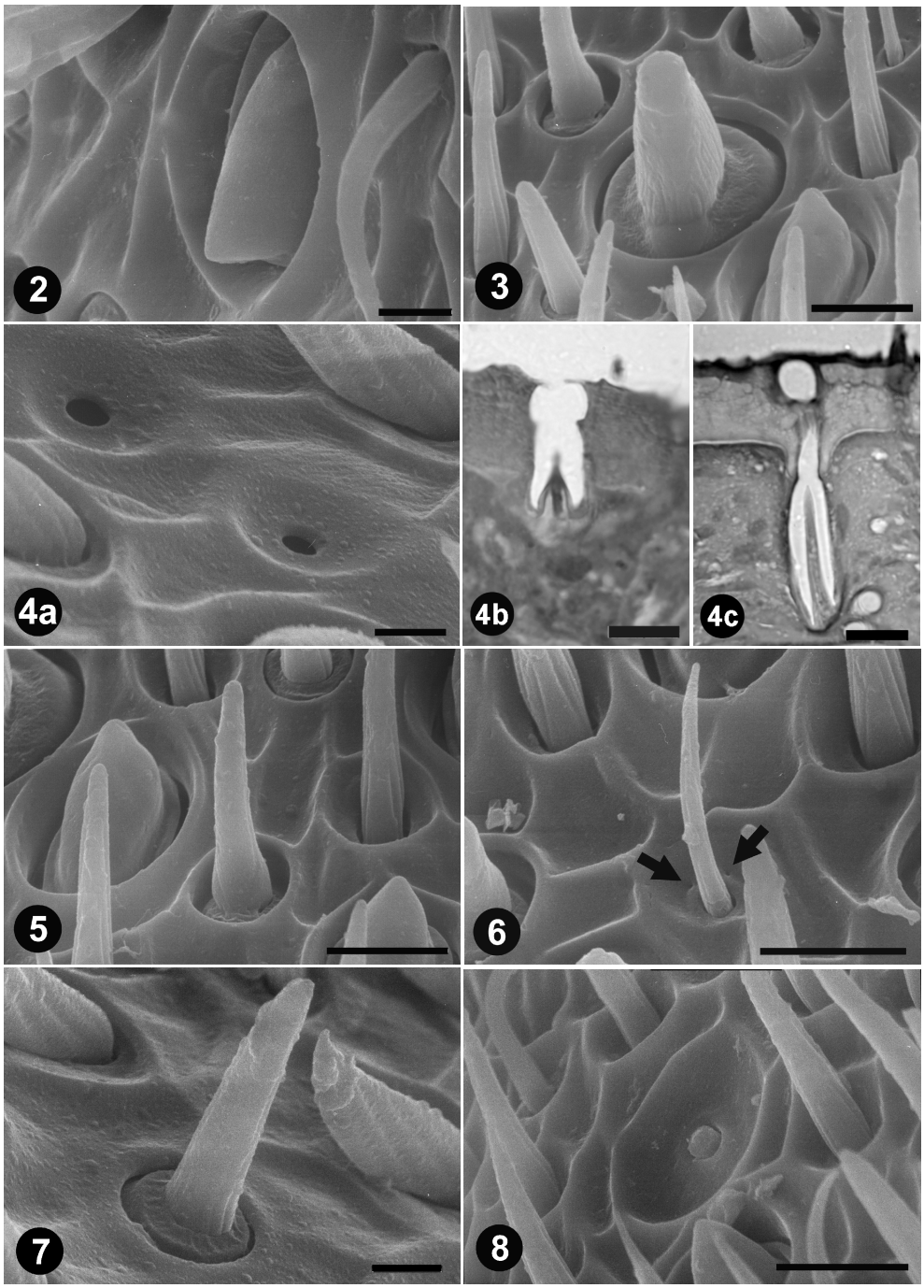

Figure 5.2: Male, 2nd annulus, dorsal side. Sensillum placodeum. Bar is 2 µm.

Figure 5.3: Female, 10th annulus, dorsal side. Large sensillum basiconicum (note wide socket). Bar is 5 µm.

Figure 5.4: Pit organs. *a.* female, 10th annulus, ventral side. Bar is 2 µm. *b.* sensillum coeloconicum (note the short peg, encircled by folds). Bar is 10 µm. *c.* sensillum ampullaceum (note the long, slender peg). Bar is 10 µm.

Figure 5.5: Female, 10th annulus, dorsal side. Sensillum trichodeum A. Bar is 5 µm.

Figure 5.6: Female, 9th annulus, dorsal side. Sensillum trichodeum B. Arrows point to little holes at the base of the sensillum. Bar is 5 µm.

Figure 5.7: Male, 10th annulus, dorsal side. Sensillum trichodeum C/D. Bar is 2 µm.

Figure 5.8: Male, 11th annulus, dorsal side. Sensillum coelocapitulum. Bar is 5 µm.

Sensilla coelocapitula (Fig. 5.8). The outer morphology of the s. coelocapitulum is very distinct with an oval concavity (8-8.6 µm across) having a central opening and a button-like protrusion with an irregular surface (1.3-1.4 µm in diameter) from the opening. This sensillum type occurs on the ventral side of male and female antennae, as well as on the very tip of the flagellar apex. It is scattered over the antenna in small numbers and it is usually found in the vicinity of pit organs. The s. coelocapitula occur on most annuli in males and females.

Grooved peg sensilla (Fig. 5.9). We found one sensillar type that can be assigned to the so-called grooved peg sensilla (Hawke and Farley 1971, McIver 1974, Altner und Prillinger 1980, Zacharuk 1980, Keil 1999). It can be found in small numbers in males and females, but only on the dorsal side and only from annulus 6-11 in males and 6-10 in females. The peg rises about 8.5-9.5 µm above the surface and is 2.36-2.5 µm wide. The base of the peg is sunken below the surrounding cuticular surface and not clearly visible. Deep longitudinal furrows (or grooves) are characteristic for this sensillar type.

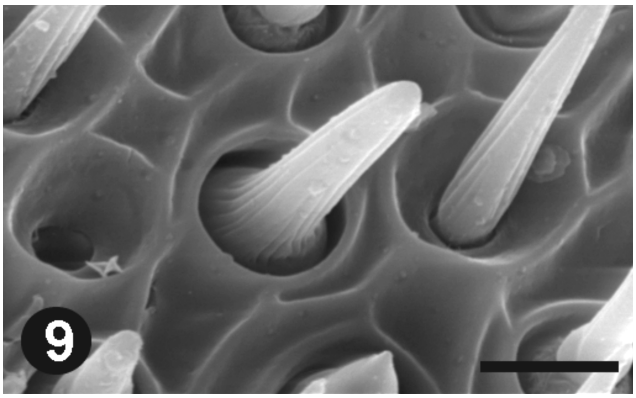

Figure 5.9: Female, 7th annulus, dorsal side. Grooved peg sensillum. Bar is 5 µm.

Setae. Setae are present on all annuli plus the scape and pedicel in males and females. They are found all around the annuli, but are most numerous on the ventral surfaces. They do not appear in one particular shape, but form a divers class of hairs. Most setae have deep longitudinal or spiral furrows, some are rather thick, especially on the distal annuli. One form is smooth and sabre-shaped and connected to the cuticle almost over its whole length.

The setae on the first annulus are thin and longer than the ones on all other annuli. The longest setae (up to four times longer than the ones found on the annuli), however, are located

on the scape. The setae at the apex of the antennae are often hooked. The tips of the setae are always tapered sharply, the sockets not prominent.

5.5 Discussion

5.5.1 General morphology

The antennal form of *P. triangulum* is similar to that in Apidae (Esslen and Kaissling 1976, Ågren 1977, 1978, 1989, Ågren and Svensson 1982). However, whereas honeybees bend their flagella downwards in flight, beewolves fly with their antennae held straight. Furthermore, in beewolves the flagellum is thickened possibly due to the presence of complex glands in females that have a function in communication between the mother and her progeny (Strohm and Linsenmair 1994/95).

An area almost free of sensilla on the ventral side of the most distal annulus has also been reported for *Cerceris rybyensis* (Ågren 1989) that belongs to the subfamily beewolves (Philanthinae), in many species of *Sphecodes* bees (Ågren and Svensson 1982), some Halictidae (Wcislo 1995) and *Apis mellifera* (Esslen and Kaissling 1976). The function of this conspicuously unarmed area is not known.

5.5.2 Sensilla types

The most conspicuous sensillar characteristic in the European beewolf is the bulbous form of the s. placodea and the stout appearance of the large s. basiconica, which seem to be typical for the family Sphecidae (Martini 1986a, b, Ågren 1989). The short peg-like s. placodea of *P. triangulum* closely resemble those of two other philanthine species *Cerceris quinquefasciata* (Martini 1986b) and *C. rybyensis* (Ågren 1989) and are still very similar to other sphecids like *Bembix rostrata*, *Psenulus concolor*, *Argogorytes fargei*, and *A. mystaceus* (Martini 1986b, Ågren 1989). In *Apis mellifera* (Esslen and Kaissling 1976) and many *Bombus* species (Ågren and Hallberg 1996) as well as in some species of Halictidae, Andrenidae, and Colletidae (Ågren 1977, 1978, Ågren and Svensson 1982) the s. placodea do not rise above the antennal surface. Martini (1986b) designated the s. placodea of other sphecid species, e. g. *C. quinquefasciata*, to the group of multiporous, single-walled sensilla with an olfactory

function. In *Apis mellifera* s. placodea are known to be olfactory receptors (Lacher 1964, Esslen and Kaissling 1976).

The closest similarity of the stout, cylindrical s. basiconica of the European beewolf, with their wide membranous sockets, can again be found in other sphecids, like *C. rybyensis* (Ågren 1989) or *P. concolor* (Martini 1986a). In the Apidae the s. basiconica are typically rather slender and long pegs with small sockets, a form that is considered more derived than the one found in the Sphecidae (Walther 1983, Ågren 1989). In *P. triangulum* only females possess the large s. basiconica. This is also the case in the sphecid wasps *Psenulus concolor* and *Dolichurus corniculus* (Martini 1986a) as well as in *Argogorytes fargei* and *A. mystaceus* (Ågren 1989). In contrast, males of *Cerceris rybyensis* and *Bembix rostrata* (Ågren 1989) as well as *Sceliphron spirifex*, *Trypoxylon attenuatum*, *Ectemnius cavifromis* (Martini 1986a) do possess s. basiconica. In *A. mellifera*, male antennae do not bear this sensillum (Esslen and Kaissling 1976). Considering the sensillar pore equipment and observations made in different species, the large s. basiconica may function as olfactory or gustatory sensilla (Slifer and Sekhon 1961, Martini 1986a, Ågren 1989, Gnatzy et al. 1990). The fact that in the European beewolf s. basiconica are only found in females suggests that they are involved in the location and identification of their only prey, honeybee workers. In the digger wasp *Liris niger* the sensilla basiconica have been reported to be essential for prey recognition (Gnatzy et al. 1990).

The morphologies of the s. trichodea A, B, C/D, the s. coelocapitula, s. ampullacea, s. coeloconica do not deviate considerably from those observed in *A. mellifera* (Esslen and Kaissling 1976, Yokohari et al. 1982). The base of the s. trichodea A is more tuberculoid in *P. triangulum* than in honeybee workers. S. trichodea A have a similar appearance in *B. rostrata* and *C. rybyensis* (Ågren 1989). In other species these sensilla have been described as single-walled hairs with wall pores, which suggests an olfactory function (Slifer and Sekhon 1961, Ågren and Hallberg 1996).

The thin s. trichodea B are found either straight or bent towards to the antennal surface. Since no other morphological difference can be found, we do not differentiate between the s. trichodeum B1 and B2 like Esslen and Kaissling (1976) did in *A. mellifera*. This variation in curvature of the s. trichodea B has also been reported in Halictidae (Wcislo 1995). The function of the two little holes situated in the socket of each s. trichodeum B in *P. triangulum*

is not clear. The holes could be pores or release sites of antennal glands as described by Isidoro et al. (1996). Bin et al. (1989) describes a close association of glands with sensilla in the parasitoid wasp *Trissolcus basalis* (see also Bartlet et al. 1994). According to Lacher (1964) and McIver (1975) the s. trichodea B have a mechanosensitive function.

The two types s. trichodea C and D as identified in *A. mellifera* by (Esslen and Kaissling) 1976 were not distinguishable by SEM. Sizes of the s. trichodea C/D vary considerably. A similar variation in sensillum size has also been recorded in *Apis* (Esslen and Kaissling 1976), some *Bombus* species (Ågren and Hallberg 1996), *Bembix rostrata* (Ågren 1989), and *Sphecodes* bees (Ågren and Svensson 1982). From structural characteristics and electrophysiological investigations a combined mechanosensory-gustatory function has been inferred (Lacher 1964, Esslen and Kaissling 1976, Ågren and Hallberg 1996).

The pit organs s. ampullacea and s. coeloconica could not be distinguished by characters at the surface of the antenna alone. The cuticular openings of the two pit organs do not differ very much in diameter and there is too much variation to assign a sensillum to one or the other pit organ type unambiguously. The same problem has been described for the leaf cutter ant *Atta sexdens* (Kleineidam 1999, Kleineidam et al. 2000) and two *Andrena* species (Ågren 1978). In *A. mellifera* (Dietz and Humphreys 1971), some *Bombus* species (Ågren and Hallberg 1996), as well as the ants *Cataglyphis bicolor* and *C. bombycinus* (Riedl 1995) the diameter of the external opening of the s. ampullacea is much smaller than that of the s. coeloconica, which allows a clear differentiation of the two types by their outer appearance.

Not all insect species possess two types of pit organs (Ågren 1989). The semi-thin sections of antennae of the European beewolf revealed that the anatomy of the s. ampullacea and s. coeloconica is similar to these sensilla in the ants *Formica rufa* (Walther 1981), *Atta sexdens* (Kleineidam 1999), *Cataglyphis bicolor,* and *C. bombycinus* (Riedl 1995). In other insect species the peg of the s. coeloconica protrudes over the antennal surface or is at least clearly visible from the outside (e.g. in the bees *Augochlora pura*, Wcislo 1995, and *Andrena vaga*, Ågren 1978, and the ant *Formica rufa*, Walther 1981). Kleineidam (1999, 2000) identified the s. ampullacea as CO_2-receptors in *Atta sexdens* by electrophysiological methods. A thermo-, hygro- and CO_2- receptive function has been proposed for s. ampullacea in Coleoptera (Guse and Honomichl 1980) and Diptera (McIver 1982). An olfactory function has been assigned to

the coeloconic sensilla in *Locusta migratoria* (Boeckh 1967, Altner and Prillinger 1980, Altner et al. 1981).

The sensilla coelocapitula do occur on the antennae of several other hymenopteran species like the honeybee *Apis mellifera* (Dietz and Humphreys 1971, Esslen and Kaissling 1976, Yokohari et al. 1982, Yokohari 1983), bumblebees (Ågren and Hallberg 1996), *Andrena tibialis* and *A. vaga* (Ågren 1978), *Bembix rostrata* (Ågren 1989), *Cerceris rybyensis* (Ågren 1989), eleven species of Sphecodes bees (Ågren 1982), and the two ant species *Cataglyphis bicolor* and *C. bombycinus* (Riedl 1995). They were not found in *Argogorytes fargei* (Ågren 1989) and in the eleven taxa of Halictidae studied by Wcislo (1995).

In most of these cited works these sensillar types have been referred to as sensilla campaniformia (Dietz and Humphreys 1971, Esslen and Kaissling 1976, Ågren 1978, Ågren 1989, Riedl 1995). Yokohari and coworkers (1982), however, reidentified this type of sensillum as a coelocapitular sensillum because it does not resemble the true campaniform sensillum described by Moran et al. (1971), which has a dome-shaped (convex) central protrusion. The coelocapitular sensilla on the antennae of *P. triangulum* are relatively large compared to the ones in *Apis mellifera* (Yokohari et al. 1982). The association of s. coelocapitular with pit organs (s. coeloconica and/or s. ampullacea) have also been reported in the honeybee (Dietz and Humphreys 1971, Yokohari et al. 1982), *Bembix rostrata* (Ågren 1989), *Andrena tibialis* and *A. vaga* (Ågren 1978), and some Sphecodes bees (Ågren 1982). The sensilla coelocapitula are hygro-and thermoreceptors in *Apis mellifera* (Yokohari et al. 1982, Yokohari 1983) [whereas the campaniform sensilla have been shown to function as proprioceptive mechanoreceptors (Iwasaki et al. 1999)].

The grooved peg sensilla are very variable in their appearance. They range from small, peg-like structures (Lewis 1971, McIver 1974) to large hairs (Lambin 1973, Hawke and Farley 1971). In *P. triangulum* they are relatively long and thick. These sensilla either stand on a small socket (Hallberg 1979), arise directly from the antennal surface (McIver 1974) or are sunken slightly below the surrounding cuticular surface with their base, as described here for *P. triangulum*. The characteristic morphological feature visible in SEM-studies are the deep longitudinal grooves ranging from near the base (as in *P. triangulum*) or about the middle of the peg to the tip. The grooved pegs seem to be common in Diptera like e.g. the fly *Stomoxys calcitrans* (Lewis 1971), the mosquito *Aedes aegypti* (McIver 1974, Cribb and Jones 1995),

the malaria mosquito *Anopheles gambiae* (Meijerink et al. 2001), and several syrphid flies (Henderson and Wellington 1982). It also occurs in the cockroach species *Periplaneta americana* (Altner et al. 1977), *Blaberus craniifer* (Lambin 1973), and *Arenivaga sp*. (Hawke and Farley 1971). The migratory locust *Locusta migratoria* (Steinbrecht 1969), the blood sucking bug *Triatoma infestans* (Guerenstein and Guerin 2001), the mealworm beetle *Tenebrio molitor* (Harbach and Larsen 1977), as well as many other species of Coleoptera and Homoptera also possess grooved pegs. Despite the wide distribution of this sensillar type in many different taxa, to our knowledge, it has not been described in hymenopterans so far. The grooved peg sensilla are presumably double-walled wall-pore sensilla (DW-WP in Altner 1977) with an olfactory function (e.g. Zacharuk 1980, Steinbrecht 1996, Keil 1999, Guerenstein and Guerin 2001). Sometimes the s. coeloconica are also denominated grooved pegs, since they belong to the DW-WP sensilla and function as olfactory receptors (Altner and Prillinger 1980). Keil (1999) distinguished between DW-WP sensilla sunken in pits (= s. coeloconica) and those with a hairlike structure above the cuticular surface (= grooved pegs).

The different types of sensilla could not always be unequivocally distinguished by SEM. There seem to be transitional forms between some types of sensilla. It is also not clear whether all the different types of setae are uninnervated hairs. TEM and electrophysiological investigations will reveal more morphological details and the function of the different types of sensilla.

In conclusion, beewolves possess a large number of different sensilla and a high density. Five of the nine sensillar types possibly have an olfactory function. With regard to our initial question about differences between the sexes in the equipment with sensilla there was a qualitative difference in that males lack the large s. basiconica. This suggests that the s. basiconica have a function in location or identification of the prey of the females, honeybee workers.

It has also to be taken into account that the functional diversity of sensilla is much greater than the number of morphological types (Zacharuk 1980, Steinbrecht 1996). Morphologically similar sensilla may have different numbers of sensory cells, different specificities and different response characteristics, not only in different insect species, but also between the sexes of the same species (Davies 1977, Städler 1978). Hence, differences in olfactory abilities of males and females might be more pronounced than suggested by differences in

morphology of the sensilla. Also, most recent results show a sexual dimorphism in the antennal lobe of beewolves, involving the numbers of glomeruli as well as the size of identified homologous glomeruli in the brains of males and females (J. Rybak, personal communication).

5.6 References

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CHAPTER 6

(*S*)-2,3-DIHYDROFARNESOIC ACID, A NEW COMPONENT IN CEPHALIC GLANDS OF MALE EUROPEAN BEEWOLVES *PHILANTHUS TRIANGULUM*

Thomas Schmitt, Erhard Strohm, Gudrun Herzner, Carlo Bicchi, Gerhard Krammer, Frank Heckel & Peter Schreier

6.1 Summary

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 reanalyzed 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.

6.2 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., 2001). 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 reanalyzed 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.

6.3 Material and Methods

6.3.1 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, unpublished 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 4 hr. 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 fibers through dissected cephalic glands.

6.3.2 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 MD800 quadrupol mass detector. The GC was equipped either with a J&W DB-5 fused silica capillary column (30m x 0.25mm ID; df=0.25μm; J&W, Folsom, CA, USA; temperature program: from 60 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 to 150°C at 10°C/min, from 150 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 ionization voltage of 70 eV and a source temperature of 220°C.

Chemical ionization 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 ionization gas, and the source temperature was 150°C.

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

6.3.3 Capillary Gas Chromatography – Fourier Transform Infrared Analysis (HRGC-FTIR)

HRGC-FTIR spectra were obtained using an HP 5890 GC (Agilent Technologies, Böblingen, Germany) coupled to an 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 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 to 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.

6.3.4 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 to 160°C at 2°C/min and from 160 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/sec. 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 analyzed.

6.3.5 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 synthesized (see below).

6.3.6 Synthesis of (Z)-10-Nonadecen-2-one

This compound was synthesized 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 hr. The reaction mixture was 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).

6.3.7 Dimethyl Disulfide (DMDS) Derivatizations

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

6.3.8 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) was added, and 1 μl of the mixture was injected into the GC without further treatment.

6.4 Results

Qualitative differences among the extracts made with hexane, dichloromethane, or SPME fibers 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., unpublished data). In the following, minor components that were found only in some males will not be reported. The heads of 25 males from the laboratory population were individually analyzed. A typical total ion chromatogram of an hexane extract of the glands of an individual male is shown in Figure 6.1. The mean (±SD) total amount of the entire pheromonewas 353±167μg. The mean relative amount of each constituent and its standard deviation is given in Figure 2.

6.4.1 (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).

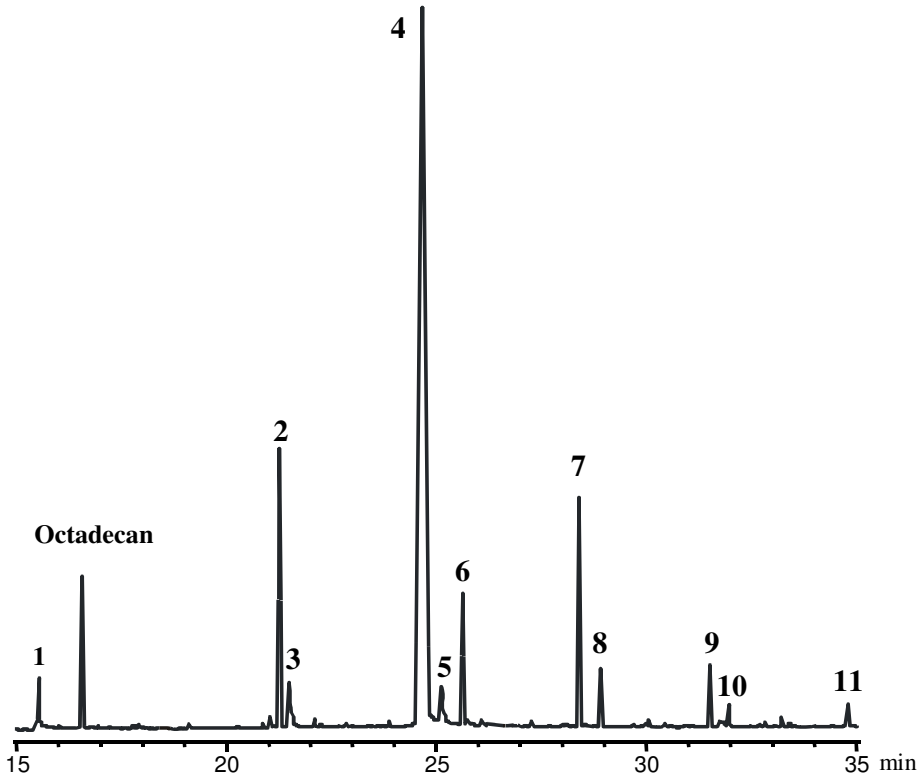

Figure 6.1: Gas chromatogram of a hexane extraction of the cephalic gland of an individual beewolf male (**1** = (*S*)-2,3Dihydrofarnesoic acid , **2** = (*Z*)-10-nonadecen-2-one, **3** = 1-octadecanol, **4** = (*Z*)-11-eicosen-1-ol, **5** = (*Z*)-9-tricosene, **6** = tricosane, **7** = (*Z*)-9-pentacosene, **8** = pentacosane, **9** = (*Z*)-9-heptacosene, **10** = heptacosane, **11** = nonacosane).

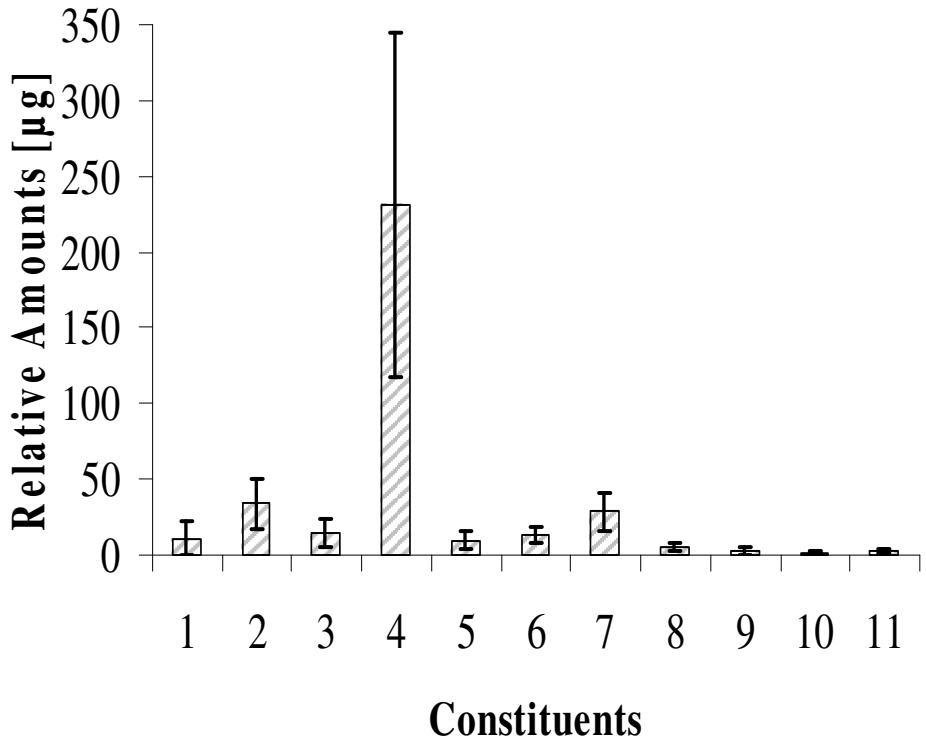

Figure 6.2: Relative amounts of the constituents of the male cephalic secretion of *Philanthus triangulum*, means (± SD) of 25 beewolf head extracts. Numbers of components are the same as in Figure 1.

6.4.2 (Z)-10-Nonadecen-2-one

Peak **2** of the cephalic gland extract was identified as (*Z*)-10-nonadecen-2-one. After derivatization with DMDS, it showed characteristic EI-MS peaks at 173 and 201 and MC 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´).

6.4.3 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).

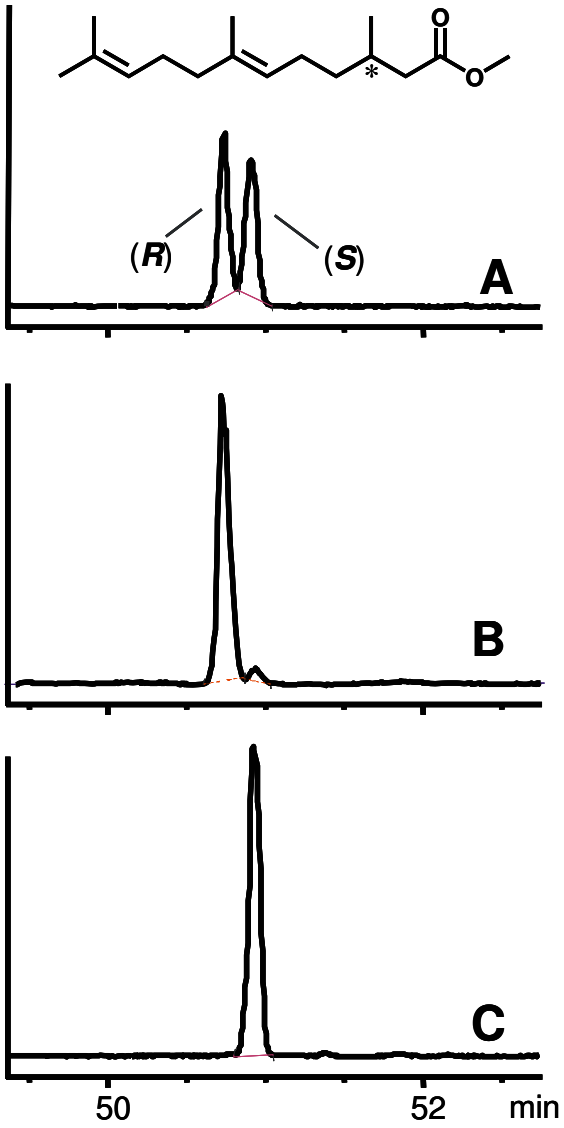

Figure 6.3: Assignment of the absolute configuration of methyl-2,3-dihydrofarnesoate by enantioselective gas chromatography. **(A)** racemic, **(B)** synthetic methyl-(*R*)- and **(C)** methyl-(*S*)-2,3-dihydro-farnesoate from male beewolf cephalic secretion.

6.4.4 (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 derivatization. MC of the derivatized compound was 390, and the major mass peaks were found at 173 and 217. The GC-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 (----CH2 ----OH), 721 (*cis* compound of RCH==HCR´).

6.4.5 Alkanes

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

6.4.6 (Z)-Alkenes

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

6.5 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 analyzed 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 honey bee, *Apis mellifera*. Its function as a component of the alarm pheromone and its attractiveness to conspecifics has already been shown (Free et al., 1982, 1983; Pickett et al., 1982). 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., unpublished data, Strohm 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 arthropods. 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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CHAPTER 7

LET ME BE YOUR HONEYBEE: EVOLUTION OF THE SEXUAL SIGNAL IN MALE EUROPEAN BEEWOLVES *PHILANTHUS TRIANGULUM* (HYMENOPTERA: CRABRONIDAE)

Gudrun Herzner, Thomas Schmitt, K. Eduard Linsenmair & Erhard Strohm

7.1 Summary

The prevalent sexual asymmetry in direct parental investment leads to differential reproductive rates between the sexes. As a consequence, males compete over the access to receptive females and invest greatly in elaborate courtship signals to advertise their presence and quality. The sensory trap model of signal evolution emphasises that male signals are shaped by sexual selection through female choice in such a way that they release favourable behavioural responses of the females that had previously been established in other contexts, like e.g. predator detection or foraging. Here we test the hypothesis that the pheromone of male European Beewolves, *Philanthus triangulum*, functions as a sensory trap for females. Beewolf females identify their prey, honeybee workers, olfactorily via a substance that is present on the prey in only very small amounts: (Z)-11-eicosen-1-ol. Beewolf females therefore seem to be highly sensitive to this compound. (Z)-11-eicosen-1-ol, as well as several other components of the prey odour, also occur in the pheromone of male European Beewolves and might, thus, function as a sensory trap. In behavioural assays, beewolf females did not show a hunting response when offered the male pheromone in the hunting context. Thus, we reject the sensory trap hypothesis for the beewolf system. Since the case of our model species is instructive with regard to the question how receiver bias models should be tested, we discuss different methods in detail. Based on these considerations and the strong odour congruence between the females' prey and the male pheromone we conclude that the male pheromone of *P. triangulum*, although not representing a sensory trap, has evolved to exploit a female (sensory) bias that originated in the context of prey hunting.

7.2 Introduction

The enormous variety of male sexual signals and the corresponding female preferences have long been an intriguing field of research (Cronin 1991, Andersson 1994). Darwin (1871) already proposed that sexual selection for female choice may lead to exaggerated sexual ornaments and behaviours in males. He could not explain, however, why males typically court and females choose. Trivers (1972) offered the explanation, when he pointed out that the differential parental investments of males and females lead to an asymmetry in the intensity of sexual selection. Females usually invest significantly more in their progeny than males and their reproductive success is limited by the resources that determine offspring fitness. As a consequence, the operational sex ratio becomes male-biased and females become a limiting resource over which males have to compete. Whereas females benefit from being choosy, i.e. maximizing the quality of their mates, males can increase their reproductive success by maximizing the quantity of their matings (see also Andersson and Iwasa 1996). Males therefore invest predominately in elaborate courtship traits to attract many receptive females.

Receiver bias models of signal evolution (e.g. West-Eberhard 1984, Christy 1995, Endler and Basolo 1998, Ryan 1998) recognize the inequality in parental investment as a major driving force in the evolution of mating signals. They assume that the selection on the male signal and the female response may be asymmetric, with the male signal tracking the female response over evolutionary time ('asymmetric tracking', Phelan 1992, 1997). Thus, a female preference for a male signal is thought to exist before the signal itself has evolved. The preference may arise either as an epiphenomenon of the receiver's perceptive or cognitive system (Ryan et al. 1990, Enquist and Arak 1993), or evolved in contexts other than sexual communication for mate choice (e.g. predator avoidance or foraging; West-Eberhard 1984, Christy 1995).

The concept of receiver biases encompasses three related models: pre-existing bias, sensory exploitation, and sensory trap (for a review see Endler and Basolo 1998). All these models share the view that the evolution of male sexual signals is influenced by pre-existing characteristics of the females' sensory or neural systems. The sensory trap model emphasizes that the receiver response evolved in a context unrelated to sexual selection. Male signals are thought to mimic model stimuli that stimulate female biases that evolved under selection pressures in ecological, social, and physiological contexts (or that did so in the past) (Wickler 1965, West-Eberhard 1984, Christy 1995). The sensory trap model predicts that the mimic

stimulus (male signal) elicits the same female response as the model stimulus (e.g. prey cue) when contextually transposed (Christy 1995).

There is evidence for receiver bias processes for visual, acoustic and mechanical courtship signals (e.g. Ryan et al. 1990, Basolo 1990a, 1995 Proctor 1991, 1992, McClintock and Uetz 1996, Hebets and Uetz 2000, Greenfield and Weber 2000, Rodd et al. 2002, Stålhandske 2002, Madden and Tanner 2003, Christy et al. 2003a, b, Smith et al. 2004, MacLaren et al. 2004), but amazingly little is known about the validity of such models in chemical communication systems (West-Eberhard 1984). This is surprising, since chemicals seem to be the most universal of stimuli and most – if not all – species seem to make use of chemical stimuli either for foraging or communication or both (see Dusenbery 1992). Here we investigate whether the male pheromone of the European Beewolf *Philanthus triangulum* (Hymenoptera: Crabronidae) might have evolved to capitalize on the females' pre-existing bias for certain chemical stimuli.

Table 7.1: Comparison of the compounds found in the pheromone glands of male European Beewolves and on the cuticle of honeybees, the prey of female European Beewolves.

	P. triangulum pheromone[1]	*A. mellifera* cuticle[2]
(*S*)-2,3-dihydrofarnesoic acid	+	-
(Z)-10-nonadecen-2-one	+	-
1-octadecanol	+	-
(Z)-11-eicosen-1-ol	+	+
(Z)-9-tricosene	+	+
tricosane	+	+
(Z)-9-pentacosene	+	+
pentacosane	+	+
(Z)-9-heptacosene	+	+
heptacosane	+	+
nonacosane	+	+

Sources: [1] Schmitt et al., 2003; [2] Schmitt et al., in prep.

Female *P. triangulum* capture exclusively worker honeybees, *Apis mellifera*, as food for their larvae (Strohm 1995). Male *P. triangulum* are territorial and scent-mark vegetation inside their territories (like many other species of *Philanthus*: see Alcock 1975, Gwynne 1978, Evans and O'Neill 1988), to attract conspecific females for mating purposes (Simon Thomas and Poorter 1972, Evans and O'Neill 1988). When a female alights in a territory, the male approaches her and the copulation immediately takes place without any further courtship (personal observation).

There is a remarkably high similarity between the cuticular substances of the honeybee prey (Francis et al. 1985; Salvy et al. 2001, Schmitt et al., in prep.) and the pheromone of beewolf males (Schmitt et al. 2003) (Table 7.1). Eight of the eleven substances of the male pheromone also occur on the honeybee cuticle. These are various long-chain aliphatic alkanes and alkenes (saturated and unsaturated) as well as (Z)-11-eicosen-1-ol. Alkanes and alkenes are almost ubiquitous in insects (Hymenoptera: e.g. Ayasse 1991, Oldham et al. 1994, Paulmier et al. 1999, Schiestl et al. 1999, Krokos et al. 2001, Clarke et al. 2001, Bartelt et al. 2002, Panek et al. 2001, Ruther et al. 2002, Diptera: Ishii et al. 2001, Coleoptera: Nelson et al. 2002, Lepidoptera: Guo & Blomquist 1991, Heteroptera: Drijfhout & Groot 2001) and therefore possibly no reliable cues for beewolf females for the identification of prey (Herzner et al., in prep.) or males. (Z)-11-eicosen-1ol, in contrast, is a rare compound in insects. In the distribution range of *P. triangulum* it has, apart from the male pheromone of the European Beewolf (Schmidt et al. 1990, Schmitt et al. 2003) and the honeybee *A. mellifera* (Free et al. 1982, 1983; Pickett et al. 1982, Schmitt et al., in prep.) not been described for any species.

An earlier study revealed that (Z)-11-eicosen-1-ol is a necessary cue for prey identification and that the females' sensitivity to this substance is remarkably high (Herzner et al., in prep.). This extreme sensory sensitivity for (Z)-11-eicosen-1-ol, that evolved by natural selection in the context of prey hunting, might make females highly susceptible to 'exploitation' by male sexual signals. Thus, we hypothesized that males have adopted this substance as a pheromonal component to make their territories more conspicuous for females.

In this study we tested whether the resemblance between the honeybee odour and the male pheromone is sufficient to really catch females in a sensory trap, i.e. whether it elicits an out-of-context response of females. If this was the case, females should show the normal hunting response when offered the male pheromone in the hunting context.

7.3 Material and Methods

7.3.1 Beewolves

For a detailed description of the European Beewolf and its behaviour see Strohm (1995). For the present study, females were either collected at different field sites in Würzburg or obtained from a laboratory population reared at the Biocenter of the University of Würzburg (F1-generation of field caught females). They were brought into an environmental chamber and individually housed in sand-filled breeding cages (60x18x18 cm) to which foraging partitions (15x18x18 cm) were attached. These were lit by neon lamps (14h/10h light/dark 26/22°C day/night cycle). 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 honeybee dummies.

7.3.2 Training and test

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). Only females that learned to accept the dead bees as prey were used for the bioassays described below.

To reduce visual stimuli we used honeybee dummies as prey items instead of real but manipulated honeybees. The dummies were made of dark-grey Teflon and attached to thin metal rods. They were cylindrical in shape and had the approximate size of honeybees (1.5 x 0.6 mm). As demonstrated in an earlier study (Herzner et al., in prep.), the dummies can easily be scented by the application of hexane extracts to their surface and are – if they carry the right odour –readily accepted, i.e. attacked, by beewolf females.

We tested whether the male pheromone of *P. triangulum* operates as a sensory trap for conspecific females. As shown above (Table 1) the odour correspondence between the honeybee, the females' prey, and the male pheromone is remarkably strong. If the pheromone was a real mimetic signal, we would expect that females attack dummies coated with male pheromone as readily as dummies with honeybee odour, since the male pheromone should elicit the same female response as the prey odour (independent of the context, Christy 1995).

A honeybee extract was obtained 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 by a gentle stream of nitrogen and applied to an odourless dummy with a pipette immediately before each test to avoid a premature volatilization of substances. After the solvent had evaporated (after 1 min), the scented dummies were used for the bioassay. Pheromone extracts were obtained by leaching 10 heads of beewolf males (that had previously been cut open to open the gland reservoirs) in 1 ml distilled hexane overnight. From this quantity of pheromone extract, 50 µl were given on one dummy for each experiment. As control, 50 µl of pure hexane were applied on dummies and presented to beewolf females.

We compared the number of attacks on dummies carrying the honeybee odour or the male pheromone and odourless dummies with Fisher's exact test (one-tailed) using the statistics program BIAS for Windows (#7.07).

7.3.3 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.

7.4 Results

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.

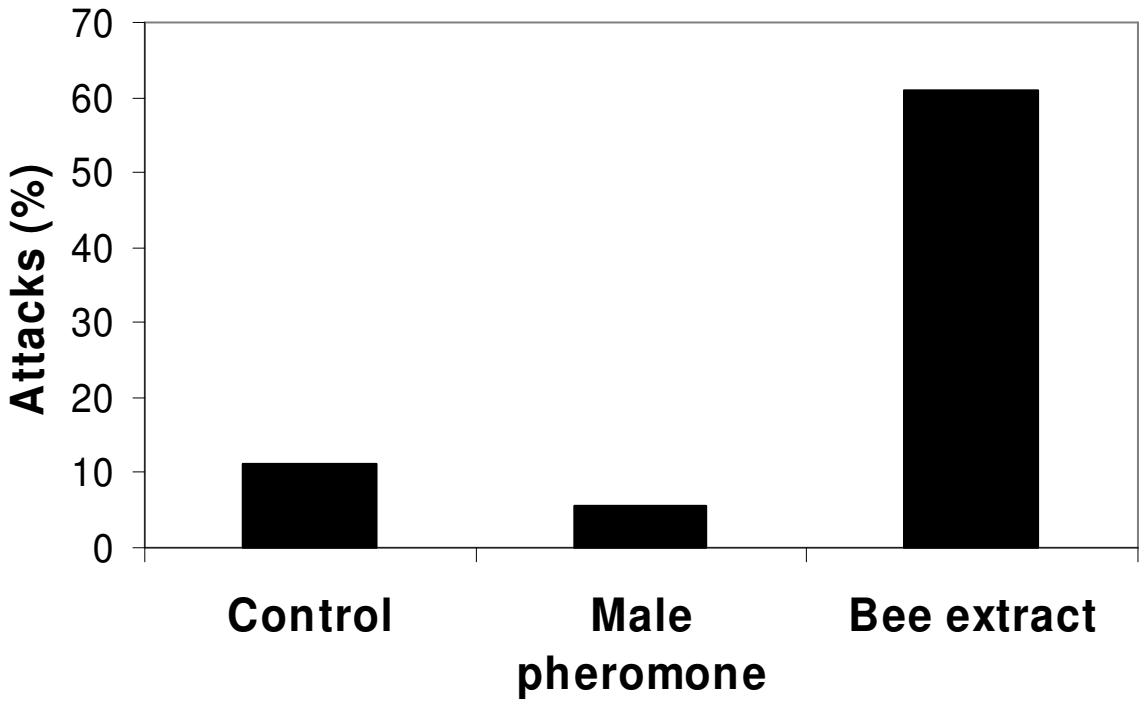

Figure 7.1: Proportion of attacks by beewolf females on dummies treated with honeybee extracts and extracts of male pheromone. Dummies treated with honeybee extract were accepted as prey and attacked (difference to control: Fisher's exact test, $p=0.001$). Dummies carrying extracts of the beewolf male pheromone did not elicit significantly more attacks than the odourless controls (Fisher's exact test, $p>0.5$ for both extracts).

When confronted with the normal honeybee extract, females displayed the typical hovering behaviour before they pounced at the dummies. Extracts of the male pheromone applied to dummies were not attractive to females at all and did not even trigger the hovering behaviour. Figure 7.1 shows the proportion of attacks for the different kinds of dummies. Dummies bearing honeybee extract (n=23) were significantly more often attacked than odourless dummies (n=18) (Fisher's exact test, p=0.001). Extracts of the male pheromone on dummies (n=18) did not evoke significantly more attacks than odourless dummies (Fisher's exact test, p>0.5).

7.5 Discussion

7.5.1 Rejection of the sensory trap hypothesis

The remarkable odour-correspondence between the females' prey and the male pheromone in the European Beewolf *P. triangulum* (see Table 7.1), suggests that males have evolved the pheromone to exploit a pre-existing receiver bias in the females. Our results show, however, that the male pheromone does not function as the bait of a sensory trap for conspecific females. In contrast to dummies carrying the original prey odour, dummies scented with the male pheromone were not treated as prey by females in the hunting context. Females did not even display the typical hovering behaviour, which is the step of the hunting sequence in which females inspect the prey-like object olfactorily and decide whether to attack or to ignore it (Herzner et al., in prep.). The fact that females did not enter this phase suggests that the similarity between the male pheromone and the honeybee cuticle is not sufficiently high to catch females in a sensory trap. The differential female response is most probably due to three components that occur only in the male pheromone: (*S*)-2,3-dihydrofarnosoic acid, (Z)-10-nonadecen-2-one, and 1-octadecanol (Schmitt et al. 2003). Furthermore, the honeybee cuticle carries many more substances (alkanes, methylalkanes and alkenes) that cannot be found in the male pheromone (Schmitt et al., in prep.) and the proportions of compounds differ vastly.

There are two important reasons why it might not be adaptive for males of the European Beewolf to mimic the honeybee odour perfectly. First, (Z)-11-eicosen-1-ol is present in large amounts in the alarm pheromone of honeybees (Pickett et al. 1982, Wagner and Breed 2000). Even though it does not seem to be an aggression releasing substance (Free et al. 1982, Wagner and Breed 2000), its presence in large concentrations and in combination with the

"hydrocarbon background" of honeybees, signals a honeybee alarm situation and females should not respond to this blend of substances by approach. Therefore, a perfect mimic would have a deterrent effect on females, if these substances were not accompanied by the additional beewolf specific components.

The second reason why males should olfactorily be distinguishable from honeybees is that the attacks of beewolf females happen very fast and accurately and a male that gets mistaken for a honeybee would most probably be killed. There are reports of predation on conspecific males in other *Philanthus* species (*P. basilaris*: O'Neill and Evans 1981, *P. pulchellus*: Asis et al. 1996). Thus, cannibalism does occur in beewolves and natural selection will favour males with courtship signals that are both effective and nevertheless unambiguously distinguishable from prey (see McClintock and Uetz 1996, Hebets and Uetz 2000). Consequently, the male pheromone is unlikely to function as a true sensory trap in *P. triangulum*, because males would either deter females or could be killed before they even had the chance to mate and transfer their sperm. This does not mean, however, that males cannot make use of the pre-existing female bias at all.

7.5.2 Receiver bias

Interspecific comparisons

The pheromone composition of other beewolf species differs considerably from *P. triangulum*. Preliminary analysis of the male pheromone of *P. venustus*, a species that occurs sympatrically with *P. triangulum* over a large part of its geographical range (Bitsch et al. 1997), revealed carboxylic acids, esters, and aldehydes as major compounds (and various hydrocarbons as additional compounds; E. Strohm, unpublished data). One of their prey species, *Lasioglossum malachurum*, carries various hydrocarbons, carboxylic acids, esters, and lactones on its cuticle (Ayasse 1991). The classes of compounds found in the pheromones of some North American *Philanthus* species (Schmidt et al. 1985, McDaniel et al. 1987, 1992) clearly differ from those of the European Beewolf and show no odour correspondence with the honeybee *A. mellifera* (with the exception of low amounts of (Z)-11-eicosen-1-ol in *P. crabroniformis*). Thus, the strong similarity of pheromonal components of *P. triangulum* with the cuticular substances of *A. mellifera* can hardly be explained by chance but has, most probably, evolved to exploit the females' pre-existing bias for honeybee odour. To verify this

scenario, the pre-existence of the female preference has to be shown (Ryan et al. 1990, Basolo 1990b). This is generally accomplished either by phylogenetic tests or by revealing the evolutionary processes that have resulted in the establishment of the bias.

The phylogenetic method

Ryan (1990) and Shaw (1995) proposed phylogenetic methods for testing the sensory exploitation hypothesis (see also Sherman and Wolfenbarger 1995). In applying these methods, preferences and traits are mapped onto phylogenies to reveal the pre-existence of the preference (Fig. 7.2). These cladistic tests have some shortcomings, however, and an impracticality to apply them to a system must not necessarily lead to a rejection of the sensory exploitation hypothesis (Christy and Backwell 1995, Basolo and Endler 1995, Jennions and Brooks 2001, Wiens 2001). Since many authors still regard these tests as necessary to prove cases of receiver biases, I will deal with the associated difficulties in detail here.

One important criterion in this regard is the "evolvability" of a receiver bias or pre-existing preference, i.e. its potential to change during cladogenesis. Depending on their origin, receiver biases may be static (not or slow evolving) or variable (fast evolving) within a higher taxon. Slow evolving or fixed biases may occur due to two reasons: First, a bias may arise as a primarily non-functional epiphenomenon of the sensory or cognitive system and therefore lack genetic variation (Arak and Enquist 1993, Enquist and Arak 1993, Basolo 1998), e.g. the preference for male low frequency calls of female Túngara frogs (Ryan et al. 1990). Second, a bias might be caused by strong selection in fundamentally important natural selection contexts like e.g. predator avoidance in wax moths (Greenfield and Weber 2000) and fiddler crabs (Christy et al. 2003 a, b), egg sac care in spiders (Stålhandske 2002), and egg fertilization in damselflies (Córdoba-Aguilar 2002). Such systems are amenable to cladistic tests of a preference pre-existence, since the preference would represent a plesiomorphic trait of a larger taxonomic unit (Fig. 7.2 a).

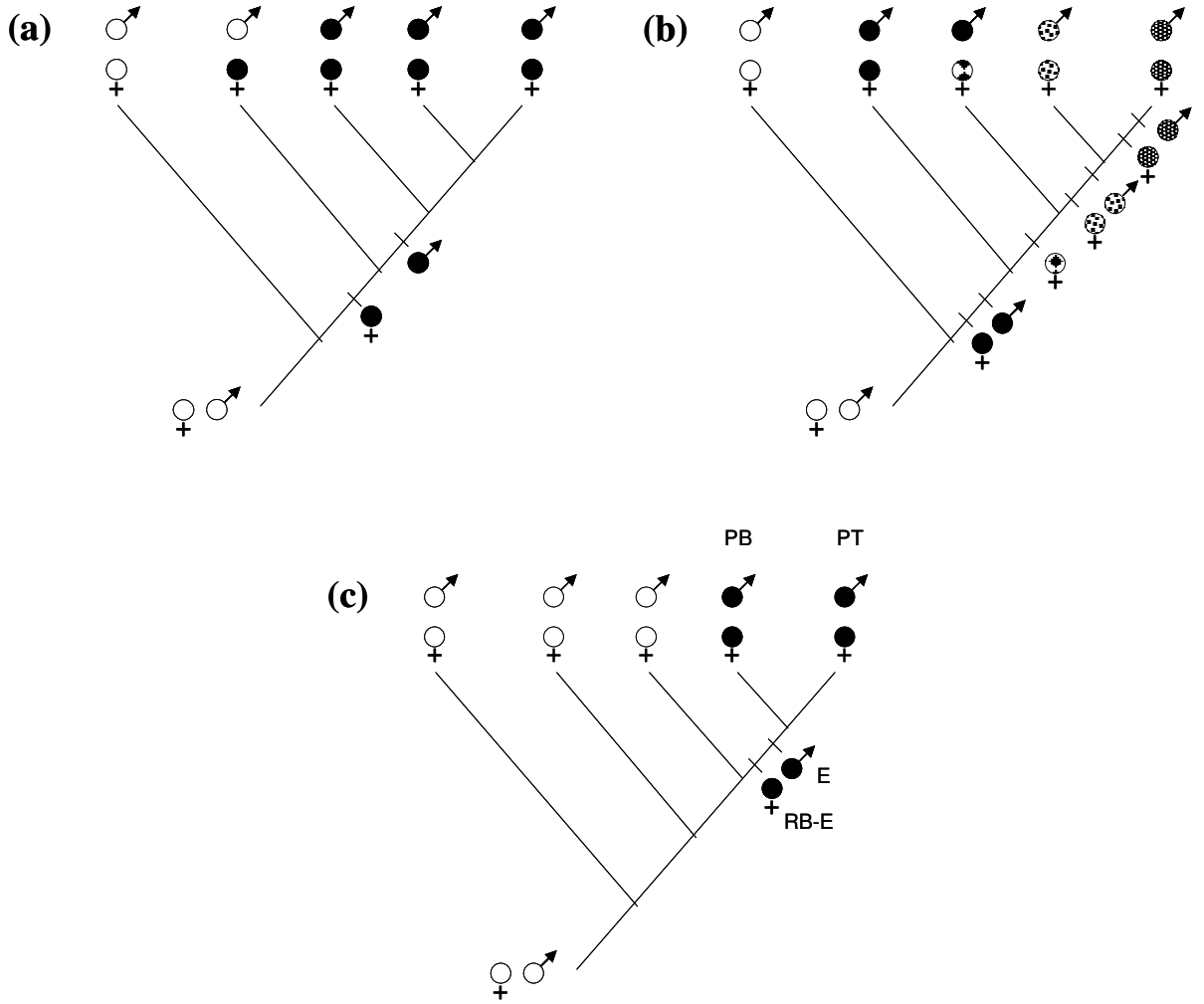

Figure 7.2: Cladograms resulting from the receiver bias model. **(a)** The receiver bias evolves early in a taxon and is slow evolving. In this case its pre-existence to the male trait can easily be inferred from the cladogram. **(b)** The female receiver bias evolves relatively fast (e.g. due to species specific specialization on different prey). The respective male trait also evolves rapidly and is assumed to reflect the female preference in most species. Therefore, the pre-existence of the female preference cannot be inferred from the cladogram. **(c)** Hypothetical cladogram for the genus *Philanthus*. Females of the more basal species have broad prey spectra and probably no pronounced receiver biases towards unique prey stimuli. Therefore sensory exploitation with regard to prey cues is not likely. Females of the more recently diverged *P. triangulum* (PT) and *P. basalis* (PB) are highly specialized on only one prey species, *Apis mellifera* and *A. cerana*, respectively. Because these prey species are closely related and both have (Z)-11-eicosen-1-ol, the female bias towards (Z)-11-eicosen-1-ol (RB-E) and the incorporation of this substance into the male pheromone, due to sensory exploitation (E), might represent an apomorphic trait of these two *Philanthus* species.

Receiver biases may, however, also evolve in contexts characterised by much higher rates of evolutionary change and might therefore vary among closely related species. Female biases that originated in a foraging or hunting context, for example, may be subject to comparatively fast evolutionary change caused by a diversification of the food or prey niches or by changing

environmental regimes (sensory drive; Endler 1992). Examples include preferences for (nuptial) coloration in guppies (Rodd et al. 2002) and sticklebacks (Smith et al. 2004) and for colourful bower decorations in bower birds (Madden and Tanner 2003). Such rapid gains and losses of preferences should cause the corresponding male traits to be changed or lost. This leads to an irregular distribution of preferences and traits within a phylogeny making an interpretation in the framework of pre-existing biases difficult, if not impossible (Fig. 7.2 b; see also Christy and Backwell 1995, Wiens 2001).

The high sensitivity of female *P. triangulum* toward (Z)-11-eicosen-1-ol arose as a side-effect of selection in a foraging context (Herzner et al., in prep. and below). It is implausible to assume that other *Philanthus* species show the same bias for (Z)-11-eicosen-1-ol, since it is a substance characteristic of *A. mellifera* and the European Beewolf *P. triangulum* is the only species specialized on hunting *A. mellifera*. Almost all other beewolf species studied so far have very broad prey spectra (up to 81 species representing various families and genera of Hymenoptera are used as prey, Evans and O'Neill 1988, Asis et al. 1996). It is not known which - if any - olfactory cues are used by females of these other beewolf species to recognize their various prey species. The great prey diversity suggests that more general cues, like e.g. body size (Evans and O'Neill 1988), or various different chemical cues are used for prey hunting, which makes receiver biases difficult to exploit by males and/or difficult to detect for researchers. There is, however, one Asian beewolf species, *P. basalis*, that has been reported to be a specialist hunter of the Indian honeybee *Apis cerana indica* (Krombein 1981). The venom of *A. cerana* has been reported to contain (Z)-11-eicosen-1-ol (Schmidt et al. 1997). It would be intriguing to analyse the male pheromone of *P. basalis* and to test for sensory exploitation in this Asian beewolf system (Fig. 7.2 c).

The base of the bias – another context

The above considerations demonstrate that our system does not lend itself well to a phylogenetic analysis. A different approach to elucidate a possible pre-existence of a bias is to reveal the evolutionary processes that caused the bias (Christy 1995, Proctor 1991, 1992, Rodd et al 2002, Madden and Tanner 2003). In female *P. triangulum*, natural selection pressures on prey hunting abilities seem to have shaped the female preference (Herzner et al., in prep.). The most parsimonious interpretation of our results is, thus, that the female

sensitivity for (Z)-11-eicosen-1-ol has evolved in the foraging context and was subsequently exploited by males with their pheromone.

This view is a corollary of the asymmetry in parental investment, with females (who most probably mate only once; personal observation) providing more parental investments than males. As a consequence females become the limiting resource over which males, who primarily invest in mate attraction and encounter, have to compete. Thus, it is the male courtship signal that is tracking the female response over evolutionary time and it will be designed to match the characteristics of the females' perceptual system (Phelan 1992, 1997). The reverse scenario would be hardly plausible, since it is unlikely that female beewolves should specialize on and hunt only insects whose odour is similar to the male pheromone. The same is e.g. true for water mite females, who cannot be expected to specialize on prey species that match the male courtship signal (Proctor 1991, 1992).

As mentioned above, very little is known about the role of receiver bias processes for the evolution of chemical courtship signals. West-Eberhard (1984) reports personal observations of a possible sensory trap in the pheromone communication of *Xylocopa* bees, but does not support them with actual data. Cases of male insects sequestering substances from their larval food sources, which are the food or host plants of the females, to later incorporate them (or parts of them) in their pheromones (Baker and Cardè 1979, Nishida et al. 1985, Krasnoff and Dussourd 1989, Shelly 2001) can also be interpreted as sensory traps (Christy 1995). The present study, however, is to our knowledge the first reporting on the exploitation of a female receiver bias by a *de novo* synthesized male pheromone.

7.5.3 Conclusion

Since the physiological level of the pre-existing female bias (sensory or higher) has not been specified, we assign our findings to the more general category of receiver biases (Arak and Enquist 1995, Basolo 1990a). Yet, the amazing ability of beewolf females to recognize honeybees based on trace amounts of (Z)-11-eicosen-1-ol (Herzner et al., in prep.) points to an extreme sensitivity of the olfactory system to this substance. It is, thus, tempting to speculate that the male pheromone of *P. triangulum* constitutes a case of sensory exploitation.

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CHAPTER 8

BROTHERS SMELL SIMILAR: SEX PHEROMONE VARIATION IN MALE EUROPEAN BEEWOLVES AND ITS IMPLICATIONS FOR INBREEDING AVOIDANCE

Gudrun Herzner, Thomas Schmitt, Peter Schreier & Erhard Strohm

8.1 Summary

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 complementary 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 crabronid 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%.

8.2 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 a female shares 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 50 and 100% depending on whether the mother was outbreeding or was also inbreeding (accordingly, the proportion of diploid males varies between 25 and 50% of the diploid progeny). 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 cephalic 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 finding and 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.

8.3 Material and Methods

8.3.1 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 18 mm) 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.

8.3.2 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.

8.3.3 Data analysis

The peaks of 10-nonadecen-2-one and 1-octadecanol were not clearly separated in all chromatograms and were thus 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.

8.4 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.

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'-$\lambda = 0.356$, $\chi^2 = 23.26$, $df = 4$, $p < 0.0001$; Fig. 8.1). 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 8.1). 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.

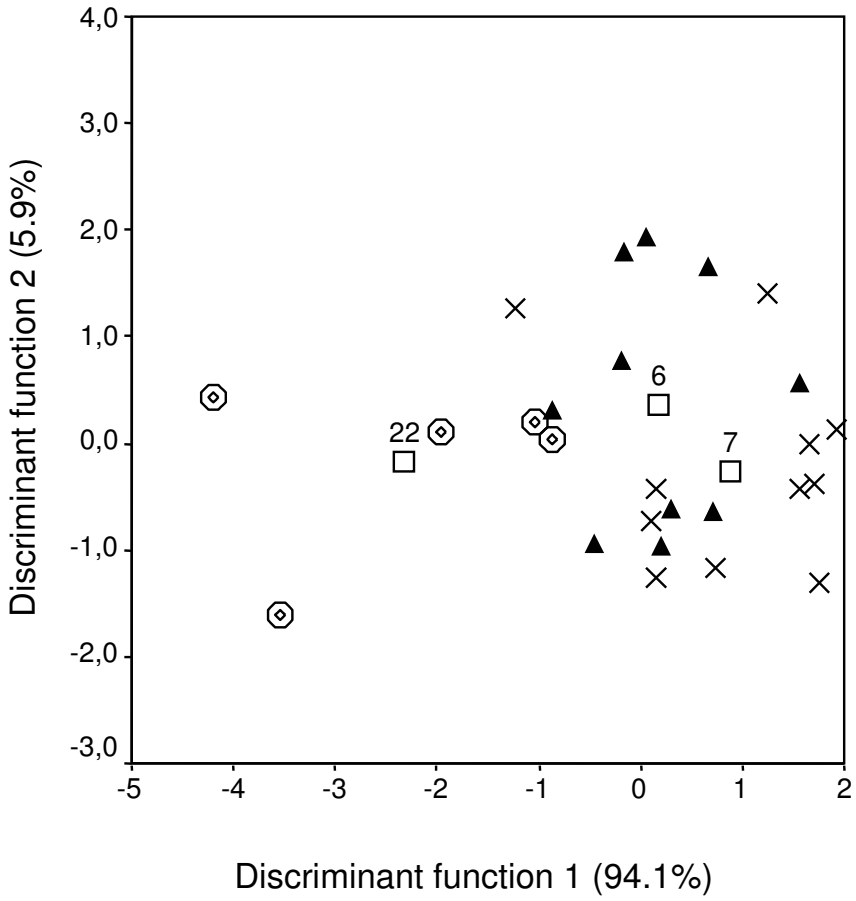

Figure 8.1: Discriminant analysis of analysis group A (3 families, 26 individuals). Despite some overlap, the families are separated significantly on the basis of the relative areas of eight pheromone peaks (Wilks'-λ = 0.356, χ^2 = 23.26, df = 4, $p < 0.0001$) (see also Table 1 and text for results of 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 8.3, 8.5).

Table 8.1: Classification results of the discriminant analysis for analysis group A. Given are the proportions of classifications to the different families (in %).

	Predicted family		
Family	**6**	**7**	**22**
6	60	40	0
7	18	82	0
22	40	0	60

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. 8.2). Discriminant function 1 accounts for 63.4% of the total variance extracted by the PCA and separated all but the families 8 and 12 from each other. The families 8 and 12 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 8.2). 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.

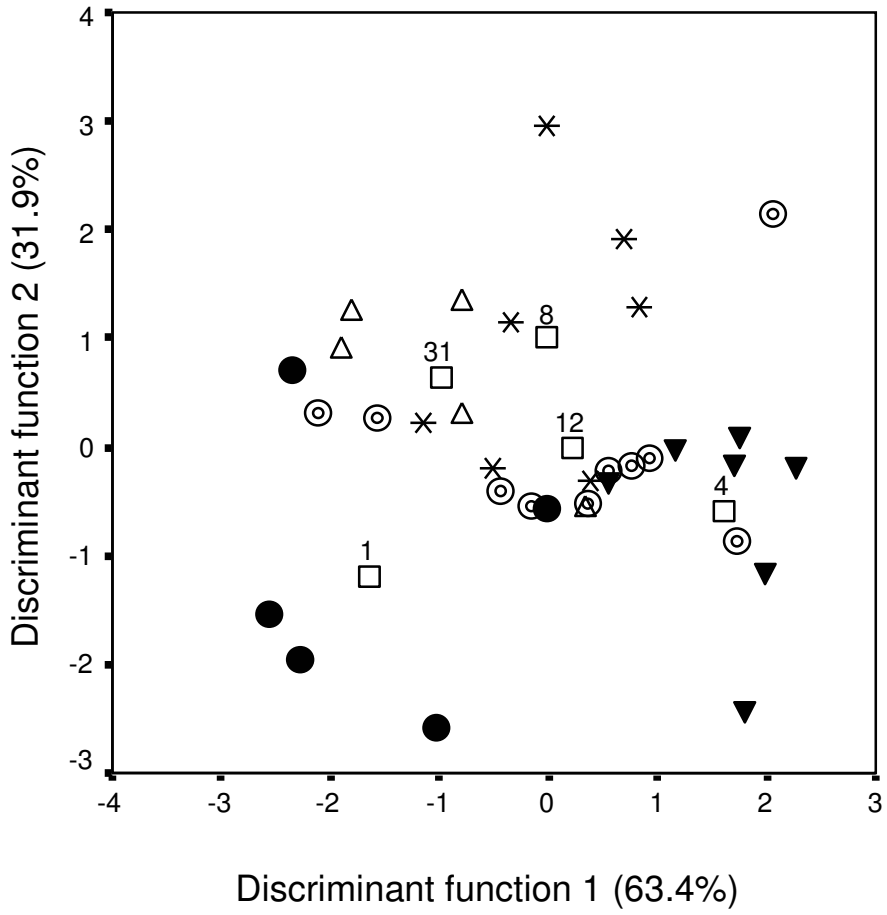

Figure 8.2: Discriminant analysis of analysis group B (5 families, 34 individuals). Despite some overlap, the families are separated significantly on the basis of the relative areas of eight pheromone peaks (Wilks'-$\lambda = 0.246$, $\chi^2 = 40.73$, df = 12, $p < 0.0001$) (see also Table 2 and text 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 8.4, 8.5).

Table 8.2: Classification results of the discriminant analysis for analysis group B. Given are the proportions of classifications to the different families (in %).

	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

8.5 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 (the paradox of the lek, Kirkpatrick and Ryan 1991). 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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8.7 Appendix

Table 8.3: 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**	**h^2 ± 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 8.4: 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	**Mean [µg] ± sd**	**h^2 ± 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 8.5: 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.

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.

CHAPTER 9

GENERAL DISCUSSION

In the present thesis we could gather substantial evidence for different selection pressures acting on the evolution of the male pheromone and the corresponding female preference in European Beewolves, *Philanthus triangulum*. Females have evolved an olfactory system that allows them to effectively hunt honeybees as food for their progeny. They possess an extreme sensitivity for one essential component of the odour of honeybees, their exclusive prey (chapters 3 to 5). The design of the male pheromone shows a remarkable similarity to the honeybee odour, but also distinct differences (chapters 3, 6, and 7). The pheromone is not a perfect mimic of a honeybee and, thus, females are not caught in a 'sensory trap'. This is most probably due to an array of - sometimes opposing - natural and sexual selection pressures (chapter 7). Based on careful theoretical considerations, our empirical results, and interspecific comparisons we conclude that, although not setting a sensory trap, beewolf males have exploited a pre-existing bias of females towards their prey's odour to effectively attract females for mating (Chapter 7). With regard to the signal's content, we propose that female choice for genetic compatibility may subsequently have influenced the evolution of the male pheromone (Chapter 8). This is, of course, a very much simplified portrayal of the evolutionary scenario and detailed discussions on the various different aspects and some peculiarities of our model system are given in the respective chapters. Here I will focus on more general considerations concerning the evolution of sexual communication systems.

The origin and maintenance of sexual signals can be best understood by realizing two basic principles: First, the asymmetry in parental investment leads to an asymmetry in sexual selection (Phelan 1992, 1997). Second, sexual signals (and signals in general) comprise two distinct components: design and content (Guilford and Dawkins 1991, Endler 1992, 1993, Chapter 1). Males are selected to design their signals in a way to achieve maximum stimulation of the females' sensory systems and, as a consequence, elicit favourable female responses that increase male fertilization rates (Christy 1995). Females, on the other hand, are selected to assess males based on information contained in their signals and to choose the best available male as a mate. Female sensory abilities and preferences largely determine male signal evolution, because they cause selective pressures on the signals. It is important to note

that females' sensory characteristics and the resulting species specific preferences may initially arise due to processes unrelated to sexual communication for mate choice (Chapter 1).

European Beewolves present an instructive model system to investigate how receiver biases in females evolve and how these sensory or cognitive properties of the females affect male signal design. As beewolf females rely primarily on their chemical sensory modality for prey hunting (Chapter 5, Tinbergen 1935), males are expected to use chemical signals, i.e. pheromones as sexual attractants. Further on, the female's sensory system is tuned to (Z)-11-eicosen-1-ol and other characteristic volatiles of honeybees. Males that incorporate these substances into their pheromone to exploit the female sensory-response system will increase the number of their mating opportunities and will thus be favoured by selection. Although our bioassays show that females can perceive very small amounts of (Z)-11-eicosen-1-ol, this substance is present in exceptionally large amounts in the male pheromone. Such large amounts of uncommon substances in male pheromone blends have to be interpreted, in the same way as the colourful wings of butterflies or the elaborate songs of birds, as exaggerated male advertisement signals. There is evidence form a variety of species that females prefer stronger stimuli, e.g. brighter colours, louder sounds, faster motion, or stronger chemical signals (see Ryan and Keddy-Hector 1992). Several hypotheses have been put forth to explain these female mating biases and the respective amplification of the male signals.

9.1 Signal design

Fisher's runaway sexual selection is the classical explanation for the evolution of such exaggerated male traits (Fisher 1930). The genetic correlation of the male trait and female preference leads to an ever-accelerating and self-reinforcing process of co-evolution, with males amplifying their signals and females preferring males with ever more amplified signals, because these signals indicate male superiority under natural selection (the Fisher-Zahavi model; Eshel et al. 2000, Kokko et al. 2002). A more parsimonious explanation is provided by the sensory bias models (West-Eberhard 1984, Ryan and Rand 1993, Endler and Basolo 1998). They assume that pre-existing characteristics of the female sensory system may drive male courtship signals towards greater exaggeration, because exaggerated traits are superior stimulators of sensory processes (Guilford and Dawkins 1991, Kirkpatrick and Ryan 1991,

Ryan and Rand 1993, Arak and Enquist 1995). Females will respond more strongly with increasing signal intensity, even if it extends substantially beyond the natural phenotypic range; that is, females have preferences for supernormal stimuli as has been shown for many stimuli by classical ethology (Tinbergen 1948). In this scenario there is no need for a genetic correlation, i.e. a step-by-step co-evolutionary change of the male signal and the female perceptual system or response. Furthermore, the male trait does not have to be (but can be) correlated with male quality under natural selection.

For *P. triangulum* both scenarios result in directional selection for larger amounts of (Z)-11-eicosen-1-ol in the pheromone blend and both are plausible. The exaggerated male signal may arise in response to the naturally selected sensory bias of females towards (Z)-11-eicosen-1-ol and the supernormal stimulus effect. Or, given that the production of (Z)-11-eicosen-1-ol is costly and its amount in the pheromone blends is genetically correlated with fitness-enhancing traits, (Z)-11-eicosen-1-ol may be a true indicator of male quality. In this case female choice for indirect benefits may account for the exaggerated quantities of (Z)-11-eicosen-1-ol in the pheromone.

9.2 Signal content

This last notion indicates that the pheromone of *P. triangulum* may convey information about male quality (signal content). Active female choice can only operate on traits that vary between male phenotypes. Male *P. triangulum* exhibit considerable individual variation in their pheromone composition that might provide a suitable basis for female mate choice. We could show that the pheromone varies with familiar affiliation and may thus contain information about the degree of relatedness between a female and her potential mate. If females use this information to discriminate among males and to avoid sib-matings, they reduce the danger of matched matings, i.e. matings with males that carry the same allele at the single sex-determination locus (Cook and Crozier 1995, Butcher et al. 2000). Via this so-called choice for genetic compatibility (Zeh and Zeh 1997, Tregenza and Wedell 2000, Colegrave et al. 2002) females can increase their reproductive success, because no (or less) sterile diploid males are produced.

Studies featuring choice for genetic compatibility usually face two problems: First that the source of the incompatibility is unknown and, second, that no indicators of male genotype are found (Zeh and Zeh 1997, Tregenza and Wedell 2000, Colegrave et al. 2002). An additional difficulty – integral to all studies of female choice – lies in demonstrating the adaptive value of female mate choice behaviour (see Andersson 1994, Kokko et al. 2002, 2003). In *P. triangulum* the idiosyncrasy of the special mode of sex determination (single-locus complementary sex determination, see chapter 8) may lead to occasional genetic incompatibility between mating partners and the production of diploid males (M. Kaltenpoth, unpublished data). Female beewolves mate only once and need to assess male genetic compatibility prior to mating (in contrast to multiple-mating species, where post-copulatory cryptic choice is commonly observed, Eberhard 1996, Birkhead and Møller 1998, Birkhead and Pizzari 2002, Colegrave et al. 2002). Females may avoid the fitness penalties caused by inbreeding by choosing males based on their pheromone composition, which may function as an indicator of relatedness. Since diploid males are disproportionally costly but do not contribute genetically to the next generation, choosy females will gain considerable fitness benefits. This provides us with a powerful, unambiguous test of the effects of particular male phenotypes on female reproductive success.

9.3 Synthesis

Although the distinction in signal design and content, as exemplified above, may prove extremely valuable for understanding signal evolution, it is conceivable that design and content may often be two sides of the same coin. Only those signals that are designed to allow a successful reception and perception by the female can be subject to sexual selection by female choice based on signal content (Endler 1992, 1993, Guilford and Dawkins 1993). Female European Beewolves seeking indirect fitness benefits most probably make use of the information content of the male sexual signal to choose the most appropriate male as a mating partner. They can only do so, however, because the male pheromone is designed to match the characteristics of the female sensory system.

Our observations on the complexity of mate preferences and the favoured sexual signals mirror those of other studies. In fiddler crabs (genus *Uca*), for example, males build mud pillars near their burrows to attract receptive females for mating. Female crabs generally

orient towards vertical structures that project above the horizontal line, a behaviour selected as an anti-predator response (Christy et al. 2002). This sensory bias of females favoured males hat construct mud pillars at the entrances to their burrows. Here, females get caught in a sensory trap due to the specific design of the male signal (Christy et al. 2003a, b). There is evidence that the ability to build mud pillars is costly and condition-dependent (Backwell et al. 1995). Thus, the mud pillar structure may also indicate male condition. If condition is heritable, females who base their mating decision on this information (signal content) will receive indirect fitness benefits.

In freshwater poeciliid fishes of the genus *Xiphophorus*, females of sworded swordtails and of swordless platyfishes both prefer males with swords, and female responses increase with male sword length (Basolo 1990a, b, 1995, Haines and Gould 1994, Rosenthal and Evans 1998). Phylogenetic and empirical evidence suggests that a pre-existing female preference has played a major role in the evolution of male swords. Recent results demonstrate that long swords elevate the energetic costs of routine and courtship swimming (Basolo and Alcaraz 2003). Thus, males that can perform the characteristic courtship swimming despite the long swords may be of superior intrinsic quality. Females exercising mate choice based on male sword length and courtship behaviour may thus gain indirect benefits in the form of 'good genes'.

The *Xiphophorus* system has a further community with European Beewolves. The sword, like the beewolf pheromone, is a composite trait consisting of different components (Basolo 1995, 1998). Each one of these components may be subject to different selection pressures and may contain different information about its bearer. For a recent review on the use of multiple cues in mate choice see Candolin (2003). Chemical stimuli are distinguished from all other stimuli with regard to the number of distinctive messages that they can convey (e.g. Dusenbery 1992). Pheromones are prime examples of multiple component – or composite – signals, since they usually consist of blends of different kinds of substances. Some substances may be more costly to produce than others, bestowing them different values as honest indicators of male intrinsic quality. Composite signals also increase the amount of potential information available to an animal in one single signal. Although these features greatly complicate research, they may allow us to identify the different, sometimes conflicting, selection pressures affecting different components of one and the same signal.

In *P. triangulum* the 'bee-like' part of the pheromone most probably resulted from a sensory exploitation process. The three pheromonal substances that are not found on bees may have evolved to enhance the detectability of the signal, as species recognition cues, or to avoid cannibalism (chapter 7). Furthermore, genetic differences in the relative amounts of different compounds allow kin recognition based on pheromone composition (chapter 8). There is also evidence that the amounts of two pheromone compounds vary regularly with age, possibly indicating the age of a potential mate to the choosing female (E. Strohm, unpublished data). Thus, the different components of the complex pheromone of male European Beewolves might serve different functions.

With regard to the fundamental understanding of sexual communication systems one of the most significant advances has been the recent appreciation that the distinctions between the various proposed models are somehow artificial, as they deal with different perspectives rather than different biological principles (Ryan 1990, Endler and Basolo 1998, Eshel 2000, Kokko et al. 2002, 2003). It is hard to imagine sexual selection operating without receiver bias processes and *vice versa*. The Fisher-Zahavi process predicts that female choice generates directional selection on male traits that convey the quality of their holders; it does not, however, make predictions about the actual design of male signals. Receiver biases can determine the sensory modality and the specific design of courtship signals, e.g. the actual colour of a plumage, the frequency of a call, or the chemical composition of a pheromone, and set the initial direction of the Fisher process. Thus, receiver bias models aid in explaining between-species diversity in the design of animal signals, but do not make predictions about within-species variability of signals and their potential information content. The focus of the Fisher-Zahavi model is the intraspecific variance in signals. It encompasses both, signal design and content; as yet most studies have focussed on signal content only.

9.4 Conclusion

Collectively, the results of the present project strongly suggest that different natural and sexual selection pressures acting on males and females may interact and jointly contribute to the evolution of sexual communication systems. The majority of these different pressures can be explained by various sexual selection models. These models are not mutually exclusive, i.e. different mechanisms may operate concurrently, nor is the distinction between them

always so clear-cut (Ryan 1990, Eshel 2000, Kokko et al. 2002, 2003). Since all sensory systems have biases, all models for the evolution of sexual signals (direct benefits, indirect benefits) may initially require some degree of sensory exploitation (West-Eberhard 1984, Kirkpatrick and Ryan 1991, Arak and Enquist 1995, Christy 1995). It is evident, therefore, that studies on the evolution of male signals and female preferences should always incorporate investigations of both, historical processes as well as current effects.

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CHAPTER 10

OVERALL SUMMARY

Darwin's theory of sexual selection explains the evolution of flamboyant male traits through female choice. It does not, however, address the question why males typically court and females choose. This asymmetry is now thought to be the result of the dichotomy in reproductive expenditures: Females invest primarily in parental care and males invest predominantly in mate attraction or competition.

Based on this view, several hypotheses for the origin and maintenance of female preferences have been proposed. They include the classical sexual selection models, i.e. female choice for direct and indirect benefits as well as the more recent concepts of female choice for genetic compatibility and receiver bias models. The complementary choice scenario assumes that females choose mates with regard to genetic compatibility. The receiver bias concept views male traits and female preferences within the framework of communication theory and encompasses various more or less distinct models, two of which are sensory exploitation and sensory trap. Both models postulate that the 'design' of male signals evolved in response to pre-existing perceptual biases of females. The sensory trap hypothesis additionally emphasizes that pre-existing female preferences for certain cues evolved in non-sexual contexts, like e.g. predator avoidance or foraging. Males mimic these cues to elicit favourable out-of-context responses by females that increase their reproductive success.

Though mostly imperceptible to human observers, pheromone plumes of male insects are – like the very conspicuous visual or acoustic sexual signals of e.g. male birds and frogs – effective and often exaggerated male advertisement signals. This thesis examines the evolution of the pheromone communication in the European Beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae). Beewolf females are specialized hunters of honeybee workers, *Apis mellifera* (Hymenoptera, Apidae), and provision their progeny with paralyzed prey. Male beewolves establish and scent mark territories with a pheromone from a head gland to court females. The concordant occurrence of the otherwise rare long-chain alcohol (Z)-11-eicosen-1-ol in the male pheromone and in the alarm pheromone of honeybees, the exclusive prey of

the females, suggests a sensory trap process as an explanation for the evolution of the male pheromone in *P. triangulum*.

According to this hypothesis, we tested three predictions: First, foraging honeybees should smell of (Z)-11-eicosen-1-ol. Via chemical analysis we could show that honeybee workers, *A. mellifera*, in fact smell of (Z)-11-eicosen-1-ol during foraging. The occurrence of (Z)-11-eicosen-1-ol on the cuticle and in the headspace of honeybees is a new finding.

Second, beewolf females should use (Z)-11-eicosen-1-ol as a cue for prey detection or identification. Using behavioural assays, we demonstrated that prey recognition in *P. triangulum* females is accomplished by olfactory cues and that (Z)-11-eicosen-1-ol is an essential cue in this process. The sensory sensitivity of beewolf females to (Z)-11-eicosen-1-ol must be extremely high, since they perceive the trace amounts present in the head space of honeybees. This sensitivity may be due to specialized olfactory receptors on the antennae of beewolf females. An inventory of the flagellar sensilla of both sexes showed that females carry one type of sensillum that is missing in males, the large sensillum basiconicum. This presumably chemo-sensitive sensillum most likely plays a role in prey recognition.

The third prediction is that beewolf males incorporate bee-like substances, including (Z)-11-eicosen-1-ol, into their pheromone, and possibly catch females in a sensory trap. A reanalysis of the male pheromone revealed, among others, (Z)-11-eicosen-1-ol and several alkanes and alkenes as pheromonal compounds. Our own analyses of the chemical profiles of honeybee workers and the beewolf pheromone disclosed a surprisingly strong resemblance between the two. Eight of the eleven substances of the male pheromone are also present on the cuticle and in the headspace of honeybees. Notwithstanding this similarity, the male pheromone does not function as a sensory trap for conspecific females. Nevertheless, the extensive congruence between the odour bouquets of the females' prey and the male pheromone strongly suggests that the male signal evolved to exploit a pre-existing female sensory bias towards bee odour, and, thus represents a case of sensory exploitation.

In addition to the above described scenario concerning mostly the 'design' of the male pheromone, we addressed possible indirect benefits female beewolves may gain by basing their mating decisions on signal 'content'. We show that the pheromone of male *P. triangulum* varies between families and may, thus, contain information about the degree of

relatedness between the female and a potential mate. Females could use this information to choose genetically complementary males to avoid inbreeding and the production of infertile diploid sons.

Collectively, our results provide strong evidence for a receiver bias process in the evolution of the male pheromone of *P. triangulum*. They further indicate that the pheromone composition may subsequently have been influenced by other natural or sexual selection pressures, like e.g. complementary female choice. The current thesis is one of only a few studies taking a comprehensive approach to the origin and maintenance of male signals and female preferences and trying to integrate historical pathways as well as current effects into the broader picture of signal evolution by female choice.

CHAPTER 11

ALLGEMEINE ZUSAMMENFASSUNG

Darwins Theorie der Sexuellen Selektion deutet die Evolution auffälliger und übersteigerter Männchenmerkmale als Ergebnis der Weibchenwahl. Sie erklärt jedoch nicht, warum Männchen normalerweise um Weibchen werben und Weibchen unter den werbenden Männchen wählen. Man glaubt heute, daß dieser Unterschied auf eine Asymmetrie im reproduktiven Aufwand zwischen den Geschlechtern zurückzuführen ist: Während Weibchen überwiegend in elterliche Fürsorge investieren, investieren Männchen vor allem in Konkurrenzfähigkeit und Balzsignale.

Aufgrund dieser Erkenntnis wurden mehrere Hypothesen zur Evolution weiblicher Präferenzen bei der Partnerwahl vorgeschlagen. Hierzu gehören die klassischen Modelle der Sexuellen Selektion, wie Weibchenwahl aufgrund von direktem und indirektem Nutzen sowie die neueren Konzepte der Weibchenwahl aufgrund genetischer Kompatibilität und die 'Receiver Bias' Modelle (Empfänger Prädisposition). Das 'Receiver Bias' Konzept betrachtet Männchenmerkmale und Weibchenpräferenzen im Rahmen der Kommunikationstheorie and umfasst einige mehr oder weniger unterschiedliche Modelle wie z. B. 'Sensory Exploitation' (sensorische Ausbeutung) und 'Sensory Trap' (sensorische Falle). Beide Modelle postulieren, dass das ‚Design' von Männchensignalen in Anpassung an bereits existierende Präferenzen in der sensorischen Verarbeitung der Weibchen entsteht. Die 'Sensory Trap' Hypothese betont darüber hinaus, dass diese sensorischen Präferenzen der Weibchen für bestimmte Signale unabhängig von der Partnerfindung in einem der natürlichen Selektion unterliegenden Kontext entstanden, so z. B. zur Räubervermeidung oder zum Auffinden von Nahrung. Männchen imitieren diese Signale um vorteilhafte Reaktionen der Weibchen auszulösen und so ihren Reproduktionserfolg zu erhöhen.

Obwohl Pheromone von Insekten für uns Menschen meist nicht wahrnehmbar sind, sind sie, ähnlich wie die sehr auffälligen visuellen oder akustischen sexuellen Signale mancher Vögel oder Frösche, sehr effektive und oft übersteigerte Werbesignale. Die vorliegende Dissertation untersucht die Evolution der Pheromonkommunikation des Europäischen Bienenwolfs *Philanthus triangulum* (Hymenoptera: Crabronidae). Bienenwolfweibchen sind

hochspezialisierte Jägerinnen von Honigbienen, *Apis mellifera* (Hymenoptera, Apidae), und versorgen ihre Nachkommen mit gelähmten Beutetieren. Bienenwolfmännchen etablieren Reviere und markieren diese mit dem Pheromon aus einer Kopfdrüse, um Weibchen anzulocken. Das übereinstimmende Vorkommen des sonst sehr seltenen, langkettigen Alkohols (Z)-11-Eicosen-1-ol sowohl im Pheromon der Männchen als auch im Alarmpheromon der Honigbienen, der ausschließlichen Beute der Weibchen, deutete darauf hin, dass es sich bei dem Pheromon von *P. triangulum* Männchen um eine 'Sensory Trap' für Weibchen handeln könnte.

Entsprechend dieser Hypothese testeten wir drei Vorhersagen: Erstens, furagierende Honigbienen sollten nach (Z)-11-Eicosen-1-ol riechen. Mit Hilfe chemischer Analysen konnten wir zeigen, dass Honigbienenarbeiterinnen während des Sammelns tatsächlich nach (Z)-11-Eicosen-1-ol riechen. Das Vorkommen von (Z)-11-Eicosen-1-ol auf der Kutikula und im Luftraum um die Honigbiene war bisher nicht bekannt.

Zweitens sollten Bienenwolfweibchen (Z)-11-Eicosen-1-ol für die Auffindung und Identifikation ihrer Beute nutzen. Wie wir in Verhaltenstests zeigen konnten, verlassen sich Bienenwolfweibchen für die Beuteerkennung auf olfaktorische Signale, und (Z)-11-Eicosen-1-ol ist eine notwendige Duftkomponente für die Identifizierung der Honigbienen. Die sensorische Empfindlichkeit der Weibchen für (Z)-11-Eicosen-1-ol scheint extrem hoch zu sein, da sie diese, nur in Spuren im Luftraum um Honigbienen vorhandene Substanz, wahrnehmen können. Diese hohe sensorische Empfindlichkeit der Weibchen könnte durch spezialisierte olfaktorische Rezeptoren auf deren Antennen bedingt sein. Die Analyse der antennalen Sensillen beider Geschlechter zeigte, dass Bienenwolfweibchen einen Sensillentyp besitzen, der bei Männchen nicht vorkommt: die Großen Sensilla basiconica. Diese vermutlich chemosensitiven Sensillen könnten eine entscheidende Rolle bei der Beuteerkennung spielen.

Die dritte Vorhersage ist, dass Bienenwolfmännchen die für Honigbienen typischen Substanzen, darunter auch (Z)-11-Eicosen-1-ol, in ihr Pheromon integrierten, um Weibchen in einer 'Sensory Trap' zu fangen. Eine Neuanalyse des Männchenpheromons zeigte, u. a. (Z)-11-Eicosen-1-ol und einige Alkane und Alkene als Pheromonbestandteile. Unsere Analysen zeigten eine überraschend deutliche Übereinstimmung der chemischen Profile von Honigbienenarbeiterinnen und Bienenwolfmännchen. Acht der elf Substanzen des

Männchenpheromons finden sich auch im Duft sammelnder Honigbienen. Trotz dieser erstaunlichen Ähnlichkeit fungiert das Männchenpheromon nicht als 'Sensory Trap' für Weibchen. Die ausgeprägte Kongruenz der Düfte von Weibchenbeute und Männchenpheromon deutet dennoch darauf hin, dass bei der Evolution des Männchenpheromons eine bereits existierende sensorische Präferenz der Weibchen für Bienenduft ausgenutzt wurde, d.h. dass es sich dabei um einen Fall von 'Sensory Exploitation' handelt.

Das oben beschriebene Szenario für die Evolution des Männchenpheromons betrifft hauptsächlich das Design dieses Männchensignals. Darüber hinaus haben wir den möglichen Informationsgehalt des Pheromons untersucht. Wir konnten zeigen, dass das Pheromonmuster beim Europäischen Bienewolf mit der Familienzugehörigkeit variiert und das Pheromon somit Information über den Verwandtschaftsgrad zwischen einem Weibchen und einem potentiellen Paarungspartner enthalten könnte. Weibchen könnten diese Information nutzen, um sich nur mit genetisch kompatiblen Männchen zu paaren und auf diese Weise Inzucht und die Produktion infertiler, diploider Söhne zu vermeiden.

Zusammengenommen liefern die vorliegenden Ergebnisse starke Evidenzen für einen 'Receiver Bias' Prozess bei der Evolution des Männchenpheromons von *P. triangulum*. Sie deuten außerdem darauf hin, dass die Zusammensetzung des Pheromons in der Folge durch weitere Selektionsdrücke, wie z.B. Weibchenwahl für genetische Kompatibilität, beeinflusst wurde. Damit ist die vorliegende Dissertation eine von nur wenigen Studien, die in einem umfassenden Ansatz sowohl historische Prozesse als auch gegenwärtige Effekte in die Analyse der Signalevolution durch Weibchenwahl integriert.

CURRICULUM VITAE

Gudrun Herzner
Diplom-Biologin
geboren am 22.09.1973
in Nürnberg

Hochschulausbildung

Seit Sept. 2000	Promotionsstudium Mit Schwerpunkt Chemische Ökologie, Verhaltensökologie und Evolutionsbiologie an der Universität Würzburg
Okt. 94 – Aug. 2000	Studium der Biologie an der Universität Würzburg Studienschwerpunkte im Hauptstudium: Tierökologie, Tierphysiologie, Biochemie und Humangenetik
1996	Vordiplom
2000	Diplom Diplomarbeit: „Variables Elterliches Investment und Phänotypische Plastizität des Dungkäfers *Onthophagus taurus* Schreber, 1759 (Coleoptera: Scarabaeidae)“
Okt. 93 – Juli 94	Studium der Geologie und Paläontologie an der Universität Erlangen/Nürnberg

Schulbildung

1993	Allgemeine Hochschulreife
1984-1993	Gymnasium: Maria-Ward-Schule in Nürnberg

Auslandsaufenthalte

Apr. 97 – Sept. 97	Forschungsprojekt in Venezuela Aufenthalt an einer Forschungsstation und Mitarbeit in einem international besetzten Team des Center for Tropical Conservation, USA, und der Universidad Simón Bolívar, Caracas
März 98 – Mai 98	Forschungspraktikum in der Elfenbeinküste, Westafrika
Aug. 98 – Aug. 99	Integriertes Auslandsstudium an der Duke University, North Carolina, USA Stipendium des Deutschen Akademischen Austauschdienstes (DAAD) und der Duke University Graduate School Eigenes Forschungsprojekt zur Anfertigung der Diplomarbeit

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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 22. September 2004