Protective bacteria and attractive pheromones

Symbiosis and chemical communication in beewolves (*Philanthus spp.*, Hymenoptera, Crabronidae)

Dissertation zur Erlangung

des naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

Martin Kaltenpoth

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Eingereicht am: …………………………………………………………………….….………..…

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Tag des Promotionskolloquiums: ………………………………………………..…………..…..

Doktorurkunde augehändigt am: ……………………………………………..……………………

“We are symbionts on a symbiotic planet, and if we care to, we can find symbiosis everywhere.”

Lynn Margulis, “Symbiotic Planet” (1998)

“There is a theory which states that if ever anyone discovers exactly what the universe is for and why it is here, it will instantly disappear and be replaced by something even more bizarre and inexplicable. There is another theory which states that this has already happened.”

Douglas Adams, “The Restaurant at the End of the Universe” (1980)

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List of Publications

This thesis is based on the following manuscripts:

Kaltenpoth, M., Goettler, W., Herzner, G. & Strohm, E. 2005. Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479 (chapter 2).

Kaltenpoth, M. 2005. Bakterien schützen Wespen-Nachwuchs vor Pilzbefall. *Naturwissenschaftliche Rundschau* 58: 329-330 (chapter 3).

Goettler, W., Kaltenpoth, M., Herzner, G. & Strohm, E. (submitted) Morphology and ultrastructure of a bacteria cultivation organ: The antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure and Development* (chapter 4).

Kaltenpoth, M., Goettler, W., Dale, C., Stubblefield, J.W., Herzner, G., Roeser-Mueller, K. & Strohm, E. 2006. *'Candidatus* Streptomyces philanthi', an endosymbiotic streptomycete in the antennae of *Philanthus* digger wasps. *International Journal of Systematic and Evolutionary Microbiology* 56 (6): 1403-1411 (chapter 5).

Kaltenpoth, M., Schmitt, T. & Strohm, E. (in preparation) Chemical composition of the antennal gland secretion of female European beewolves, *Philanthus triangulum* (chapter 6).

Strohm, E., Herzner, G., Kaltenpoth, M., Boland, W., Schreier, P., Peschke, K. & Schmitt, T. (in preparation) The chemistry of the postpharyngeal gland of female European beewolves (Hymenoptera, Sphecidae) supports a homology with this gland in ants (chapter 7).

Kaltenpoth, M. & Strohm, E. (in press) The scent of senescence: age-dependent changes in the composition of the marking pheromone of male European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae). *Journal of Insect Science* (chapter 8).

Kaltenpoth, M., Kroiss, J. & Strohm, E. (submitted) Geographical variation in the marking-pheromone of male European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae). *Journal of Animal Ecology* (chapter 9).

Kaltenpoth, M., Strohm, E. & Gadau, J. 2004. Polymorphic microsatellite markers for a solitary digger wasp, the European beewolf (*Philanthus triangulum*; Hymenoptera, Sphecidae). *Molecular Ecology Notes* 4: 589-592 (chapter 10).

CHAPTER 1

GENERAL INTRODUCTION

Interactions between organisms of different species and the communication among conspecific individuals constitute central themes in both behavioral and evolutionary ecology. The present thesis addresses two facets of these research areas in a hymenopteran model organism, the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae): first, the specialized association with endosymbiotic bacteria and, second, the intersexual chemical communication and its potential for female choice. The following paragraphs provide a short review of the current literature on symbiosis as well as chemical communication and mate choice and give an overview of the biology of the European beewolf.

1.1 SYMBIOSIS

Symbioses are enormously important components of the natural world and have probably shaped the evolution of every living organism (Margulis 1998). The ecological significance of symbiosis is immediately evident from the enormous variety of symbiotic interactions among animals, plants, fungi, eubacteria and archaea. Most modern terrestrial ecosystems are critically dependent on symbiosis: About 90 percent of land plants are mycorrhizal, and virtually all mammalian and insect herbivores would starve without their cellulose-digesting symbionts (Margulis & Fester 1991). In marine environments, symbiotic interactions likewise play an important role for many eukaryotic and prokaryotic organisms and have facilitated the colonization of inhospitable environments (Buchner 1921; McFall-Ngai 1991; Saffo 1991; Hentschel *et al.* 2003).

The evolutionary importance of symbiosis is especially apparent in the origin of eukaryotic cell organelles. After the first proposition of the 'serial endosymbiosis theory' for the evolution of eukaryotic cell organelles from fusion events of several different bacterial cells (Sagan 1967; Margulis 1970), it has taken several decades of fierce dispute and rejection before the theory was widely accepted among biologists (Margulis 1998). Today, there remains little doubt that the eukaryotic cell constitutes a symbiotic union of several primitive prokaryotic ancestors (Margulis 1970, 1981, 1992, 1998; Gray *et al.* 1999; Dyall *et al.* 2004). Even after the evolution of eukaryotic cells, bacterial symbionts have apparently played a major role for numerous radiation events in plants and animals (Price 1991), and symbiosis has been suggested as a

major factor driving speciation and evolutionary innovation (Maynard-Smith 1989; Margulis & Fester 1991; Sapp 1994; Bordenstein 2003; Leonardo & Mondor 2006).

Originally, the term 'symbiosis' has been defined as the living together of different organisms over significant parts of their life span (De Bary 1879). This definition includes mutually beneficial relationships as well as commensalism and parasitism (Douglas 1994). Since the outcomes of symbiotic interactions for the different partners are often difficult to assess and constitute a continuum, a clear-cut distinction between mutualism, commensalism and parasitism is often impossible (Dettner 1999). In this thesis, as in most current biological literature, symbiosis is used in a stricter sense encompassing only mutualistic interactions between different organisms (Margulis & Fester 1991; Douglas 1994).

1.1.1 Insect-bacteria symbiosis

Insects probably constitute the most diverse and abundant animal class on earth (May 1988), and they are associated with an amazing variety of symbiotic microorganisms (Buchner 1921). In some cases, symbioses seem to be facultative and of relatively recent evolutionary origin, whereas other associations exist for several hundred million years and are characterized by a high degree of mutual interdependence (Moran *et al.* 1993; Bandi *et al.* 1995; Ishikawa 2003). Regarding the localization of the symbionts, two major types are distinguished: ektosymbionts live outside of the host or on its outer surface, whereas endosymbionts reside within the insect's body cavity, digestive tract or specialized organs (Dettner 1999). Endosymbiotic organisms can live either extracellularly, e.g. in the gut lumen or in specialized gland reservoirs, or intracellularly, often in specialized cell types that are called mycetocytes or bacteriocytes (Ishikawa 2003).

Symbiotic microorganisms can provide a wide range of benefits to their insect hosts (e.g. Margulis & Fester 1991; Douglas 1994; Dettner 1999; Moran & Baumann 2000; Bourtzis & Miller 2003; Douglas 2003b). The most common and best-known symbioses are interactions for the exchange of essential nutrients between host and symbionts (e.g. Breznak *et al.* 1973; Breznak 1984; Margulis & Fester 1991; Sasaki & Ishikawa 1995; Schafer *et al.* 1996; Douglas 1998; Moran & Baumann 2000; Douglas 2003b; Dillon & Dillon 2004; Zientz *et al.* 2004; Douglas 2006). In other cases, microorganisms provide compounds that are used by the host as pheromone components (Dillon *et al.* 2000, 2002), affect hydrocarbon profiles that are important for nestmate recognition (Matsuura 2003), or defend the host or the host's nutritional basis against pathogens (Currie *et al.* 1999b; Hu & Webster 2000; Imamura *et al.* 2001; Gebhardt *et al.* 2002a; Gebhardt *et al.* 2002b; Currie *et al.* 2003a; Piel 2004; Dillon *et al.* 2005) or parasitoids (Oliver *et al.* 2003; Moran *et al.* 2005d). At the boundary between symbiosis and

parasitism, a special group of microorganisms associated with insects are bacteria causing reproductive alterations in the host, the most famous and widespread member of which is *Wolbachia* (Oneill *et al.* 1992; Rousset *et al.* 1992; Hurst 1997; Stouthamer *et al.* 1999; Jiggins *et al.* 2001; Lawson *et al.* 2001; Zchori-Fein *et al.* 2001; Zchori-Fein & Perlman 2004; Provencher *et al.* 2005). In the following, nutritional and defensive interactions between insects and bacteria will be discussed in more detail.

1.1.2 Nutritional interactions

One important factor contributing to the evolutionary success of insects is their ability to exploit a wide variety of food sources. Symbiotic microorganisms have apparently played an essential role for the evolution of such diverse feeding habits (Ishikawa 2003). Bacterial endosymbionts are especially common in specialist feeders whose diet is often deficient in essential nutrients (Buchner 1921; Douglas 1994; Dettner 1999; Ishikawa 2003). This is particularly true for phloem-sucking, wood-feeding, and blood-sucking insects, and many of these have been found to be engaged in highly derived and obligate symbiotic interactions with bacteria (e.g. Harington 1960; Hill *et al.* 1976; Nogge 1981; Sasaki *et al.* 1991; Sasaki & Ishikawa 1995; Schafer *et al.* 1996; Lilburn *et al.* 2001; Akman *et al.* 2002; Aksoy 2003; Zientz *et al.* 2004). However, many cockroaches, ants and several other generalist feeders have also been discovered to harbor symbiotic microorganisms that play important roles in the nutrition of the host (Buchner 1921; Dillon & Dillon 2004; Zientz *et al.* 2004).

Aphids and other phloem-feeding insects are associated with a variety of different endosymbiotic bacteria. The aphids' primary endosymbionts of the genus *Buchnera* belong to the γ–subdivision of the proteobacteriaceae and are located intracellularly in specialized bacteriocytes (McLean & Houk 1973; Douglas & Dixon 1987; Munson *et al.* 1991a). The bacteria-containing cells aggregate to form a bilobed structure within the body cavity called bacteriome or mycetome (Buchner 1921; McLean & Houk 1973). Extensive studies on the physiology of aphids and their primary symbionts have demonstrated that *Buchnera* provides its hosts with essential amino acids that the aphids can neither synthesize themselves nor obtain in sufficient quantities from their diet (Sasaki *et al.* 1991; Sasaki *et al.* 1993; Sasaki & Ishikawa 1993, 1995; Douglas 1998; Zientz *et al.* 2004; Douglas 2006).

The long evolution as intracellular symbionts of aphids has resulted not only in the obligate dependence of aphids on their primary endosymbionts, but also of the endosymbionts on their aphid hosts (Shigenobu *et al.* 2000; Tamas *et al.* 2002; van Ham *et al.* 2003). In *Buchnera* and other obligate endosymbionts, this is reflected in significant changes in genome size and structure (Shigenobu *et al.* 2000; Akman *et al.* 2001; Akman *et al.* 2002; Gil *et al.* 2002; Tamas

et al. 2002; Gil *et al.* 2003; Rio *et al.* 2003; van Ham *et al.* 2003; Moran & Plague 2004; Degnan *et al.* 2005). Many regulatory genes and genes involved in DNA repair and recombination or coding for non-essential amino acids and cell-surface components have been lost in *Buchnera*, thus rendering life outside the host cells impossible (Shigenobu *et al.* 2000; Tamas *et al.* 2002; Tamas & Andersson 2003; van Ham *et al.* 2003; Wilcox *et al.* 2003; Moran *et al.* 2005a; Moran & Degnan 2006). Thus, the aphid-*Buchnera* symbiosis is a closed system, in which the bacteria are obligately dependent on their host cell and therefore have to be transmitted vertically from mother to offspring via the eggs (Buchner 1921; Moran *et al.* 1993; Douglas 2003a). Over evolutionary timescales, strictly vertical transmission results in coevolution and cospeciation, which is reflected in congruent phylogenies of hosts and symbionts (Moran & Baumann 1994). Phylogenetic congruence has been demonstrated for aphids and their primary endosymbionts (Munson *et al.* 1991b; Moran *et al.* 1993; Moran & Baumann 1994; Moran *et al.* 1994; Clark *et al.* 2000; Martinez-Torres *et al.* 2001) as well as for other obligate insect-bacteria symbioses (Aksoy *et al.* 1997; Sauer *et al.* 2000; Clark *et al.* 2001; Moran *et al.* 2003; Degnan *et al.* 2004; Thao & Baumann 2004; Baumann & Baumann 2005; Moran *et al.* 2005b).

In addition to the primary endosymbionts, aphids harbor a variety of accessory bacteria or 'secondary symbionts' from different bacterial groups (Baumann & Moran 1997; Fukatsu *et al.* 1998; Douglas 2003a; Haynes *et al.* 2003). The prevalence and infection rate of these accessory bacteria varies among aphid species (Sandstrom *et al.* 2001; Tsuchida *et al.* 2002; Haynes *et al.* 2003). Although vertical transmission has been demonstrated for several secondary symbionts (e.g. Fukatsu *et al.* 2000; Darby *et al.* 2001; Fukatsu *et al.* 2001; Tsuchida *et al.* 2005), horizontal transfer events appear to be common in the field (Sandstrom *et al.* 2001; Russell *et al.* 2003; Russell & Moran 2005; Tsuchida *et al.* 2005) and can also be demonstrated experimentally by the injection of bacteria from a 'donor' to a symbiont-free (aposymbiotic) 'recipient' (Chen *et al.* 2000; Fukatsu *et al.* 2001). In contrast to their requirement for *Buchnera*, aphids do not seem to be dependent on the presence of these accessory bacteria (Douglas 2003a). However, it has been shown that host plant specialization, temperature range, parasitoid and fungal resistance can be affected positively or negatively by the presence of secondary symbionts (Chen *et al.* 2000; Oliver *et al.* 2003; Ferrari *et al.* 2004; Tsuchida *et al.* 2004).

A well-studied insect-bacteria symbiosis involving generalist feeders is the *Camponotus*-*Blochmannia* association that shares many characteristics with the aphid-*Buchnera* symbiosis. Harvester ants of the genus *Camponotus* (Hymenoptera, Formicidae) harbor gram-negative bacteria in specialized bacteriocytes of the midgut epithelium (Blochmann 1892; Buchner 1921; Schroder *et al.* 1996). The bacteria belong to the γ-subclass of Proteobacteria (Schroder *et al.* 1996; Sauer *et al.* 2000; Degnan *et al.* 2004), and they were recently named '*Candidatus* Blochmannia' in honour of their discoverer (Sauer *et al.* 2000). The bacteria are apparently

transmitted vertically from the queen's ovaries to the eggs (Blochmann 1892; Buchner 1921; Schroder *et al.* 1996; Sauer *et al.* 2002). Phylogenetic analyses of hosts and symbionts revealed concordant phylogenies with minimal conflict between hosts and symbionts, suggesting an evolutionarily stable symbiosis without horizontal transfer of the symbionts (Schroder *et al.* 1996; Sauer *et al.* 2000; Degnan *et al.* 2004).

Like the aphid-*Buchnera* symbiosis, the association between *Camponotus* and *Blochmannia* seems to be obligate for both partners (Zientz *et al.* 2004). Although aposymbiotic adult ants are apparently able to survive for several months without any obvious negative effects (Sauer *et al.* 2002), the endosymbionts seem to be important in earlier phases of the host's life-cycle (Wolschin *et al.* 2004). Especially during pupation, the bacteria have been suggested to play a role in supplying the ants with aromatic amino acids that are needed in large amounts for sclerotization of the cuticle (Wolschin *et al.* 2004). Additionally, genome analyses revealed that *Blochmannia* might be involved in nitrogen, sulphur and lipid metabolism of the host (Gil *et al.* 2003; Zientz *et al.* 2004; Zientz *et al.* 2005). The bacteria, on the other hand, are obligately dependent on their host due to the reduction of genes coding for enzymes involved in basic metabolic and regulatory pathways (Gil *et al.* 2003; Zientz *et al.* 2004; Degnan *et al.* 2005; Zientz *et al.* 2005). Surprisingly, the bacterial replication seems to be completely under the control of the host organism, since *Blochmannia* apparently lacks the gene for the replication initiation protein DnaA (Gil *et al.* 2003). Thus, in both the aphid-*Buchnera* and the *Camponotus-Blochmannia* symbiosis, a long coevolutionary history has led to a high degree of specialization and mutual interdependence of hosts and symbionts.

1.1.3 Defensive symbioses

In addition to playing an important role in the nutrition of many insects, bacterial symbionts are sometimes involved in the defense of the host against pathogen attack. In several insect taxa, symbiotic gut bacteria have been shown to provide colonization resistance against invading pathogens (Charnley *et al.* 1985; Takatsuka & Kunimi 2000; Dillon *et al.* 2005), either by producing antimicrobial secondary metabolites (Dillon & Charnley 1995), or by exploiting the limiting nutrient resources more efficiently and thereby outcompeting potential invading pathogens (Godfray *et al.* 1999; Dillon & Dillon 2004). In some cases, antimicrobial metabolites have been identified from bacteria isolated from insect guts (Jigami *et al.* 1986; Gebhardt *et al.* 2002a; Gebhardt *et al.* 2002b), but these studies neither tried to locate the bacteria within the insect tissue nor demonstrated their symbiotic nature. In *Paederus* beetles, however, extensive studies provided strong evidence that symbiotic gamma-Proteobacteria of the genus *Pseudomonas* are involved in the production of the defense toxin pederin that the

beetles use for protection against predators (Kellner 1999, 2001a, 2001b, 2002b, 2002a; Piel 2002, 2004; Piel *et al.* 2005).

Symbiotic bacteria cannot only confer protection against pathogenic microorganisms and predators but in some cases also improve the resistance of the host against eukaryotic parasitoids. This fascinating phenomenon has been found in aphids and their secondary endosymbionts (Oliver *et al.* 2003; Ferrari *et al.* 2004). In a controlled genetic background, bacteria of the enterobacterial candidate species *Hamiltonella defensa* (Moran *et al.* 2005c) have been demonstrated to cause a high mortality of parasitoid wasp larvae within developing aphids, thereby allowing the host to develop to the adult stage and reproduce (Oliver *et al.* 2003). Recently, a third player in this symbiosis has been identified: a bacteriophage that appears to be an obligate component of the life cycle of *H. defensa* (Moran *et al.* 2005d). This phage contains intact homologs of a gene encoding a toxin that is known from several mammalian pathogens to interrupt the eukaryotic cell cycle (for review see Yamasaki *et al.* 2006). Thus, the phage may be responsible for the resistance of *H. defensa*-infected aphid host against parasitoid attack (Moran *et al.* 2005d).

Leaf-cutter ants (Hymenoptera, Formicidae, Attini) engage in a highly specialized defensive symbiosis with bacteria. These ants are well-known for the cultivation of fungus gardens that are used for the nutrition of the larvae and the adult ants, which evolved about 50 million years ago (Mueller *et al.* 1998; Mueller *et al.* 2001; Mueller *et al.* 2005). Phylogenetic studies revealed that the ants and their usually vertically transmitted fungal cultivars show a congruent branching pattern at the deeper phylogenetic splits (Chapela *et al.* 1994; Hinkle *et al.* 1994; Mueller *et al.* 1998; Currie *et al.* 2003c), while experiencing extensive lateral transfer of cultivars at more recent phylogenetic levels (Chapela *et al.* 1994; Mueller *et al.* 1998; Bot *et al.* 2001; Green *et al.* 2002; Currie *et al.* 2003c). The ants' fungus-gardens can be infested by parasitic fungi of the genus *Escovopsis* (Ascomycota) that show a similar coevolutionary history with the fungal cultivars (Currie *et al.* 2003c). *Escovopsis* is highly specialized on the fungal cultivars (Gerardo *et al.* 2004) and can seriously reduce the fitness of attine ant colonies (Currie *et al.* 1999a; Currie 2001a; Reynolds & Currie 2004). As a defense against this parasitic fungus, the ants engage in a symbiosis with actinomycete bacteria of the genus *Pseudonocardia* (Currie *et al.* 1999b, 2003b; Cafaro & Currie 2005).

The attine ants' symbiotic bacteria are cultivated in elaborate cuticular crypts that have been highly modified during the ants' evolutionary history (Currie *et al.* 1999b; Currie *et al.* 2006). The symbionts apparently occur in all fungus-growing ants, suggesting an ancient infection with *Pseudonocardia* (Currie *et al.* 2006). The bacteria seem to be vertically transmitted via the queens (Currie *et al.* 1999b), although horizontal transfer occasionally occurs (Poulsen *et al.* 2005). Bioassays demonstrated that the bacteria produce antibiotic substances that specifically

inhibit growth of the parasitic fungus *Escovopsis*, thereby providing protection to the ants' fungal cultivars (Currie *et al.* 1999b; Currie *et al.* 2003a). The bacteria apparently benefit from the association by obtaining an unoccupied and lasting ecological niche and an ensured transmission route (Currie 2001b). Furthermore, two pieces of evidence suggest that they are additionally supplied with nutrients by the host: First, the cuticular crypts are supported by exocrine glands that enable nutrient transfer from the ant to the bacteria (Currie *et al.* 2006), and second, morphological and physiological studies demonstrate that the bacteria impose metabolic costs on the ants (Poulsen *et al.* 2002; Poulsen *et al.* 2003).

1.2 CHEMICAL COMMUNICATION AND MATE CHOICE

1.2.1 Sexual selection and mate choice

When Darwin (1859) first proposed the theory of evolution by means of natural selection, one of the greatest problems he faced concerned the evolution of conspicuous male traits, such as song and other display, bright colors, and horns and other weapons. These traits would seem to reduce rather than enhance survival and should therefore be eliminated by natural selection (Andersson 1994). Darwin (1859; 1871) solved this paradox by proposing sexual selection as an additional selective force complementing the effect of natural selection: "Sexual selection […] depends, not on a struggle for existence, but on a struggle between the males for possession of the females; the result is not death to the unsuccessful competitor, but few or no offspring" (Darwin 1859, p.88).

Sexual selection is the result of the asymmetry between the sexes in the amount of resources that are directly invested in the offspring: Females produce few large eggs, and they usually invest much time and resources in brood care, whereas males produce many small sperm cells and often do not contribute any resources to rear the offspring (Trivers 1972). This asymmetry generally leads to competition among males for females, and males are expected to maximize their fitness predominantly by attracting and mating with as many females as possible (Bateman 1948; Trivers 1972; Andersson 1994). Females, on the other hand, are usually limited by resources for offspring production and brood care, so they should be choosy and mate with the best available male (Trivers 1972; Gould & Gould 1997).

To attract receptive females, males evolved a variety of advertisement signals that often provide a set of information on species affiliation and mate quality and thus enable females to choose adaptively among potential mates (Droney & Hock 1998; Jones & Hamilton 1998; Lopez *et al.* 2003; O'Loghlen & Rothstein 2003; Slater 2003). By choosing a high-quality male, females can

either benefit directly, if males vary in the ability to provide essential resources to the females (Halliday 1983; Vahed 1998), or indirectly, if offspring quality depends on the genetic background of the male. Several models have been proposed to explain female choice based on indirect benefits, the most prominent of these being the "good genes" model that predicts the occurrence of certain males with good genes that are the best choice for all females (Andersson 1994; Johnstone 1995; Wilkinson *et al.* 1998; Moller & Alatalo 1999; Tomkins & Simmons 1999; Hine *et al.* 2002; Kokko *et al.* 2002), and the model of the "best compatibility/complementarity" assuming that one particular male is the best choice for a particular female (Halliday 1983; Johnsen *et al.* 2000; Tregenza & Wedell 2000; Colegrave *et al.* 2002; Reinhold 2002).

The genetic compatibility of a mate depends – among other factors – on the degree of relatedness which ranges from strict inbreeding to maximal outbreeding. Both in- and outbreeding have certain advantages (Partridge 1983) and disadvantages for reproduction (Bateson 1983; Pusey & Wolf 1996). According to the model of optimal outbreeding, females should choose a mate of a certain genetic difference to balance the costs of in- and outbreeding (Bischof 1972; Alexander 1977; Bateson 1983), avoiding both inbreeding depression and the break-up of local adaptations.

In hymenoptera, deleterious mutations, which have severe consequences in inbred diploid organisms, usually disappear quickly due to the haploidy of males (Goldstein 1994; Smith 2000; Henter 2003). Nevertheless, inbreeding may have especially high costs in most hymenoptera owing to the predominant mechanism of sex-determination, the single-locus complementary sex-determination (sl-CSD) (Cook 1993; Haig 1998; Beye *et al.* 2003). Normally, unfertilized (haploid) hymenopteran eggs develop into males, whereas fertilized (diploid) eggs develop into females. However, diploid animals that are homozygous at the sex-determination locus develop into diploid males, which are usually sterile (Cook 1993; Owen & Packer 1994; Cook & Crozier 1995). Since inbreeding increases the proportion of homozygosity and therefore the occurrence of diploid males, matings between close kin should be strongly selected against in hymenoptera with sl-CSD. Thus, inbreeding avoidance should be an especially important factor in the context of female choice in hymenoptera.

1.2.2 Pheromones and mate choice

In species with female choice, indicator mechanisms must be present that allow the assessment of a potential mate's quality. To avoid cheating, these signals have to be honest, which is usually the case if they inflict costs on the males, because this leads to a correlation between signal production and mate quality (Zahavi 1975). Many studies have demonstrated adaptive

mate choice based on visual or acoustical displays (e.g. Ryan 1983; Burkhardt & Delamotte 1988; Andersson 1994; Moller & Alatalo 1999; Klappert & Reinhold 2003; Tallamy *et al.* 2003). However, olfactory signals as potential indicators for mate quality have as yet received comparatively little attention (Sappington & Taylor 1990c, 1990a, 1990b; Eisner & Meinwald 1995; Moore 1997; Van Dongen *et al.* 1998; Hine *et al.* 2002). This is especially surprising because chemical signals have the potential for carrying a large amount of information for mate choice, due to the quantitative and qualitative variability in multicomponent semiochemicals (Hölldobler 1995; Ayasse *et al.* 2001) and the extreme sensitivity of olfactory systems (Kaissling 1971; Angioy *et al.* 2003).

Many insects heavily rely on pheromones for inter- and intraspecific communication. Although insect sex pheromones are predominantly produced by females (Alexander *et al.* 1997), male sex pheromones occur in a number of taxa (Shelly & Whittier 1997). Several studies have demonstrated that male pheromones can communicate mate qualities to females (Sappington & Taylor 1990c, 1990a, 1990b; Thornhill 1992; Eisner & Meinwald 1995; Moore 1997; Droney & Hock 1998), and there is some evidence for adaptive female choice on the basis of male sex pheromones (Jones & Hamilton 1998; Jones *et al.* 1998; Jones *et al.* 2000; Hine *et al.* 2002). However, studies addressing both "good genes" effects and genetic compatibility in a species with olfactory communication are lacking.

1.3 BIOLOGY OF *PHILANTHUS TRIANGULUM* (HYMENOPTERA, CRABRONIDAE)

1.3.1 Systematic position and distribution of the genus *Philanthus*

The genus *Philanthus* (Hymenoptera, Crabronidae) comprises about 135 species that are mainly distributed over the Ethiopian, Palearctic and Nearctic regions, with a few species being Oriental or Neotropical (Bohart & Menke 1976). The Neotropical species occur in Cuba and Central America. South America and Australia are not inhabited by any *Philanthus* species (Bohart & Menke 1976). The genus *Philanthus* is paraphyletic with respect to the most closely related genus, *Trachypus* (Alexander 1992), which is distributed in Central and South America (Bohart & Menke 1976). Together with the genus *Philanthinus*, these two genera represent the tribe Philanthini, which is the sister group to a clade comprising the Cercerini and the Aphilantopsini; together, these three tribes constitute the subfamily Philanthinae (Alexander 1992).

The European beewolf, *Philanthus triangulum*, has a wide distribution, ranging from Skandinavia in the north to South Africa in the south, reaching out to the Near and Middle East

(Arnold 1925; Erlandsson 1962; Bohart & Menke 1976; Hansen 1997; Blösch 2000; Ebrahimi 2005). It prefers warm and sandy areas and represents a pioneer species that frequently colonizes new habitats under anthropogenic influence, a strategy that has also been described for many other digger wasps (Evans 1974). Although usually sparsely distributed, female nest aggregations can sometimes comprise several hundred nests (Tinbergen 1932; Simon-Thomas & Simon-Thomas 1980). In central Europe, beewolves are active from June to September in one or two generations, depending on the weather conditions (Strohm 1995; Blösch 2000).

1.3.2 Behavior of female European beewolves

Female European beewolves construct nest burrows in sandy soil, hunt honeybees (*Apis mellifera*), paralyze them by stinging and carry the prey to the nest in flight (Fig. 1.1) (Strohm 1995). The hunting behavior seems to be guided by a combination of visual and olfactory cues (Tinbergen 1932; Herzner 2004; Herzner *et al.* 2005). After discovering a honeybee worker visiting a flower, the beewolf female hovers at a distance of approximately 10 cm usually at the downwind side of the prey. This hovering flight seems to be an important step in the attack sequence, during which a female decides to attack or to ignore a potential prey (Herzner *et al.* 2005). Recent studies have shown that the final attack is then elicited by olfactory cues from the honeybee (Herzner 2004; Herzner *et al.* 2005). In this context, an essential component of the honeybee odor is (*Z*)-11-eicosen-1-ol, a long-chain unsaturated alcohol (Herzner *et al.* 2005). Interestingly, this compound also constitutes the main component of the male beewolves' marking pheromone (Schmidt *et al.* 1990; Schmitt *et al.* 2003). This supports the hypothesis that males use (*Z*)-11-eicosen-1-ol to exploit a high pre-existing sensory sensitivity of the females that evolved in another context to attract females according to the sensory exploitation model (Herzner 2004; Herzner *et al.* 2005).

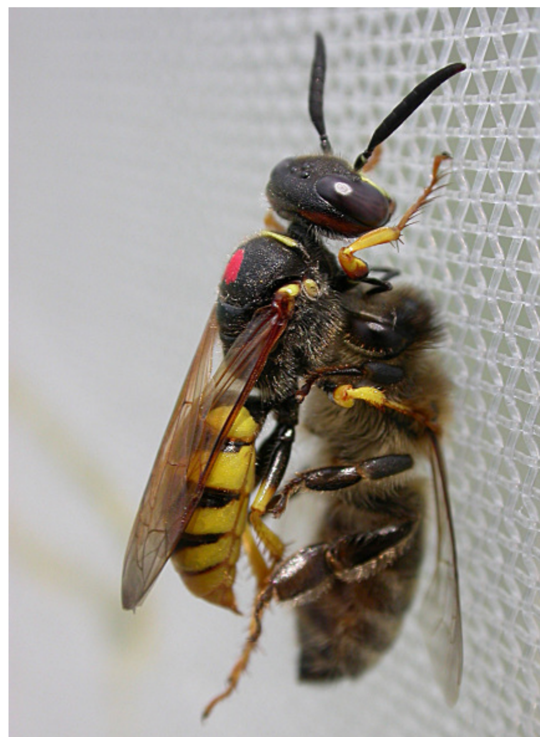

Figure 1.1: Female European beewolf with paralyzed honeybee prey.

The paralyzed honeybees serve as larval provisions in the self-excavated underground nest burrow (Tinbergen 1932; Simon-Thomas & Veenendaal 1978; Strohm 1995). The nest consists of a main burrow with several horizontal side burrows, each of which ends in one terminal

brood cell (Simon-Thomas & Veenendaal 1978; Strohm 1995). One to five honeybees are provisioned as larval food within each brood cell (Fig. 1.2a). After oviposition, the female fills the side burrows connecting the brood chamber and the main burrow with sand, presumably as a protection against parasitoids (e.g. chrysidid wasps and sarcophagid flies) (Evans & O'Neill 1988). The larva feeds on the prey and spins a cocoon that is attached with its basal part to the distal wall of the brood cell (Fig. 1.2b,c) (Olberg 1953; Strohm 1995; Strohm & Linsenmair 1995). Larvae mostly overwinter and emerge in the next summer (Fig. 1.2d).

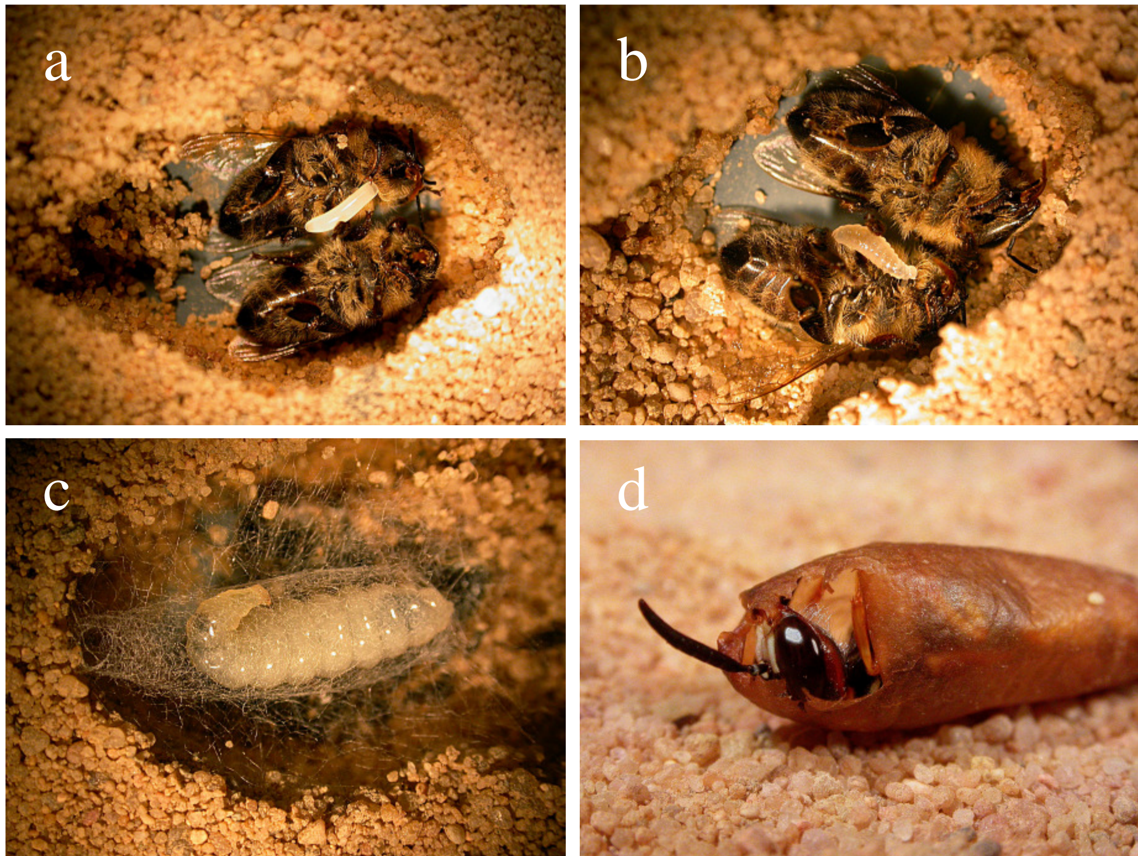

Figure 1.2: Development of a European beewolf within the brood cell. **(a)** Egg on paralyzed honeybees. **(b)** Young larva feeding on the paralyzed prey. **(c)** Larva spinning its cocoon. **(d)** Adult female emerging from the cocoon.

Eclosed adult beewolves face the problem of finding their way out of the nest, since the side burrows are filled with sand (Simon-Thomas & Veenendaal 1978; Evans & O'Neill 1988; Strohm 1995; Strohm & Linsenmair 1995). Beewolves solve this problem effectively: After oviposition, the beewolf female applies a white secretion from antennal glands to the distal end of the brood cell (Strohm 1995; Strohm & Linsenmair 1995). During cocoon-spinning, the larva locates this secretion and attaches the cocoon to the distal end of the brood cell, where the white substance had been secreted (Strohm & Linsenmair 1995). Thus, the cocoon points directly towards the main burrow, and since newly exclosed beewolves start to dig forward without

changing the direction of their bodies, they eventually find the main burrow and can easily leave the nest (Strohm & Linsenmair 1995).

Since the conditions in the brood cells are humid and warm, there is a continuous threat that pathogenic microorganisms infest the larva or the provisioned honeybees, which usually results in the death of the larva (Fig. 1.3) (Strohm 2000; Strohm & Linsenmair 2001). To reduce fungal infestation of the provisioned honeybees, female beewolves lick the prey extensively, which has been shown to significantly delay fungus growth or suppress it entirely (Strohm & Linsenmair 2001). It has been suggested that the females thereby apply an anti-fungal secretion to the cuticle of their prey, but the chemical nature of the substances involved has not been reported yet (Strohm & Linsenmair 2001). Virtually nothing is known about how the larva itself is protected against pathogenic microorganisms, especially during the long and possibly very dangerous phase of overwintering in the cocoon.

Figure 1.3: Female European beewolf heavily infested and killed by fungi in the cocoon.

1.3.3 Behavior of male European beewolves

Male European beewolves establish territories (about 0.25 m^2 in size), mostly in the vicinity of the females' nest aggregations, that do not contain any resources for females (Simon-Thomas & Poorter 1972; Strohm 1995). The males mark plants in their territories with the secretion of a cephalic gland that they apply to the substrate with a clypeal brush, and they defend the territories against intruding males in combat flights without physical contact of the opponents (Fig. 1.4) (Simon-Thomas & Poorter 1972; Evans & O'Neill 1988; Strohm 1995; Strohm & Lechner 2000; Schmitt *et al.* 2003). In the field, males can survive for more than four weeks, although the apparent median life span is usually shorter since emigrations from an observation site cannot be distinguished from the death of an individual (Strohm & Lechner 2000). A male can occupy the same territory for several days and up to two weeks (Simon-Thomas & Poorter 1972; Strohm & Lechner 2000). Several pieces of evidence suggest that the cephalic gland secretion is serves as a pheromone that attracts receptive females to the male's territory (Evans & O'Neill 1988, 1991): Females of several *Philanthus* species have been observed to approach

territories of conspecific males in a zigzagging flight pattern from the downwind side, suggesting that they are orienting towards the windborne cephalic gland components (Evans & O'Neill 1988, 1991). Copulations usually occur within the males' territories (Fig. 1.5) (Simon-Thomas & Poorter 1972; Strohm 1995) and seem to be under the control of the females since they can easily repel unwanted mates by virtue of their larger body size (Evans & O'Neill 1988) or refuse copulations by bending their abdomen tip downwards (E.Strohm, pers. obs.). Territories of different males are often found close together, thereby constituting a lek situation in which females have an ideal opportunity to choose among males (Simon-Thomas & Poorter 1972; Evans & O'Neill 1988). Since the copulation is not preceded by any kind of visual display, female choice appears to be, at least predominately, based on information obtained from the male marking pheromone (E. Strohm and M. Kaltenpoth, unpubl. obs.).

Analyses of head extracts from male European beewolves revealed a complex blend of at least 11 compounds, with (*Z*)-11-eicosen-1-ol as the main component (Schmidt *et al.* 1990; Schmitt *et al.* 2003). All of these components were also found in samples of pure cephalic glands in the same relative amounts (Kroiss *et al.*, in prep.) and in extracts from male territories (E. Strohm, T. Schmitt, G. Herzner, J. Kroiss and M. Kaltenpoth, unpubl. data). Although behavioral studies on the biological activity of the single components are lacking, these compounds might be important cues for females to assess male quality and choose among potential mates.

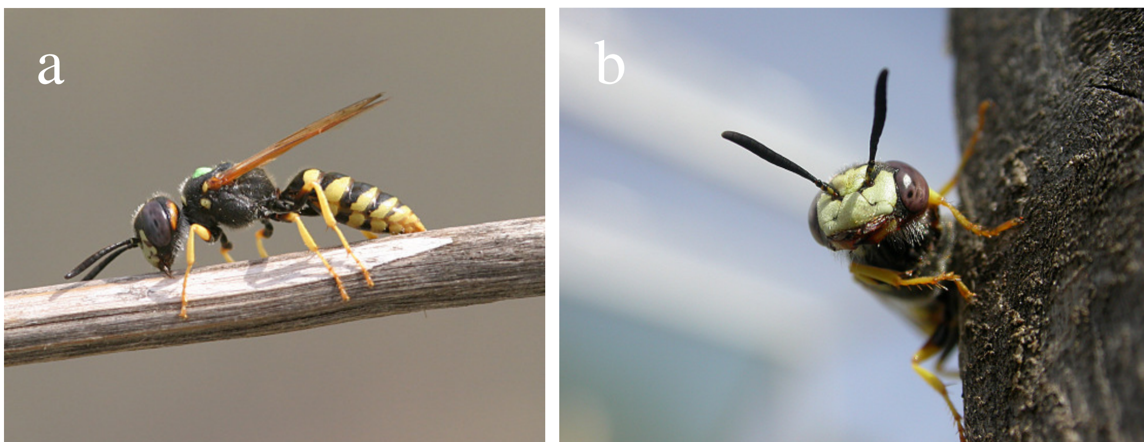

Figure 1.4: Male European beewolf **(a)** scent-marking and **(b)** guarding its territory.

Mate choice can be assumed to be of particular importance in the European beewolf. Females most probably mate only once (Evans & O'Neill 1988). Thus, choosing a low-quality male will affect all daughters (due to the haplo-diploid sex determination mechanism, male offspring are not affected, because they do not inherit genes from their mother's mate). Due to the extraordinary physiological requirements for reproduction including the fact that females have to carry the comparatively heavy prey to their nest in flight, a daughter's reproductive success heavily depends on her "quality" (Strohm & Linsenmair 1997; Strohm & Daniels 2003).

Therefore, “bad” genes from the father might affect a daughter's ability to hunt honeybees and carry the prey in flight as well as her life span. Thus, female choice for males with “good genes” could strongly influence female fitness.

Figure 1.5: Female (left) and male (right) European beewolf in copula.

1.4 OUTLINE OF THE THESIS

1.4.1 Symbiotic microorganisms in *Philanthus triangulum*

In the first part of this thesis (chapters 2-7), we investigate a unique interaction of European beewolves with endosymbiotic bacteria of the genus *Streptomyces*. Chapter 2 and 3 include the first description of this symbiotic interaction and provide evidence for a beneficial effect of the bacteria for the host by protecting the beewolf offspring against pathogen infestation. The specialized antennal organs for the cultivation of the bacteria in the beewolf female are characterized in chapter 4. In chapter 5, we provide ultrastructural, morphological and genetic data for the bacterial endosymbionts of 28 different *Philanthus* species and subspecies, and we propose the new taxon ‘*Candidatus* Streptomyces philanthi’ for the beewolf antennal bacteria.

To elucidate the ecology of an insect-bacteria symbiosis, it is necessary to investigate the effect of the interaction for both the host and the symbionts. As indicated above, there is evidence that

beewolves benefit from the association with '*Candidatus* Streptomyces philanthi' by obtaining protection against pathogens during the long and potentially very dangerous developmental phase in the brood cell (chapter 2 and 3). The benefits for the bacteria, however, are less obvious. In chapters 6 and 7, we analyzed the chemical composition of the *Streptomyces*-containing antennal reservoir exudate as well as the postpharyngeal gland content of female beewolves. The results allow us to draw some conclusions on the possibility of nutrient transfer from the beewolf host to the antennal endosymbionts.

1.4.2 The male marking pheromone as a potential cue for female choice

The marking pheromone of male European beewolves has been suggested to play an important role for female choice (Herzner 2004; Herzner *et al.* in press). Previous studies analyzed the composition of the male pheromone (Schmidt *et al.* 1990; Schmitt *et al.* 2003) and provided evidence that it evolved as an exploitation of pre-existing female preferences according to a sensory trap model (Herzner 2004; Herzner *et al.* 2005). Furthermore, it is known that the pheromone composition of close kin is more similar than that of unrelated individuals, indicating that the pheromone composition probably has a strong genetic basis (Herzner 2004; Herzner *et al.* in press). Thus, the male marking pheromone contains information that could be used by females to choose adaptively among potential mates.

The second part of this thesis (chapter 8-10) tries to complement previous studies on the potential of the male marking pheromone as an indicator for female choice. Chapter 8 investigates age-related changes in the amount and composition of the male pheromone. Age is often regarded as an important factor for mate choice (Trivers 1972; Manning 1985; Hansen & Price 1995; Brooks & Kemp 2001), thus, age-related changes in the beewolf marking pheromone might contribute to the significance of the pheromone for female choice. In chapter 9, we analyzed the marking pheromones of males on three different scales simultaneously: between families, among populations on a local scale, and between geographically distant populations. Differences in pheromone composition between families and across populations may be important for females to choose the best available mate according to the model of optimal outbreeding.

Although female choice has been postulated for the European beewolf (Herzner 2004; Herzner *et al.* in press), behavioral studies demonstrating female choice are still lacking. This is because the investigation of male reproductive success in relation to mate qualities has been hampered by the difficulty of observing beewolf matings under controlled conditions. Chapter 10 makes a contribution to circumvent this problem by obviating the need for behavioral observations: Polymorphic microsatellite markers provide the basis for genetic paternity analyses that will

allow the assessment of male reproductive success in controlled experiments with males differing in their age, size, relatedness or source population.

Figure 1.6: Male European beewolf feeding on *Eryngium campestre*.

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CHAPTER 2

SYMBIOTIC BACTERIA PROTECT WASP LARVAE FROM FUNGAL INFESTATION

Current Biology 15 (5), March 8, 2005: 475-479.

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2.1 SUMMARY

Symbiotic associations between different organisms are of great importance for evolutionary and ecological processes (Buchner 1921; Maynard-Smith 1989; Margulis & Fester 1991; Sapp 1994). Bacteria are particularly valuable symbiotic partners due to their huge diversity of biochemical pathways that may open entirely new ecological niches for higher organisms (Buchner 1921; Margulis & Fester 1991; Sapp 1994). Here we report on a unique association between a new *Streptomyces* species and a solitary hunting wasp, the European Beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae). Beewolf females cultivate the *Streptomyces* bacteria in specialized antennal glands and apply them to the brood cell prior to oviposition. The bacteria are taken up by the larva and occur on the walls of the cocoon. Bioassays indicate that the streptomycetes protect the cocoon from fungal infestation and significantly enhance the survival probability of the larva, possibly by producing antibiotics. Behavioral observations strongly suggest a vertical transmission of the bacteria. Two congeneric beewolf species harbor closely related streptomycetes in their antennae, indicating that the association with protective bacteria is widespread among philanthine wasps and might play an important role in other insects as well. This is the first report on the cultivation of bacteria in insect antennae and the first case of a symbiosis involving bacteria of the important antibiotic-producing genus *Streptomyces*.

2.2 RESULTS AND DISCUSSION

The European Beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) is a solitary digger wasp that constructs nest burrows in sandy soil. Females hunt honeybees (*Apis mellifera*) (Strohm & Linsenmair 1995) and provision one to five prey items as larval food in each brood cell. The larva feeds on the prey and spins a cocoon that is attached with its basal part to the wall of the brood cell. Larvae mostly overwinter and emerge next summer (Strohm 1995; Strohm & Linsenmair 1999). Since the conditions in the brood cells are humid and warm, there is a continuous threat of fungal or bacterial infestation of the provisions or the immature wasp. To protect the prey against microbes during the feeding period of the larvae, the females embalm the paralyzed honeybees with a cephalic gland secretion (Herzner *et al.*, unpubl. data). However, little was known on how the larva is secured from microbial attack during the nine months period of diapause in the cocoon.

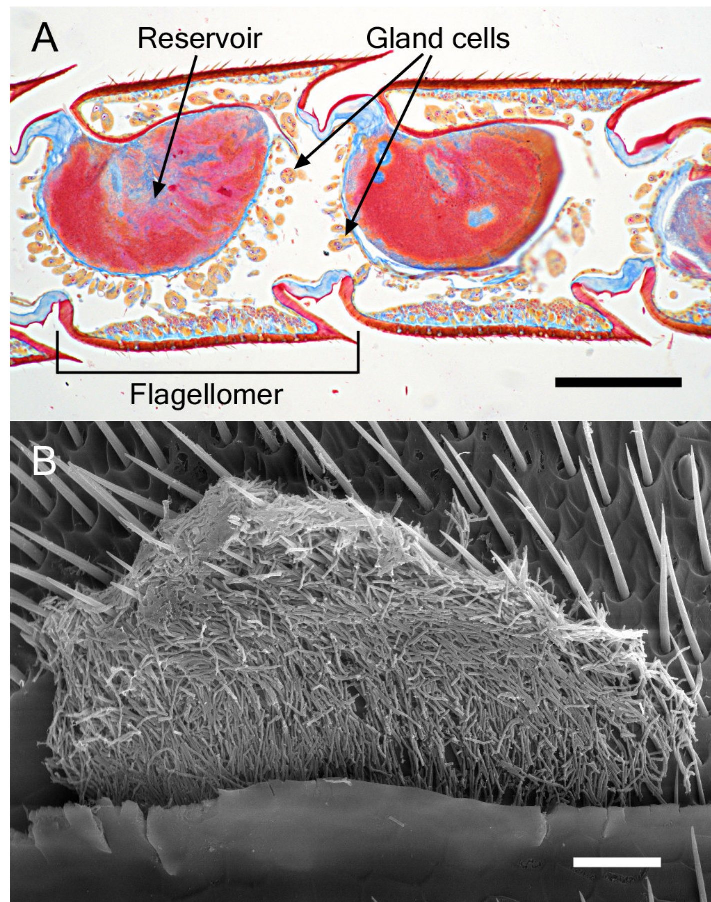

Figure 2.1: Antennae of female beewolves, *Philanthus triangulum*, with endosymbiotic *Streptomyces*. **A** Light microscopic picture of a semi-thin section of a female antenna, with endosymbiotic bacteria (red) in the reservoir of antennal glands. Scale bar 0.3 mm. **B** Scanning electron micrograph of a female antenna. White substance is secreted from the opening of the gland at the joint between two flagellomers. Scale bar 20 µm.

A promising candidate for such a protective function is a whitish substance that the female secretes into the brood cell in conspicuously large amounts prior to oviposition. The female enters the excavated brood cell and starts to move her body laterally, probably building up a high hemolymph pressure in the antennae (Strohm & Linsenmair 1995). The white substance is thus pressed out of specialized antennal glands and appears as white particles on the antennae (Fig. 2.1A). The female smears these particles on the ceiling of the brood cell. One known function of this secretion is to provide an orientational cue for the emergence of newly eclosed beewolves (Strohm & Linsenmair 1995). However, the unusually large amounts of white substance suggest a second function.

Scanning electron microscopy of newly secreted white substance revealed regularly shaped rod-like and branched structures with a diameter of about 0.5 µm (Fig. 2.1B). Using transmission electron microscopy, these structures were found to be encapsulated in biomembranes and sometimes contained circular structures consisting of several layers of membranes. We hypothesized that these structures were bacteria and that those with multiple biomembranes were spores. The overall appearance and the possible occurrence of spores suggested that these bacteria belong to the actinomycetes.

To verify the identity of these bacteria we used culture-independent molecular techniques. Isolation of DNA from antennae of female beewolves and amplification via polymerase chain reaction (PCR) with actinomycete-specific primers (Rintala *et al.* 2001; Stach *et al.* 2003) confirmed the presence of actinomycete bacteria. We sequenced about 1300 bp of the 16S rDNA and compared it to known actinomycete sequences. A phylogenetic analysis showed that the bacteria from the beewolf antennae belong to the genus *Streptomyces* (Fig. 2.2). The new type is most closely related to the *S. armeniacus* group (*S. griseocarneum, S. kasugaensis, S. lydicus, S. albulus*). Comparative genetical analyses of the 16S rDNA sequences (700-1320 bp, including the most variable regions) of endosymbionts from eleven *P. triangulum* individuals from four different populations (three in Germany and one in the Ukraine) revealed identical sequences, strongly suggesting that the association between beewolves and *Streptomyces* bacteria is obligate.

To exclude the possibility of bacterial contamination in the PCR, we designed a specific oligonucleotide probe that perfectly matched a variable region of the 16S rRNA of the putative symbiotic bacteria, while having at least two mismatches with all other *Streptomyces* 16S rRNA sequences in the Ribosomal Database Project (RDP II) (Maidak *et al.* 2001). The oligonucleotide probe was labeled with a fluorescent dye (Cy3) and used for fluorescence in-situ hybridization (FISH). The probe clearly stained large amounts of bacteria present in the white substance (Fig. 2.3) as well as in the antennal glands of female beewolves. Control strains of *Streptomyces aureofaciens* or *Bacillus subtilis* were not stained by the probe, demonstrating

the specificity of the probe for the bacterial sequences we obtained by PCR. These results confirm the presence of specialized streptomycete bacteria in the antennae of beewolf females and in the white substance secreted in the brood cells.

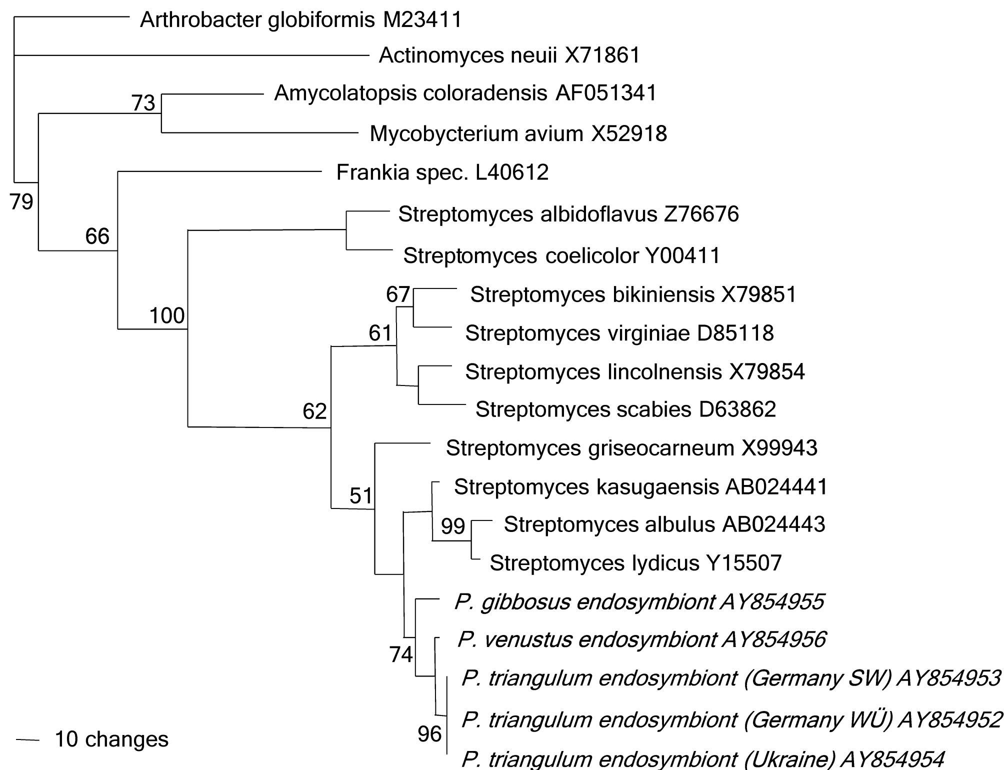

Figure 2.2: Phylogenetic position of beewolf endosymbionts within the actinomycetes: First of three equally parsimonious trees from a full heuristic search with tree bisection and reconnection (TBR) branch swapping and random addition sequence (100 replicates). Analysis is based on 1324 bp of 16S rDNA, with 219 characters being parsimony-informative. *A. globiformis* was defined as the outgroup. Values at the nodes represent bootstrap values from 1000 replicates. GenBank accession numbers are given behind the species names. *P. triangulum* specimens were collected at three different locations: in Schweinfurt (Germany SW), in Würzburg (Germany WÜ), and in the Ukraine.

Streptomycetes are filamentous high GC Gram-positive soil bacteria belonging to the actinomycetes (Kutzner 1981). The whole group is characterized by the ability to synthesize a huge diversity of antibacterial and antifungal secondary metabolites (Kutzner 1981; Behal 2000). In fact, most of the antibiotics used for medical application are produced by *Streptomyces* species (Goodfellow & Cross 1984; Behal 2000). Despite this high potential for producing antibiotics that would predestine streptomycetes as symbionts of other organisms, this is – to our knowledge – the first description of a mutualistic interaction between streptomycetes and animals, and there are only few known examples of symbioses with actinomycetes. The best-studied animal-actinomycete symbiosis is that of leaf-cutter ants and

actinomycete bacteria of the family Pseudonocardiaceae (Currie *et al.* 1999; Currie 2001; Currie *et al.* 2003; Poulsen *et al.* 2003). These ants tend fungus gardens in their nests for nutrition, and they carry the bacteria on specific regions of their cuticle (Currie *et al.* 1999). The actinomycetes produce compounds that specifically inhibit the growth of a specialized parasitic fungus of the fungus gardens (Currie *et al.* 1999; Currie *et al.* 2003). This association is widespread among attine ants, and the vertical mode of transmission points to a long coevolutionary relationship between the symbionts (Currie *et al.* 1999).

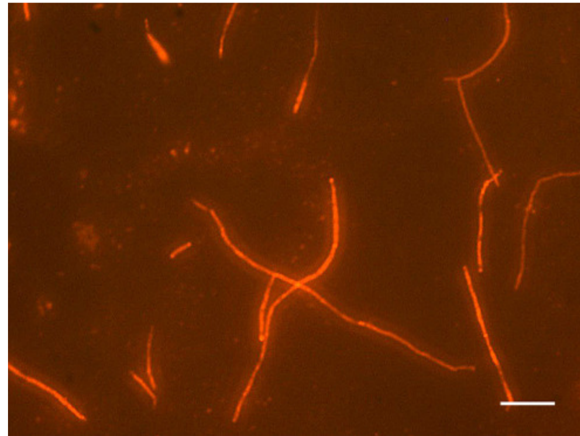

Figure 2.3: Fluorescence in-situ hybridization (FISH) of endosymbiotic *Streptomyces* in the white substance after secretion by a beewolf female. Scale bar 5 µm.

Beewolves face a high risk of fungal and bacterial infestation in the brood cell, especially during the first days after cocoon spinning, because fungi develop on the remains of the honeybee prey and may also infest the cocoon (pers. obs.). We hypothesized that – as in the attine ants – the beewolf endosymbionts may function as producers of antibiotics and protect the larva against pathogen attack. We examined cocoons for the presence of the antennal bacteria using the FISH method described above as well as transmission electron microscopy. The endosymbiotic streptomycetes were present in large numbers on the walls of the cocoon (Fig. 2.4). In fresh cocoons (1-3 weeks old), bacterial cells were conspicuously longer and covered the walls of the cocoons in higher density than in one-year old cocoons from which the progeny had already emerged. We hypothesized that the short cells on old cocoons were metabolically inactive spores. Thus, if the bacteria protect the cocoons from bacterial or fungal infestations, old cocoons should be more susceptible than fresh ones. Bioassays confirmed this hypothesis. On fresh cocoons, fungal growth was significantly delayed as compared to one-year old cocoons. This was true for the part where the cocoon is attached to the brood cell ($p = 0.0041$) and even more pronounced for the rest of the cocoon ($p = 0.0013$). Additionally, the development of fungal conidia was significantly delayed or even completely inhibited ($p = 0.0005$) (Gehan-Wilcoxon tests). The effects in fresh cocoons were independent of the presence of a larva (Gehan-Wilcoxon test, $p > 0.10$ for all comparisons).

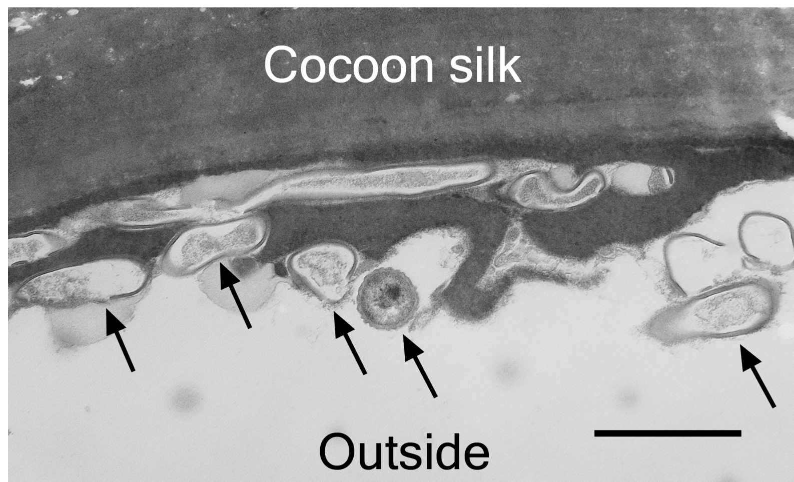

Figure 2.4: Transmission electron micrograph of a cocoon cross-section with bacteria on the outside (arrows). Scale bar 1 µm.

In a second series of bioassays, we examined the importance of the white substance for the actual survival of larvae in the brood cells. Larvae had a dramatically reduced survival probability when they had no access to the white substance (Fig. 2.5, Gehan-Wilcoxon test, $Z = 3.401$, $p = 0.00067$). Only one out of 15 individuals that had no access to the white substance survived until emergence (6.7%), whereas 15 of the 18 control individuals with white substance (83.3%) successfully emerged or survived as larvae until the end of the experiment (45 days). The experiment was terminated after 45 days, because the most critical phase after cocoon spinning was over and the surviving larvae had either emerged or entered diapause for overwintering. Taken together, the results of the bioassays strongly support the hypothesis that the *Streptomyces* bacteria protect the cocoon from fungus infestation and thereby increase the survival probability of beewolf larvae.

An important question is how beewolf females acquire the antennal bacteria. A priori, there are two alternatives: females might opportunistically take up the bacteria from the environment or they may inherit them from their mother (Moran & Baumann 2000). Observations of larvae searching for and apparently ingesting parts of the white substance in the brood cell before spinning the cocoon suggest a vertical transmission of the bacteria from mother to daughters. Further evidence for vertical transfer is provided by one beewolf female that survived until adulthood in the absence of white substance. The female failed to construct any brood cells during her- entire lifetime, and PCR-based attempts to detect endosymbionts in the antennae yielded no amplicons, strongly suggesting that this female did not harbor endosymbiotic *Streptomyces* bacteria in her antennae.

The complexity of the association including the occurrence of unique glands, uptake of the bacteria by the larva, application to the cocoon, and a probably vertical transmission make it

unlikely that this association is limited to *P. triangulum*. Therefore, we examined two congeneric species for the presence of antennal symbionts: *P. venustus* from Southern Europe and *P. gibbosus* from North America. We found streptomycetes in the antennae of both species, and comparative 16S rDNA sequence analysis revealed that they are very closely related to the endosymbionts of *P. triangulum*. In fact, the endosymbionts of the three *Philanthus* species form a monophyletic clade within the genus *Streptomyces* (Fig. 2.2). These results point to an early origin of the beewolf-*Streptomyces* mutualism possibly during the formation of the genus *Philanthus*. Further studies on the phylogenies of both hosts and symbionts are necessary to illuminate the coevolutionary patterns and to investigate whether horizontal transfer has occurred during the evolutionary history of the symbiosis.

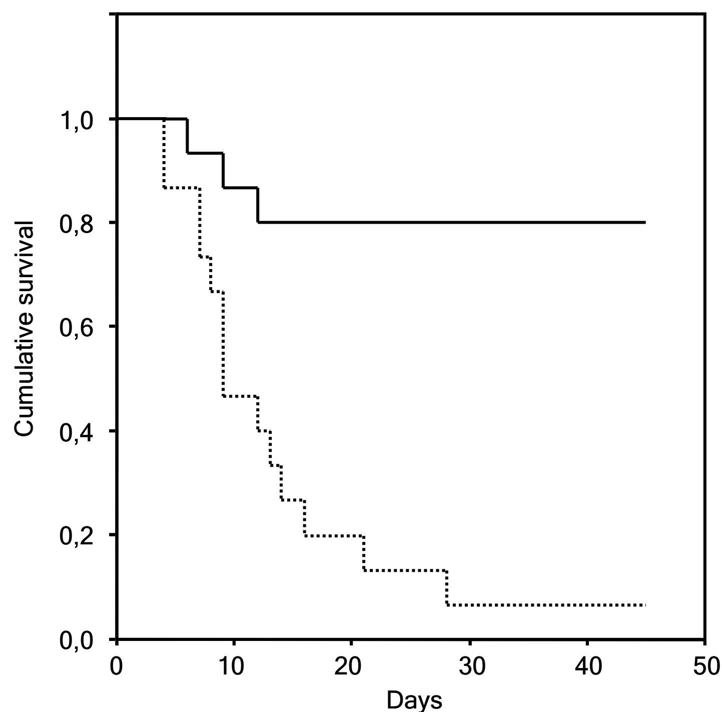

Figure 2.5: Cumulative survival of larvae with (solid line) and without white substance (dotted line) in the brood cell. The experiment was terminated after 45 days.

Soil-nesting hymenoptera and other ground-dwelling arthropods generally face a high risk of bacterial and fungal infestation of the provisions and the progeny from the surrounding soil. Therefore, one would expect high selection pressures to act on the evolution of protective mechanisms against pathogen attack. The cultivation of antibiotic-producing bacteria in specialized organs might represent a key invention to cope with the threat of pathogen infestation. So far, this is the only study providing evidence for a symbiosis between a ground-nesting wasp and protective bacteria, but associations of this kind may be much more widespread and might have played a crucial role in the evolution of ground-nesting behavior. Furthermore, assuming that the protection against microbes is mediated by chemicals, the study

of actinomycete-insect associations may provide knowledge on novel antimicrobial compounds. Since the antibiotics involved should not harm their eukaryotic hosts, they might be of particular value for medical use.

2.3 METHODS

2.3.1 PCR and sequencing

Bacterial DNA was extracted from whole beewolf antennae according to a standard phenol-chloroform extraction protocol. The following primer pairs were used for amplification of *Streptomyces*: fD1 (fwd.) (Weisburg *et al.* 1991) and StrepF (rev.) (Rintala *et al.* 2001), Act-S20 (fwd.) (Stach *et al.* 2003) and rP2 (rev.) (Weisburg *et al.* 1991). PCR amplification was performed on Eppendorf® Mastercyclers in a total reaction volume of 25 µl containing 4 µl of template, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 2.5 mM MgCl2, 240 µM dNTPs, 20 pmol of each primer, and 1 U of Taq DNA polymerase (MBI Fermentas). Cycle parameters were as follows: 3 min. at 94°C, followed by 32 cycles of 94°C for 40 sec., 65°C for 1 min., and 72°C for 1 min., and a final extension time of 4 min. at 72°C. For sequencing, we used the following primers: fD1 (fwd.), Act-S20 (fwd.), Act-A19 (rev.) (Stach *et al.* 2003), StrepF (rev.), rP2 (rev.). Sequencing was carried out on a Beckmann-Coulter CEQ 2000 XL sequencer.

2.3.2 Phylogenetic analysis

Partial 16S rDNA sequences of the endosymbionts and representative actinomycete genera from the GenBank database (accession numbers are given in Fig. 2.2) were aligned in ClustalX 1.83 using the default settings and imported into PAUP 4.0. Phylogenetic trees were constructed based on 1324 bp of 16S rDNA in a full heuristic search with tree bisection and reconnection (TBR) branch swapping and random addition sequence (100 replicates). Bootstrap values were obtained from a search with 1000 replicates.

2.3.3 FISH

The following species-specific oligonucleotide probe was designed for the endosymbiont by comparison with known sequences in the RDP II: 5`-Cy3-CACCAACCATGCGATCGGTA-3` (positions 176-196 *Streptomyces ambofaciens* nomenclature, Pernodet *et al.* 1989). The

unspecific eubacterial probe EUB 338 was used as a positive control (Amann *et al.* 1990). Secretions of the white substance from beewolf females were harvested and spread onto six-field microscope slides. Fixation and hybridization was carried out as described previously (Grimm *et al.* 1998), with minor modifications: hybridization buffer contained only 50 ng of the labeled probe, and samples were incubated for 90 min. at 45°C for hybridization. For hybridization within the antennae, fresh female antennae were cut into thin sections with a razor blade and glued onto microscope slides. Fixation and pre-treatment of the samples was done following the protocol of Sauer *et al.* (2002). Hybridization was carried out as for the bacterial samples, but with 3 hrs. of incubation with the labeled probe.

2.3.4 Fungal infestation bioassays with beewolf cocoons

Paper towels were placed in eight petri dishes and moistened with 3 ml distilled water. Three cocoons were placed in each petri dish: an empty one-year old cocoon; a fresh cocoon with larva (1-3 weeks old); and a fresh cocoon from which the larva had been removed. Petri dishes were kept in a closed box at room temperature to keep moisture approximately constant. Fungal growth was recorded daily under a Wild Heerbrugg M3B dissecting scope with 40x magnification. Usually, fungi started to grow at the basal part of the cocoon where it had been attached to the brood cell. Therefore, fungal growth was recorded separately for the attachment site and the rest of the cocoon. The time until first appearance of fungi, the time until fungi completely covered the attachment site or the whole cocoon, and the time until conidia formation were compared among groups using survival analyses (Gehan-Wilcoxon tests, software: Bias 8.05).

2.3.5 Survival of larvae with and without white substance

Newly provisioned brood cells in the nesting cages of seven females were assigned randomly to two different groups: with (control group) and without white substance (experimental group). In cells of the experimental group, the glass covering the brood cells in the observation cages was lifted and a microscope cover slip was introduced between the brood cell and the glass cover. Thus, the white substance that is applied to the ceiling of the brood cell was covered and the larva had no access to the white substance. In control cells, the glass cover was also lifted but no cover slip was introduced, so the white substance was freely accessible to the larva. Survival of the larvae was checked daily for all brood cells and compared between groups using survival analysis (Gehan-Wilcoxon test, software: Bias 8.05). Larvae that survived until the end of the experiment (45 days) and individuals that emerged successfully from the cocoon were included as censored data.

Acknowledgements

We thank Michael Steinert and Silke Killinger of the Institute for Molecular Infection Biology of the University of Würzburg for help and introduction to the FISH technique. We would like to thank Roy Gross for helpful suggestions and discussions, and Norbert Weißel for comments on the manuscript. We thank Jon Seger for providing us with *P. gibbosus* specimens, Johannes Kroiss for collecting *P. venustus* in Greece, Thomas Eltz for sending *P. triangulum* from Düsseldorf, Silke Fregin for collecting *P. triangulum* in Schweinfurt, and Protsenko Yura for sending *P. triangulum* specimens from the Ukraine.

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Partial 16S rDNA sequences from *Streptomyces* endosymbionts of *Philanthus triangulum* (from three different populations: Würzburg, Germany; Schweinfurt, Germany; and from the Ukraine), *P. venustus* and *P. gibbosus* are available at GenBank (http://www.ncbi.nlm.nih.gov/) with the accession numbers AY854952-AY854956.

CHAPTER 3

BAKTERIEN SCHÜTZEN WESPEN-NACHWUCHS VOR PILZBEFALL

Naturwissenschaftliche Rundschau 58 (6), June 2005: 329-330.

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3.1 ZUSAMMENFASSUNG

Weibchen einer solitären Grabwespe, des Europäischen Bienenwolfes, sind an einer erstaunlichen und einzigartigen Symbiose beteiligt: Sie kultivieren Bakterien in spezialisierten Antennen-Drüsen und geben diese vor der Eiablage als eine sichtbare weiße Substanz in ihre Brutzellen im Boden ab. Die Bakterien werden von der Larve aufgenommen und zur Imprägnierung des Kokons verwendet. Dadurch wird die Gefahr von Pilzbefall herabgesetzt und der Überlebenserfolg der Larve beträchtlich erhöht. Genetische Analysen deuten darauf hin, dass die Symbiose zwischen Bienenwölfen und Bakterien bereits vor langer Zeit entstanden ist.

3.2 EINLEITUNG

Symbiosen mit Bakterien haben nicht nur für die Evolution aller mehrzelligen Organismen eine herausragende Rolle gespielt, sondern sie sind auch in rezenten Ökosystemen für die meisten Tiere und Pflanzen von entscheidender Bedeutung (Buchner 1921; Margulis & Fester 1991; Sapp 1994). Viele Insekten sind beispielsweise auf symbiontische Bakterien angewiesen, die essentielle Aminosäuren oder Vitamine produzieren und ihren Wirten zur Verfügung stellen (Moran & Baumann 1994; Douglas 1998). Der Europäische Bienenwolf, *Philanthus triangulum* (Hymenoptera, Crabronidae), macht sich Bakterien in einem ganz anderem Zusammenhang zunutze, der für das Überleben der Tiere aber nicht minder wichtig ist: er setzt Bakterien zum Schutz seiner Nachkommen vor Pilzbefall ein (Kaltenpoth *et al.* 2005).

3.3 ERGEBNISSE UND DISKUSSION

Der Bienenwolf verdankt seinen Namen dem Jagdverhalten der Weibchen, die Honigbienen fangen und lähmen und sie als Lebendfutter für ihre Larven in selbstgegrabene Nisthöhlen im Boden eintragen. Schon seit mehreren Jahren ist bekannt, dass Bienenwolf-Weibchen vor der Eiablage große Mengen eines weißlichen Sekretes in die Brutkammer abgeben (Abb. 3.1), das später als Richtungsinformation für den schlüpfenden Bienenwolf dient (Strohm & Linsenmair 1995). Die Zusammensetzung dieser weißen Substanz war jedoch weitgehend unbekannt. Rasterelektronenmikroskopische Untersuchungen haben nun längliche, zellähnliche Strukturen in dem Sekret erkennen lassen, deren Form stark an bestimmte Bakterien erinnert (Abb. 3.2). Genetische Analysen bestätigten das Vorhandensein von Bakterien und ermöglichten deren Bestimmung als eine bislang unbekannte Art der Gattung *Streptomyces* (Kaltenpoth *et al.* 2005). Diese Ergebnisse sind in zweierlei Hinsicht besonders erstaunlich: erstens sind die Antennen von Insekten ein außerordentlich ungewöhnlicher Platz für endosymbiontische Bakterien, und zweitens ist dies der erste Fall einer Symbiose von Tieren mit Bakterien der interessanten und extrem wichtigen Gattung *Streptomyces*.

Abbildung 3.1: Bienenwolf-Weibchen bei der Abgabe der bakterienhaltigen weißen Substanz aus den Antennendrüsen in die Brutzelle. Foto: Erhard Strohm.

Viele Arten dieser Gattung produzieren nämlich wirkungsvolle Antibiotika gegen andere Bakterien oder Pilze, und zahlreiche dieser Substanzen werden erfolgreich in der Humanmedizin eingesetzt (Behal 2000). Ob die Bakterien der Bienenwölfe ebenfalls Schutz gegen schädliche Bakterien oder Pilze bieten, sollten Biotests zeigen, bei denen Larven der Zugang zur bakterienhaltigen weißen Substanz in der Brutkammer verwehrt wurde. Verglichen mit Kontrolltieren, die Kontakt zu den Bakterien hatten, war bei den Larven ohne Bakterien die

Mortalität tatsächlich drastisch erhöht; ihre Überlebenswahrscheinlichkeit sank dramatisch von über 80% auf knapp 7% (Kaltenpoth *et al.* 2005).

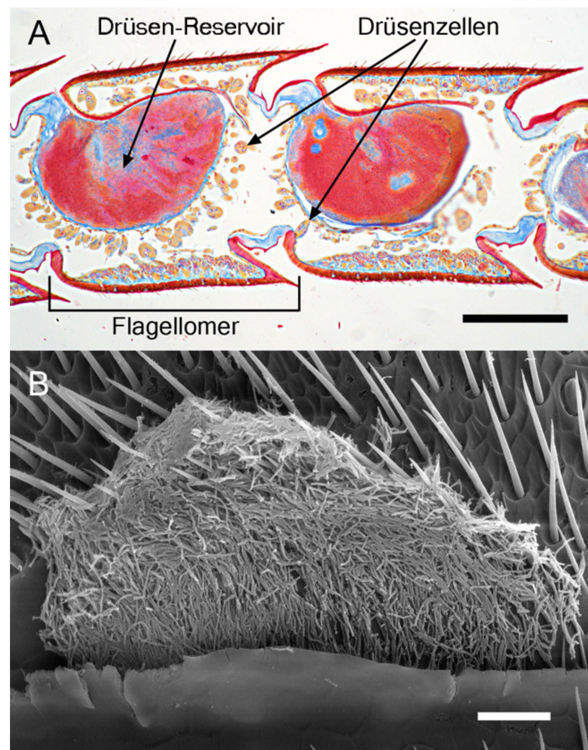

Abbildung 3.2: Antennen von Bienenwolf-Weibchen mit endosymbiontischen Bakterien.
(A) Lichtmikroskopische Aufnahme einer Weibchen-Antenne mit endosymbiontischen Bakterien (rot angefärbt) in den Reservoirs der Antennendrüsen. Maßstabsbalken: 0,3 mm.
(B) Rasterelektronenmikroskopisches Bild der Antenne eines Bienenwolf-Weibchens mit austretenden Bakterien an der Öffnung einer Antennendrüse. Maßstabsbalken: 20 µm. Verändert aus Kaltenpoth *et al.* (2005), mit freundlicher Genehmigung von Elsevier®.

Wie aber können die Bakterien den Bienenwolf-Nachwuchs beschützen? Verhaltensbeobachtungen zeigten, dass die Larven in der Brutkammer aktiv die weiße Substanz mit den Bakterien suchen und aufnehmen. Durch verschiedene spezifische Färbemethoden und Elektronenmikroskopie konnten die Bakterien außerdem auf dem Kokon nachgewiesen werden, in den sich die Larve etwa 8-10 Tagen nach ihrem Schlupf einspinnt (Kaltenpoth *et al.* 2005). Die Larven scheinen also ihren Kokon mit den aufgenommenen Bakterien zu imprägnieren. Biotests zeigten, dass sehr alte und verlassene Kokons schneller verschimmeln als frische Kokons, was vermuten lässt, dass die Bakterien auf frischen Kokons Antibiotika produzieren und sie so gegen Pilzbefall schützen, während alte Kokons keine lebenden oder zumindest keine aktiven Bakterien mehr beherbergen und so Infektionen durch Pilze schutzlos ausgeliefert sind (Kaltenpoth *et al.* 2005). Der letztendliche Nachweis und die Identifikation der wahrscheinlich beteiligten antibiotisch wirksamen Substanzen stehen allerdings noch aus.

Aus evolutionären und ökologischen Gesichtspunkten ist die Frage besonders interessant, ob die Symbiose zwischen Bienenwölfen und Bakterien obligat ist und ob die Bakterien von der Mutter an die Tochter weitergegeben werden oder ob jedes Bienenwolfweibchen sie erneut aus der Umwelt aufnehmen muss. Genetische Analysen der Endosymbionten verschiedener Bienenwolf-Individuen lassen darauf schließen, dass alle Weibchen des Europäischen Bienenwolfes dieselbe *Streptomyces*-Art kultivieren, und dass es sich somit um eine hochspezialisierte und vermutlich obligate Symbiose handelt (Kaltenpoth *et al.* 2005). Verhaltensbeobachtungen und die Aufzucht von Tieren in Abwesenheit der Bakterien deuten außerdem stark auf eine strikt vertikale Weitergabe der Endosymbionten von der Mutter an ihre Töchter hin (Kaltenpoth *et al.* 2005).

Angesichts des hohen Spezialisierungsgrades beider Symbiosepartner erscheint es unwahrscheinlich, dass diese Assoziation auf den Europäischen Bienenwolf beschränkt ist. Tatsächlich wurden mithilfe genetischer Methoden die Endosymbionten auch in den Antennen anderer Arten der Gattung *Philanthus* nachgewiesen und es wurde gezeigt, dass die Bakterien der verschiedenen Arten näher miteinander verwandt sind als mit irgendeiner anderen bekannten Art der Gattung *Streptomyces* (Kaltenpoth *et al.* 2005). Diese Ergebnisse lassen vermuten, dass die Symbiose schon vor langer Zeit entstanden ist und sich Wirte und Symbionten parallel entwickelt und in mehrere Arten aufgespaltet haben. Genauere Untersuchungen weiterer Bienenwolf-Arten sind nötig, um diese Hypothese zu testen.

Die Kultivierung Antibiotika-produzierender Bakterien zum Schutz des Nachwuchses stellt eine einzigartige Anpassung für im Boden nistende Insekten dar, um ihre Nachkommen gegen Infektionen durch omnipräsente pathogene Pilze und Bakterien zu schützen. Neben der Assoziation von Blattschneiderameisen mit Antibiotika-produzierenden Bakterien ist der Bienenwolf-*Streptomyces*-Mutualismus erst der zweite bekannte Fall einer solchen Symbiose. Inwieweit andere bodenlebende Arthropoden von Bakterien als „externes Immunsystem“ Gebrauch machen und ob die Symbiose mit schützenden Mikroorganismen einen wichtigen Schritt in der Evolution des bodennistenden Verhaltens von Grabwespen darstellt, werden weitere gezielte Untersuchungen an anderen bodenlebenden Insektenarten zeigen. Die Erforschung der dabei involvierten Bakterienarten kann außerdem zur Entdeckung neuer antibiotischer Substanzen führen und damit möglicherweise dazu beitragen der Humanmedizin neue Waffen im Kampf gegen zunehmend resistente Krankheitserreger zu liefern.

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CHAPTER 4

MORPHOLOGY AND ULTRASTRUCTURE OF A BACTERIA CULTIVATION ORGAN: THE ANTENNAL GLANDS OF FEMALE EUROPEAN BEEWOLVES, *PHILANTHUS TRIANGULUM* (HYMENOPTERA, CRABRONIDAE)

Arthropod Structure and Development, submitted.

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4.1 SUMMARY

Females of the European beewolf (*Philanthus triangulum* F.), a solitary digger wasp, cultivate symbiotic bacteria of the genus *Streptomyces* in specialized antennal glands.

They secrete the bacteria into their subterranean brood cells prior to oviposition. The bacteria are taken up by the larvae and protect them against mould fungi. We investigated the ultrastructure of the antennal glands and reconstructed the complex morphology using 3D-visualization software. The bacteria are reared in five antennomeres within large reservoirs that consist of two slightly bent lobes. Each gland reservoir is bordered by a monolayered epithelium lined with a partially reinforced cuticle and comprises about half of the antennomere's volume when it is completely filled with bacteria. The opening of the reservoir is covered by a membranous appendage of the cuticle. Approximately 400 class 3 gland units surround each reservoir and are connected to the reservoir lumen through conducting canals. The class 3 gland cells contain numerous vesicles and a high density of rough endoplasmatic reticulum. The cells of the monolayered epithelium bordering each reservoir show basal invaginations, apical microvilli and numerous vesicles. In the reservoir lumen, large numbers of symbiotic *Streptomyces* bacteria are embedded in secretion droplets. We discuss the role of the antennal glands as organs for the cultivation of the symbiotic bacteria.

4.2 Introduction

Symbiotic associations between insects and bacteria are of great ecological and evolutionary importance (e.g. Moran & Baumann 2000; Bourtzis & Miller 2003). Symbiotic bacteria occur either intracellularly or extracellularly and enhance the fitness of their hosts, e.g. by supplying essential nutrients (e.g. Buchner 1965; Douglas 1998; Paracer & Ahmadjian 2000) or by providing defence against pathogens (Charnley *et al.* 1985; Currie *et al.* 1999; Takatsuka & Kunimi 2000; Dillon *et al.* 2005). Recently, a symbioses has been described between a digger wasp, the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae), and bacteria of the genus Streptomyces (Kaltenpoth *et al.* 2005, 2006). This association is unusual with regard to the localization of the bacteria cultivation organ: the symbionts live in specialized glands in the antennae of beewolf females.

Female European beewolves construct subterranean nests in sandy habitats and provision their offspring with paralysed honeybees (*Apis mellifera*). Shortly before oviposition, the female beewolf enters the brood cell and starts to perform lateral movements with her whole body while bending its antennae slightly downwards (Strohm & Linsenmair 1995). Thereupon a white substance appears at five spots on the dorsal side of each antenna. The female applies this secretion to the distal side of the brood cell ceiling (Strohm & Linsenmair 1995; Kaltenpoth *et al.* 2005). Then the female beewolf lays an egg and closes the brood cell.

The white substance serves at least two functions: First it provides an orientational cue for the beewolf larva, which attaches its cocoon in accordance with the position of the white substance. This orientation finally facilitates the emergence of the adult beewolf from the brood cell (Strohm & Linsenmair 1995). The second function of the whitish antennal gland exudate is to inhibit microbial infestation of the cocoon during overwintering. Bioassays showed that more than 80% of the larvae with access to white substance survived inside their cocoons, whereas survival was reduced to less than 10% if access to the white substance had been experimentally blocked prior to cocoon-spinning (Kaltenpoth *et al.* 2005). The main components of the white substance are symbiotic bacteria of the genus *Streptomyces* (Kaltenpoth *et al.* 2005, 2006) that are cultivated in antennal glands of beewolf females.

In Hymenoptera antennal glands were first described in the male parasitoid wasp *Melittobia australica* (Eulophidae; Dahms 1984), and up to now antennal glands were found only in a few other taxa. In male Hymenoptera, antennal glands are known to play a role in mating and courtship behaviour by producing volatile or paste-like secretions acting as sex-pheromones. In various taxa like Cynipoidea (Isidoro *et al.* 1999), Chalcidoidea (Guerrieri *et al.* 2001), Platygastridae (Isidoro & Bin 1995), Scelionidae (Bin & Vinson 1986), Vespidae (Isidoro *et al.* 1996; Bin *et al.* 1999a; Romani *et al.* 2005), and Apidae (Romani *et al.* 2003) the male antennal

gland secretions are applied onto female antennae either by direct contact or through the air (Isidoro *et al.* 2000).

In female Hymenoptera, antennal glands have been found in the egg parasitoid *Trissolcus basalis*, Scelionidae, and four ant species, Formicidae (Billen & Buschinger 2000; Isidoro *et al.* 2000; Romani *et al.* 2006). In *T. basalis*, the secretion of the antennal glands is suspected to be used in host recognition by dissolving kairomones from host eggs (Isidoro *et al.* 1996; Bin *et al.* 1999b), whereas the function of antennal glands in ants is not yet clear (Isidoro *et al.* 2000; Romani *et al.* 2006). The antennal glands of Hymenoptera investigated so far are characterized as either class 1 or class 3 cell units secreting to the outer surface of the antennae (Isidoro *et al.* 1999), but no gland reservoirs have been described.

In the present study, we describe the ultrastructure and exceptional morphology of the antennal glands of female European beewolves, present a 3D-reconstruction based on series of histological sections and discuss the glands' role as brood pouches for the symbiotic *Streptomyces* bacteria.

4.3 Materials and Methods

4.3.1 Specimens

Female European beewolves were obtained from a laboratory population at the Biocenter of the University of Würzburg, Germany. For detailed information about the rearing conditions see e.g. Strohm and Linsenmair (1997). Females were removed from their cages at different stages of the nesting cycle. For the general denomination of the antennal segments, we follow Isidoro *et al.* (1996), counting the antennomeres from proximal to distal, including scape and pedicel. The antennae of female beewolves comprise the scape (A1), the pedicel (A2), and the flagellum with 10 antennomeres (A3-A12). For a description of the general outer antennal morphology see Herzner *et al.* (2003). *P. triangulum* females usually hold their antennae straight, slightly upwards (about 30°), and slightly laterally.

4.3.2 Semithin sections and 3D-reconstruction

To reveal the three-dimensional structure of the glands we used series of semithin sections of the five antennomeres bearing the glands. Whole antennae were fixed with alcoholic Bouin, dehydrated in a graded ethanol series and embedded in Durcupan (ACM Fluka, Deisenhofen,

Germany). Sections of 4 µm thickness were made with a diamond knife on a Reichert 2040 Microtome and stained with methylene blue/azure II, trichrom after Masson-Goldner, or AZAN after Heidenhain (Böck 1989). We also examined sections that had been prepared by W. Rathmayer (see Rathmayer 1962 for methods). Digital photos of the sections were obtained with a Nikon Coolpix 990 camera attached to a Zeiss Axiophot M45 light microscope. The image stack was transferred to a computer and the slices were manually aligned with the 3D-visualization software Amira® (Mercury Computer Systems, Berlin). In a final step, the gland reservoirs and other components of the antennae were manually marked with different colours in every slice to allow 3D-reconstruction of the antennal structures. The volume of certain structures can then be calculated.

4.3.3 Electron microscopy

For scanning electron microscopy (SEM), female beewolves were anaesthetised with CO_2 and killed with diethyl ether. The antennae were cut off and fixed in alcoholic Bouin for 3 hours at 4°C followed by dehydration in a graded acetone series. Then they were critical point dried (BAL-TEC CPD 030), sputtered with Pt/Pd (BAL-TEC SCD 005) and examined with a Zeiss DSM 962 digital scanning electron microscope at 15 kV. To investigate the interior fine structure of the glands, antennae were intersected with a razor blade before sputtering.

For transmission electron microscopy (TEM), the antennae were fixed overnight at 4 °C in a solution of 4% glutardialdehyd in 0,1 M Sörensen phosphate buffer at pH 7.4 (Sörensen 1909), followed by postfixation with 2% osmium tetroxide. After dehydration in a graded ethanol series and propylene oxide the specimens were embedded in Epon 812 (Polysciences, Eppelheim, Germany). Ultrathin sections were made with a 45° diamond knife on a Reichert Ultracut E microtome. Sections were stained with 2% uranyl acetate and Reynold's lead citrate and examined with a Zeiss EM 10 at 80 kV.

4.4 RESULTS

4.4.1 Overall morphology

The antennae of female European beewolves possess large reservoirs in the five antennomeres A4 to A8. The glands of different antennomeres show a nearly identical morphology [Fig. 4.1A]. Each reservoir consists of a large invagination of the proximal side of the antennomere. The reservoir is bordered by an epithelium that is lined with cuticle and surrounded by class 3

gland cell units (according to Noirot & Quennedey 1974). The 3D-reconstruction reveals that such a gland reservoir consists of two lobes, that more or less describe a bent figure "S" [Fig. 4.1D, F] surrounding the antennal nerve. The medial part of the reservoir is slightly shorter and comprises about 1/3, the lateral part 2/3 of the total reservoir volume [Fig. 4.1D, F].

The reservoir's opening is located dorsally in the proximal intersegmental gap of the antennomere [Fig. 4.1E, 4.2] and is visible as a small hole when the adjacent proximate antennal segment is removed [Fig. 4.2]. Longitudinal semithin sections revealed that the opening is covered by a flap-like projecting part of the intersegmental cuticle with membranous appendages [Fig. 4.1E]. From the opening, the slightly depressed cuticle of the proximal face of the antennomere forms a flat channel that leads upwards. The white substance is pressed out through this channel during delivery and finally appears at the dorsal side between adjacent antennomeres.

The reservoir's volume changes considerably with its filling status. A reservoir completely filled with white substance takes more than 50% of the antennomere volume and apparently even squeezes the antennal nerve between its two parts [Fig. 4.1B]. An empty reservoir, by contrast, appears completely collapsed with the opposing sides of the reservoir cuticle close to each other [Fig. 4.1C]. The 3D-reconstruction of filled reservoirs showed a maximum volume of 0.07 µl. Thus, the ten reservoirs of both antennae have a remarkable maximum volume of approx. 0.7 µl. Sections of antennae of females that had probably just delivered the white substance show that the reservoir is not totally empty but that some white substance remains in the rear parts of the lobes.

The reservoir lumen is bordered by a monolayered epithelium lined by cuticle. Semithin cross sections show that in both parts of the reservoir the wall of the medial side, i.e. the side pointing to the body axis is thin and membranous and appears to be slightly folded [Fig. 4.1B, C], whereas on the lateral side of the cuticle is reinforced and has a net-like structure [Fig. 4.1B, C, F, see also Fig. 4.3]. The transition between the reinforced, net-like cuticle and thin cuticle is abrupt at the dorsal and ventral side of the reservoir tubes [Fig. 4.1B, C, E]. The arrangement of both cuticle types suggests that the change in reservoir volume is accomplished by dilatation and contraction of the apparently resilient medial walls of the reservoirs. No muscles were found in the five flagellomeres (A4 to A8) bearing the glands. This is in accord with the morphology of geniculate antennae so far described, in which muscles only appear in the scape (A1) and pedicel (A2) (Snodgrass 1935).

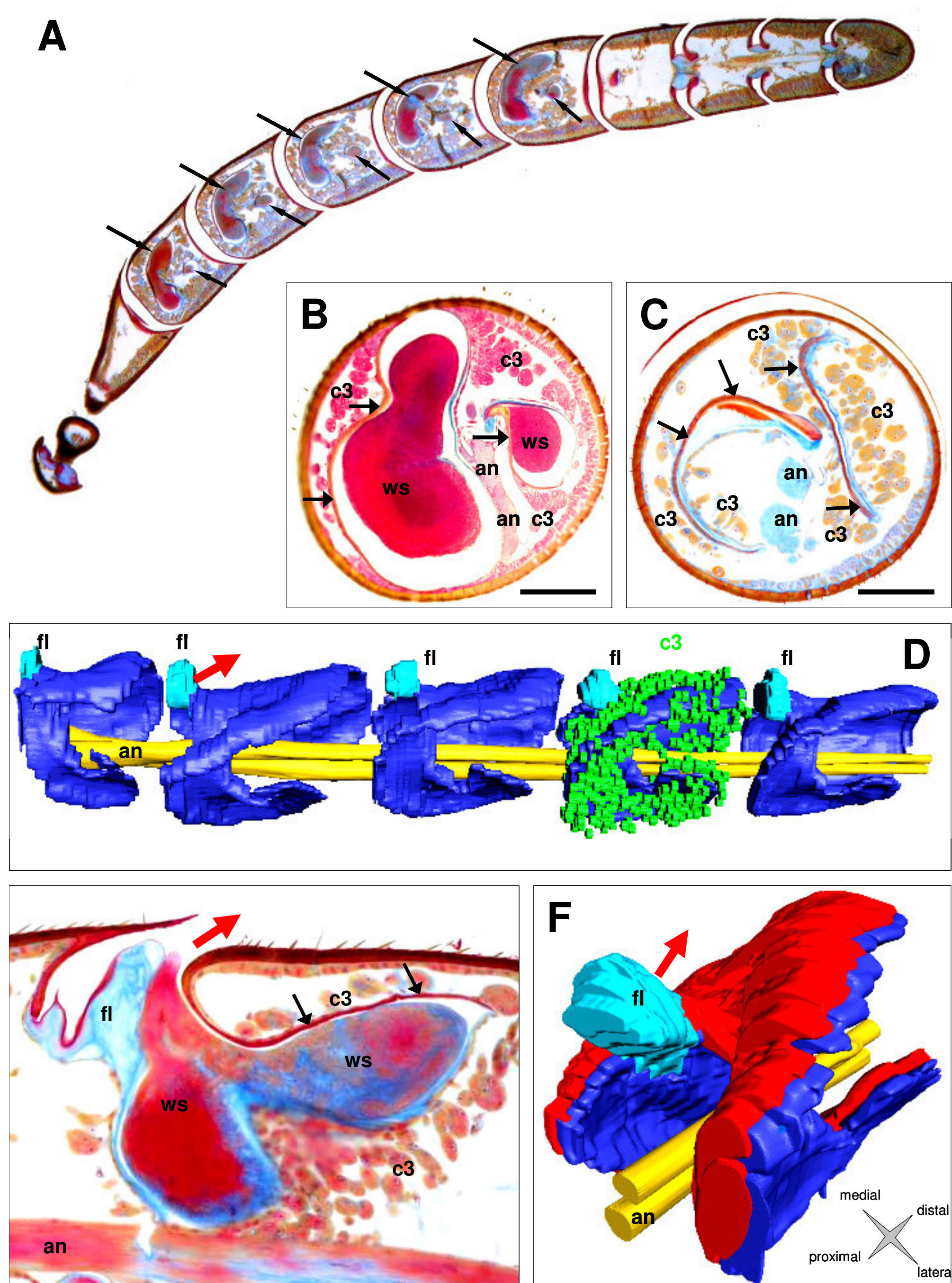

Figure 4.1: (**A**) Longitudinal semithin section through an antenna of a female European beewolf with two parts of the filled antennal gland reservoirs (arrows) in antennomeres A4 to A8.

(**B**)/(**C**) Semithin cross sections of antennomeres with filled (**B**) and empty (**C**) gland reservoir surrounded by class 3 gland units (c3). The antennal nerve in figure (**B**) is squeezed between the two parts of the

reservoir filled with white substance (ws). Black arrows indicate the reinforced net like cuticle bordering the reservoir, scale bars 100µm.

(**D**) 3D-reconstruction of nearly empty reservoirs (dark blue) in antennomere A4 to A8 surrounding the ventral antennal nerve (an, yellow). Class 3 cell units (c3, green) are only shown in A7. Due to missing slices in the semithin section series the antennomere A4 appears shorter than A5 to A8. The dorsal flaps

(fl) covering the reservoir openings are coloured turquoise (red arrow) route of reservoir content when pressed out.

(**E**) Longitudinal semithin section through antennomere showing the flap (fl) with membranous part (light blue) and projecting cuticle (*) controlling the reservoir outlet. Black arrows indicate the reinforced cuticle, scale bar 100µm. (red arrow) route of reservoir content when pressed out.

(**F**) Proximal view on 3D-reconstruction of a single reservoir showing the distribution of the reinforced cuticle (black arrows, red) bordering the lumen. (A1-A12) antennomeres; (c3) class 3 gland cell units; (fl) flaps at the dorsal opening of the reservoir. (an) antennal nerve; (ws) white substance; (red arrow) route of reservoir content when pressed out.

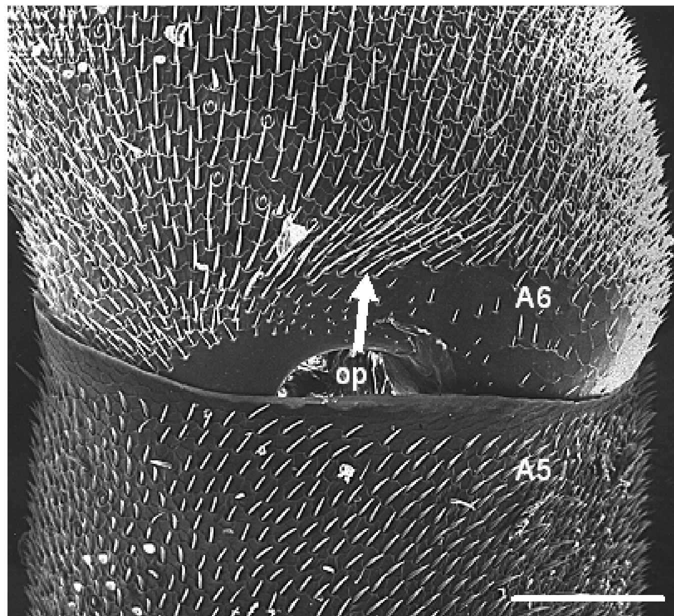

Figure 4.2: SEM micrograph of antennomeres A5 and A6 (left antenna). Antennomere A6 is bent downwards, so the dorsal opening (op) of the gland reservoir in A6 is visible, scale bar 100µm. (white arrow) route of reservoir content when pressed out

The reservoir's volume changes considerably with its filling status. A reservoir completely filled with white substance takes more than 50% of the antennomere volume and apparently even squeezes the antennal nerve between its two parts [Fig. 4.1B]. An empty reservoir, by contrast, appears completely collapsed with the opposing sides of the reservoir cuticle close to each other [Fig. 4.1C]. The 3D-reconstruction of filled reservoirs showed a maximum volume of 0.07 µl. Thus, the ten reservoirs of both antennae have a remarkable maximum volume of approx. 0.7 µl. Sections of antennae of females that had probably just delivered the white substance show that the reservoir is not totally empty but that some white substance remains in the rear parts of the lobes.

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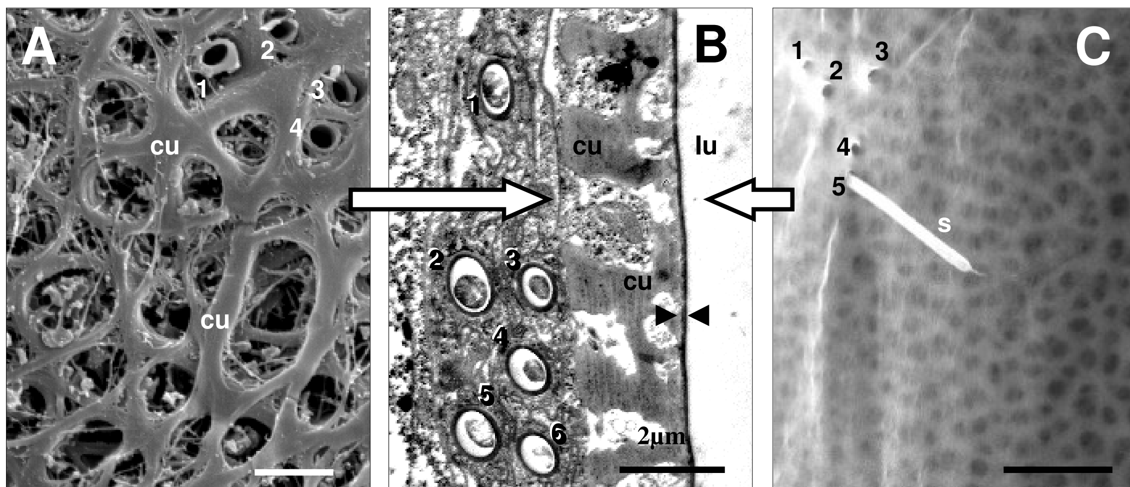

Figure 4.3: Electron micrographs of net-like cuticle bordering the reservoir.
(**A**) SEM: Exterior view of the cuticle with net-like structure (cu). The monolayered epithelium was eliminated during preparation, scale bar 2 µm. (1-4) conducting canals
(**B**) TEM: Monolayered epithelium covered with cuticle. A thin epicuticle (double arrow) separates the cuticle with net-like structure (cu) from the reservoir lumen (lu). A bundle of conducting canals (1-6) containing secretion penetrates the epithelium, scale bar 2 µm. Thick arrows indicate the line of view in (A) and (C).
(**C**) SEM: Interior view of the gland reservoir with openings of conducting canals (1-5) and filamentous secretion (s) coming out of canal 5. Note the net like structure of the reinforced cuticle shining through the thin epicuticle, scale bar 5 µm.

The gland reservoir of each antennomere is surrounded by loose groups of roughly 400 acini, each consisting of 1 to 8 class 3 gland cells [Fig. 4.1 B-E]. The acini are spherical or drop shaped with diameters up to 30 µm. They are almost evenly distributed over the surface of the reservoir, but slightly more abundant at the membranous cuticle of the medial side of the reservoir. Conducting canals connect the class 3 cells to the reservoir, whereas the canals of each acinus frequently form a bundle and open into the reservoir in groups [Fig. 4.3].

4.4.2 Ultrastructure

Scanning electron micrographs of the reservoir lumen show the canal openings as holes in the cuticle and the secretion of the gland cells appear as filamentous material emerging from the canals [Fig. 4.3C]. Where the bordering epithelium was removed during preparation the net like structure of the reinforced cuticle is clearly visible [Fig. 4.3A]. Noteworthy, this net like structure even shines through the epicuticle when scanning the inner side of the reservoir lumen [Fig. 4.3C]. This is due to the fact that the high voltage electron beam of the SEM has a penetration depth of a few micrometers, whereas the epicuticle is only about 0.2 µm thick [Fig. 4.3B].

TEM micrographs confirm the existence of a cuticular lining the reservoir lumen. The membranous reservoir cuticle is only about 1 µm, the reinforced net-like cuticle up to 4 µm thick [Fig. 4.3B, 4.4E]. The monolayered epithelium bordering the reservoir is 2 to 5 µm thick and also fills the interspaces of the net-like reinforced parts of the cuticle [Fig. 4.3B]. The cells of the epithelium are flat with comparatively large nuclei and are connected by septate desmosomes [Fig. 4.4D, F]. Especially the epithelium of the membranous cuticle contains numerous electron lucent vesicles with diameters up to 1 µm [Fig. 4.4D, E]. In the epithelium bordering the membranous projection at the opening of the reservoir there are invaginations of the basal cell membrane and microvilli at the apical side [Fig. 4.4D].

The class 3 gland cells forming the acini show a high density of rough endoplasmatic reticulum and both electron dense as well as electron lucent vesicles [Fig. 4.4A, B]. The majority of these vesicles are about 1 µm in diameter, whereas some show diameters of more than 4µm [Fig. 4.4A, B]. Some vesicles bear membrane structures which are best visible in electron dense vesicles [Fig. 4.4B]. Sometimes, the class 3 cells show invaginations of the parts of the plasmamembrane that are in contact with the hemolymph [Fig. 4.4C]. We found no conspicuous golgi apparatus in the class 3 cells.

The end apparatus in each gland cell is formed by a cuticular receiving canal associated with microvilli [Fig. 4.4A, B]. Canal cells encircle the conducting canals leading from the acini to the reservoir lumen [Fig. 4.4F]. The content of the receiving and conducting canals seems to be a mixture of electron dense and lucent secretion [Fig. 4.3B, 4.4A, B, E, F]. Apart from the conspicuous antennal nerve no axons linked to class 3 gland cells or the bordering epithelium were observed. Tracheae were found in the bordering epithelium, but they never penetrated the lumen of the reservoirs.

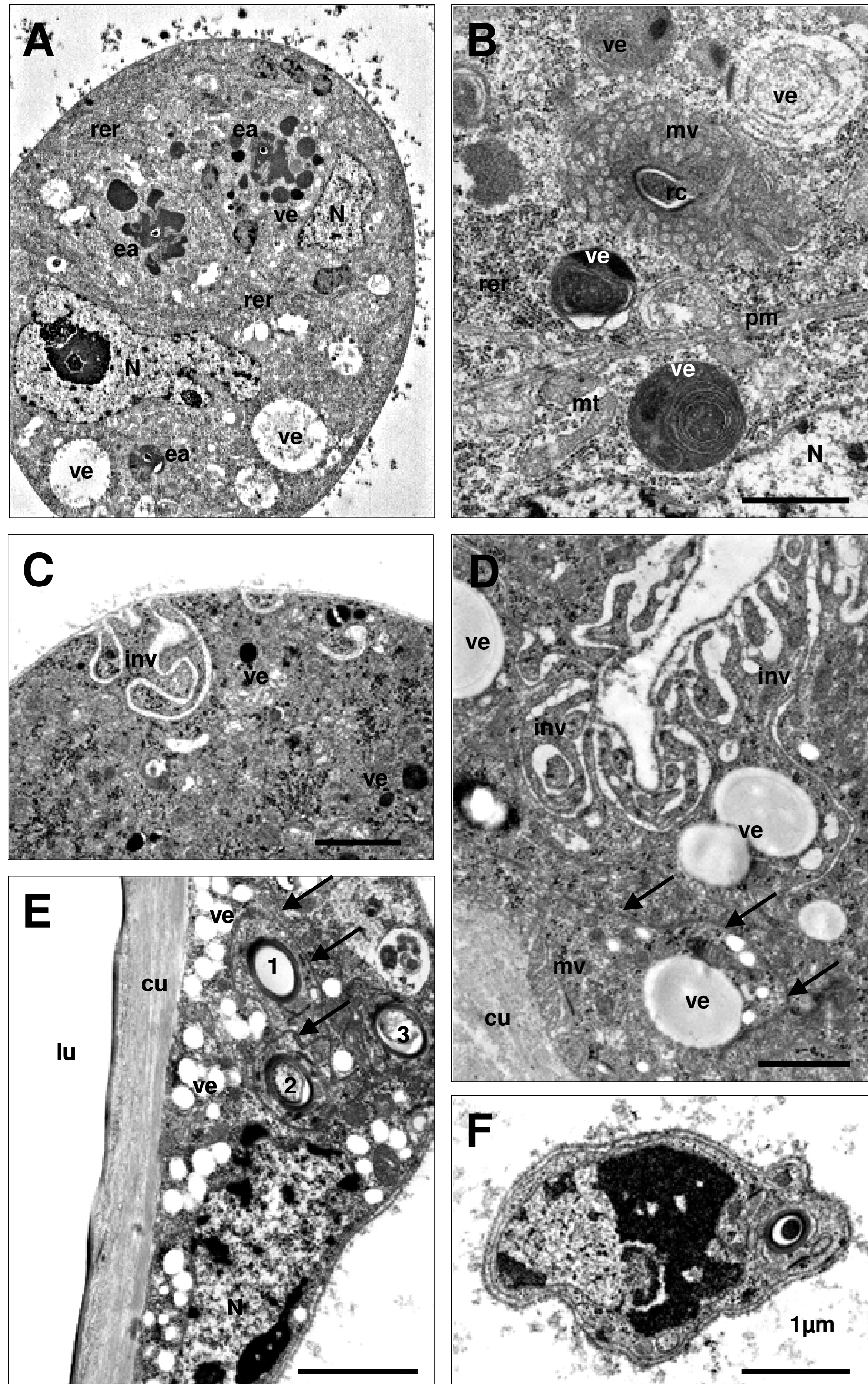

Figure 4.4: TEM micrographs of class 3 gland cells and epithelial cells surrounding the antennal gland reservoir of a female beewolf.

(**A**) Acini consisting of class 3 gland units. The cytoplasm bears many vesicles (ve), high density of endoplasmatic reticulum (rer) and end apparatuses (ea), scale bar 5 µm. (N) nucleus.

(**B**) End apparatus in class 3 gland cell consisting of receiving canal (rc) and microvilli (mv). Some vesicles (ve) bear membranous structures, scale bar 1 µm. (mt) mitochondria, (N) nucleus, (pm) plasma membrane, rer (rough endoplasmatic reticulum).

(**C**) Plasma membrane invaginations (inv) of class 3 gland cell at the hemolymph side, scale bar 5 µm. (ve) vesicles.

(**D**) Monolayered epithelium lined with membranous cuticle (cu) near the reservoir opening showing basal invaginations (inv), electron lucent vesicles (ve) and apical microvilli (mv). The epithelial cells are connected by septate desmosomes (arrows), scale bar 1µm.

(**E**) Monolayered epithelium lined by membranous cuticle (cu) with numerous electron lucent vesicles (ve). (lu) reservoir lumen, (N) nucleus, (1-3) conducting canals, (arrows) septate desmosomes, scale bar 2 µm.

(**F**) Conducting canal (cc) with electron dense content surrounded by canal cell, scale bar 1 µm. (N) nucleus.

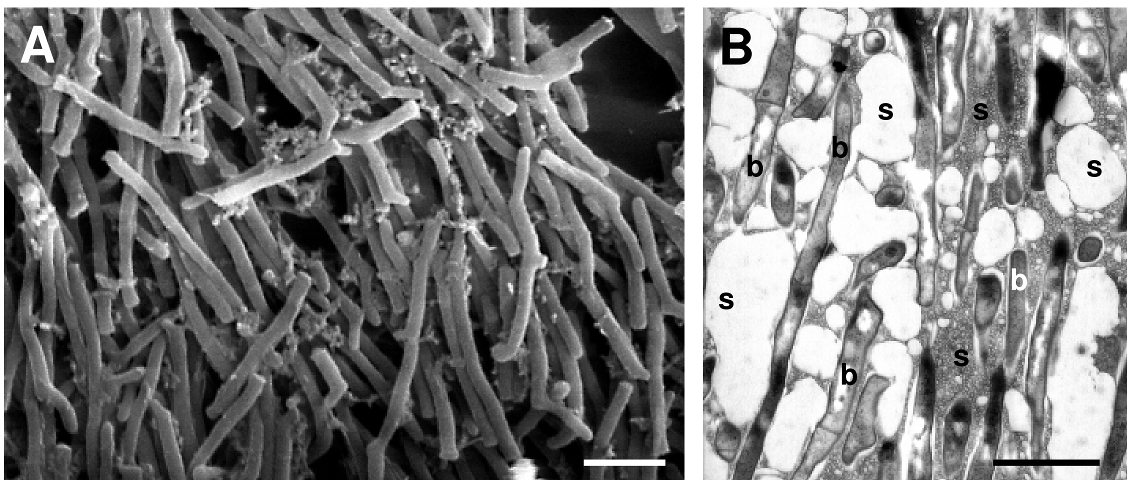

Figure 4.5: Contents of the antennal gland reservoir. (**A**) SEM micrograph of the filamentous *Streptomyces* bacteria. Due to preparation the secretion between the bacteria is reduced to some flakes, scale bar 2µm.

(**B**) TEM micrograph of the white substance inside gland reservoir. The *Streptomyces* bacteria (b) are embedded in secretion droplets (s) of various sizes. Daughter cells remain connected after cell division and form long filaments, scale bar 2µm.

4.4.3 Reservoir contents

The content of a reservoir consists of the primary secretion of the class 3 gland cells and the bordering epithelium, seen as dark and light vesicles (see above), and the symbiotic bacteria of the genus *Streptomyces* (Kaltenpoth *et al.* 2005, 2006). The bacteria are about 0.5 µm in diameter and form long and sometimes branched filaments. The branching is characteristic for actinomycetes [Fig. 4.5A, B]. The bacterial filaments are mostly aligned parallel and are embedded in electron lucent as well as electron dense secretion droplets of irregular shape and diameters of up to 5 µm [Fig. 4.5A, B].

4.5 DISCUSSION

Symbioses between insects and bacteria are often associated with the evolution of specialized organs in the insect hosts. Examples are some species of fungus-growing ants which rear antibiotic-producing bacteria in cuticular crypts that are associated with ectodermal glands (Currie *et al.* 2006) and *Tetraponera* ants which evolved bacterial pouches in their digestive tract (Billen & Buschinger 2000). Here we describe the morphology and ultrastructure of a unique bacteria cultivation organ in the antennae of female European beewolves. The lumen of the antennal gland reservoir harbouring the bacteria has two parts that make up a considerable fraction of the antennomer. The reservoir is enclosed by a monolayered epithelium with a cuticle and many class 3 gland cells that contain large numbers of rough endoplasmatic reticulum and apparently secrete compounds into the reservoir lumen.

In cross sections of empty antennal gland reservoirs of *P. triangulum*, the membranous cuticle appears slightly folded, whereas it is smooth and bulged in filled reservoirs. We thus propose that this thin cuticle is flexible, whereas the net-like cuticle is sturdy and remains mostly in place as a counter bearing. The gland reservoir could therefore be seen as a bellow with one rigid and one flexible side, which expands in response to the increase of the content. This structure, the to and fro movement of the female prior to and during the delivery of the white substance as well, as the lack of any muscles in the vicinity of the reservoir suggest that the content of the reservoir might be pressed out by increasing hemolymph pressure in the antennae. The projecting cuticle with membranous appendages that covers the reservoir's opening probably acts as a closing device to control the reservoir outlet. The transport of white substance out of the inter-antennomere space might be facilitated by the observed bending of the antennae.

Since the antennal gland reservoir is nearly empty after a female has delivered the secretion for one brood cell and females can construct and provision up to three brood cells per day (E. Strohm, M. Kaltenpoth & K. Roeser-Mueller, unpubl. data), beewolf females probably provide the symbiotic bacteria with essential nutrients to allow rapid growth and, thus, replenishment of the gland reservoir. In the reservoir, the Streptomycetes are embedded in a matrix of electron dense and electron lucent vesicles that may contain nutrients provided by the beewolf female. Noteworthy, the structure of the reservoirs with elongated lobes might ensure that a certain part of the reservoir content remains in the gland during the delivery of the white substance. These remains may facilitate the renewal of the bacterial population.

Nutrients for the symbiotic bacteria may be secreted into the reservoir by the surrounding class 3 gland cells or sequestered from the hemolymph by vesicles via the epithelial cells. The high abundance of rough surfaced endoplasmatic reticulum in the class 3 gland cells suggests protein synthesis at a high level that may provide the bacterial nutrients. Additionally, substances may

be sequestered from the hemolymph, as suggested by the invaginations of the cell membrane, stored in vesicles of the class 3 gland cells and later transported into the reservoir. Despite the numerous vesicles and membranous structures, we found no conspicuous golgi apparatus in the class 3 secretory cells.

Sequestration of substances from the hemolymph may also occur via the epithelium. Electron lucent vesicles, basal invaginations of the plasma membrane and apical microvilli strongly suggest a transport of substances from the hemolymph into the reservoir lumen. Chemical analyses of the reservoir contents using combined gas-chromatography and mass-spectrometry (GC-MS) revealed saturated and unsaturated hydrocarbons (C21-C31), branched alkanes, and ketones as the main components of the volatile fraction (Kaltenpoth *et al.* in prep.). Since these substances can also be found in the hemolymph of beewolf females in the same proportions (Strohm *et al.* in prep.), it seems likely that they are sequestered from the hemolymph and transported through the monolayered epithelium by the observed vesicles.

The evolutionary origin of the mutualism between beewolves and *Streptomyces* bacteria is not yet clear. Possibly, the ancestors of *P. triangulum* initially produced only the primary secretion of either the epithelium or the class 3 gland units in their antennal glands and delivered it as a directional cue for the cocoon alignment of the larvae (Strohm & Linsenmair 1995). At this point of evolution, the morphology of the antennal glands of beewolves may have been more similar to antennal glands like in extant formicidae (for phylogeny of the Hymenoptera see Brothers 1999; for antennal glands in ants see Isidoro *et al.* 2000). The *Streptomyces* bacteria might have secondarily invaded these glands and provided benefits for the larvae by protecting them against pathogen infestation (Kaltenpoth *et al.* 2005, 2006). Subsequently, natural selection could have rapidly changed the morphology of the antennal glands by forming large reservoirs that now function as cultivation organs for the bacterial partners.

Using genetic analysis, endosymbiotic streptomycetes have recently been found in the antennae of many species of *Philanthus*, but not in closely related species of Crabronid wasps (Kaltenpoth *et al.* 2006). It will be interesting to investigate the morphology of the respective glands in these congeneric species as well as other genera in the subfamily Philanthinae to elucidate the origin of these unique glands and the association with the *Streptomyces* bacteria. Moreover, most species of ground-nesting hymenoptera face similar threats by microbial attack of their offspring. Therefore, we predict that other hymenoptera might have evolved comparable symbioses with bacteria and corresponding structures for their cultivation to increase the survival of their progeny.

Acknowledgements

We are grateful to Prof. Dr. Georg Krohne, Daniela Bunsen and Elisabeth Meyer-Natus at the Dept. of Electron Microscopy (University of Würzburg) for their kind help with SEM and TEM preparation. We also would like to thank Angelika Kühn, Ursula Roth, Margot Schilling (Zoological Institute, University of Regensburg) for technical assistance. This study was partly supported by the German Science Foundation DFG (STR 532/2-1). This paper is dedicated to W. Rathmayer, who had independently discovered the glands in the early 1960s.

Supplemental Data

2 videos of rotating reconstructed antennal glands of female European beewolves.

Video 1: Single reconstructed antennomere.

Video 2: Five reconstructed antennomeres.

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CHAPTER 5

'CANDIDATUS STREPTOMYCES PHILANTHI', AN ENDOSYMBIOTIC STREPTOMYCETE IN THE ANTENNAE OF *PHILANTHUS* DIGGER WASPS

International Journal of Systematic and Evolutionary Microbiology 56 (6), June 2006: 1403-1411.

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5.1 SUMMARY

Symbiotic interactions with bacteria are essential for the survival and reproduction of many insects. The European beewolf (*Philanthus triangulum,* Hymenoptera, Crabronidae) engages in a highly specific association with bacteria of the genus *Streptomyces* that appears to protect the beewolf offspring against infection by pathogens. Using transmission and scanning electron microscopy, the bacteria were located in the antennal glands of female wasps, where they form dense cell clusters. Using genetic methods, closely related streptomycetes were found in the antennae of 27 *Philanthus* species (including two subspecies of *P. triangulum* from distant localities). In contrast, no endosymbionts could be detected in the antennae of other genera within the subfamily Philanthinae (*Aphilanthops, Clypeadon* and *Cerceris*). On the basis of morphological, genetic and ecological data, the new taxon '*Candidatus* Streptomyces philanthi' is proposed. 16S rRNA gene sequence data are provided for 28 ecotypes of '*Candidatus* Streptomyces philanthi' that reside in different host species and subspecies of the genus *Philanthus*. Primers for the selective amplification of '*Candidatus* Streptomyces philanthi' and an oligonucleotide probe for specific detection by fluorescence *in situ* hybridization (FISH) are described.

5.2 INTRODUCTION

Many insects have evolved associations with endosymbiotic bacteria that are essential for reproduction or survival of the host (Moran & Baumann 1994). Most of these bacteria are intracellular symbionts in specialist feeders, e.g. phloem-feeding, blood-sucking, or wood-feeding insects (Baumann & Moran 1997; Priest & Dewar 2000). Since the diets of these insects lack essential nutrients, they depend on bacteria that are able to synthesize the necessary compounds (Douglas 1998; Bourtzis & Miller 2003). In many cases, symbiotic bacteria are transmitted vertically from one generation to the next, resulting in coevolution and cospeciation of hosts and symbionts which is reflected in congruent phylogenies (Moran *et al.* 1993; Bandi *et al.* 1995; Baumann *et al.* 1997; Chen *et al.* 1999; Sauer *et al.* 2000; Lo *et al.* 2003).

The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) engages in a unique and highly specific symbiosis with bacteria of the genus *Streptomyces* (Kaltenpoth *et al.* 2005). Female beewolves construct nest burrows in sandy soil, hunt honeybees (*Apis mellifera*), paralyze them by stinging and provision one to five honeybees as larval food in each brood cell (Strohm 1995; Strohm & Linsenmair 1995). After feeding on the provisioned prey, larvae spin a cocoon in which they usually overwinter and emerge the following summer (Strohm & Linsenmair 1995). Since the conditions in the brood cells are humid and warm, there is a continuous threat that the female's investment could be destroyed due to fungal or bacterial infection of the provisions or the immature wasp (Strohm & Linsenmair 2001). Recent studies have shown that symbiotic bacteria protect beewolf offspring against fungal infection at the cocoon stage (Kaltenpoth *et al.* 2005).

The symbionts are cultivated in specialized antennal glands of the beewolf female and are secreted into the brood cell prior to oviposition (Strohm & Linsenmair 1995; Kaltenpoth *et al.* 2005). Later, they are taken up by the larva and applied to the outside of the cocoon, where they seem to serve as a protection against fungal infection, presumably by producing antifungal secondary metabolites (Kaltenpoth *et al.* 2005). A second function of the secretion is to direct the cocoon-spinning of the larva which facilitates its eventual emergence (Strohm & Linsenmair 1995). The bacteria certainly benefit from the association by obtaining an unoccupied and competition-free ecological niche and a reliable route of transmission into the next generation. They may also receive nutrients from the beewolf (M. Kaltenpoth & E. Strohm, unpubl. data). A similar symbiotic relationship for pathogen defense between insects and actinomycetes has been described for leaf-cutter ants (Currie *et al.* 1999): A species of the family Pseudonocardiaceae protects the ants' fungus gardens against a parasitic fungus by producing antibiotic substances (Currie *et al.* 1999; Cafaro & Currie 2005).

In the present study, we investigated 28 different *Philanthus* species and subspecies and several closely related genera for the presence of endosymbiotic *Streptomyces* bacteria in their antennae. Ultrastructural and genetic data (16S rRNA gene sequences) are presented that support the description of '*Candidatus* Streptomyces philanthi', including 28 ecotypes in different host species and subspecies.

5.3 METHODS

5.3.1 Specimens

Specimens of 27 *Philanthus* species including two subspecies of *P. triangulum*, two *Cerceris* species, *Aphilanthops frigidus*, and two *Clypeadon* species were collected in Germany, Greece, Oman, South Africa, Ukraine, and the USA (Table 5.1). The South African specimens were identified by comparison with voucher specimens in the collection of the Albany Museum in Grahamstown, South Africa, and the South African Museum in Cape Town, South Africa. The US species were identified according to Bohart & Grissell (1975) and Ferguson (1983a, b). Because males lack the relevant glands (Strohm & Linsenmair 1995) and the endosymbiotic bacteria have so far only been found in females (M. Kaltenpoth unpubl. data), only antennae from female specimens were used for electron microscopy and genetic analyses.

5.3.2 Electron microscopy

For scanning electron microscopy (SEM), specimens were fixed in alcoholic Bouin's fixative for 3 h and dehydrated in a graded acetone series. The objects were then critical point dried (CPD 030; BAL-TEC), sputtered with Pt/Pd (SCD 005; BAL-TEC) and examined with a digital scanning electron microscope (DSM 962; Zeiss). To investigate their interior ultrastructure, preserved antennae were cut with a razor blade before sputtering.

Specimens for transmission electron microscopy (TEM) were fixed for 2 h in a cold solution of 2% glutaraldehyde, 2.5% formaldehyde and 5% sucrose buffered in 50 mM sodium cacodylate, pH 7.2. After postfixation in 2% OsO_4 and dehydration in an ethanol series, the specimens were embedded in Epon 812. Ultrathin sections of about 70 nm thickness (MT-7000 microtome; RMC; 45° diamond knife) were stained with 2% uranyl acetate and Reynolds' lead citrate. Electron micrographs were taken with a transmission electron microscope (EM10; Zeiss) at 80 kV.

5.3.3 DNA extraction, PCR and sequencing

DNA was extracted from whole beewolf antennae according to a standard phenol/chloroform extraction protocol (Sambrook *et al.* 1989). The following primer pairs were used for amplification of *Streptomyces* 16S rRNA gene: fD1 (forward) (Weisburg *et al.* 1991) and StrepF (reverse) (Rintala *et al.* 2001), Act-S20 (forward) (Stach *et al.* 2003) and rP2 (reverse) (Weisburg *et al.* 1991). While fD1 and rP2 can be used to amplify a wide range of eubacterial 16S rRNA gene, the combination with StrepF and Act-S20 ensured that the PCR was specific for actinomycete 16S rRNA. PCR amplification was performed on Eppendorf Mastercyclers in a total reaction volume of 25 µl [containing 2 µl of template, 1x PCR buffer (10 mM Tris-HCl, pH 8.8; 50 mM KCl; 0.08% NP-40), 2.5 mM $MgCl_2$, 240 µM dNTPs, 20 pmol each primer, and 1 U *Taq* DNA polymerase (MBI Fermentas)]. Cycle parameters were as follows: 3 min at 94°C, followed by 32 cycles of 94°C for 40 s, 65°C for 1 min and 72°C for 1 min, and a final extension time of 4 min at 72°C. For sequencing, the following primers were used: fD1 (forward), Act-S20 (forward), Act-A19 (reverse) (Stach *et al.* 2003), StrepF (reverse), rP2 (reverse).

For the selective amplification of the *Philanthus* endosymbionts, the following forward primers were designed on the basis of the 16S rRNA gene sequences of the endosymbiotic *Streptomyces* and reference strains from the GenBank database: Strep_phil_fwd1: 5'-TACCGATCGCATGGTTGGTG-3', Strep_phil_fwd2: 5'-TATGACTACYGAYCGCATGG-3', Strep_phil_fwd3: 5'-CATGGTTRGTGGTGGAAAGC-3', Strep_phil_fwd4: 5'-GTGGTGGAAAGCTCCGGC-3' [binding to nucleotide positions 177-196, 170-188, 184-203, and 192-209, respectively, following the *Streptomyces ambofaciens* nomenclature (Pernodet *et al.* 1989)]. The forward primers Strep_phil_fwd1-4 were used in combination with the general actinomycete reverse primer Act-A19. Temperature gradient PCRs were performed for all primer combinations and two Mg^{2+} concentrations were used to adjust the stringency of the reaction (1.5 and 2.5 mM). Final PCR conditions were the same as described above, except that 1.5 mM $MgCl_2$ was used for Strep_phil_fwd4/Act-A19. The annealing temperature was set to 65°C for Strept_phil_fwd2/Act-A19, and to 68°C for the three other primer combinations. DNA extracts from the antennae of 27 *Philanthus* species and one subspecies, two *Cerceris* species, *Aphilanthops frigidus*, and two *Clypeadon* species (Table 5.1) were used as templates. Extracted DNA from cultures of *Streptomyces rimosus* DSM 40260^T, *S. aureofaciens* DSM 40631, and *S. venezuelae* DSM 40230^T was included to assess the specificity of the primers for *Philanthus* endosymbiont DNA.

5.3.4 Fluorescence *in situ* hybridization (FISH)

The general eubacterial probe EUB 338 (Amann *et al.* 1990) and the specific oligonucleotide probe SPT 177 (5`-Cy3-CACCAACCATGCGATCGGTA-3`) (Kaltenpoth *et al.* 2005) were used for FISH. *S. aureofaciens* DSM 40631, *S. venezuelae* DSM 40230^{T}, *S. rimosus* DSM 40260^{T} and *Bacillus subtilis* DSM 402 served as negative controls for the specific probe. The SPT177 probe is complementary to positions 177-196 of the *P. triangulum* endosymbiont 16S rRNA gene sequence (*S. ambofaciens* nomenclature; Pernodet *et al.*, 1989). Secretions of the white substance from beewolf females were harvested from brood cells and spread onto six-field microscope slides. Fixation and hybridization were carried out as described previously (Grimm *et al.* 1998), with minor modifications: the hybridization buffer contained only 50 ng labeled probe, and samples were incubated for 90 min. at 45°C for hybridization. For hybridization within the antennae, fresh female antennae were cut into sections with a razor blade and glued onto microscope slides. Fixation and pre-treatment of the samples was performed following a previously described protocol (Sauer *et al.* 2002). Hybridization was carried out as for the bacterial samples, but with 3 h incubation with the labeled probe.

5.3.5 Phylogenetic analysis

BioEdit 7.0.4.1 software was used to assemble and align sequences and to calculate DNA distances with the DNADIST 3.5c algorithm by Joseph Felsenstein. The alignment was checked by eye, and arbitrary alignment regions were excluded from further analysis. The aligned sequences were imported into PAUP 4.0. Phylogenetic trees were constructed based on 1324 bp of 16S rRNA gene sequences in a full heuristic search with tree bisection and reconnection (TBR) branch swapping and 10 random addition sequence replicates, saving no more than 100 trees with a score $\geq$ 100 per replicate. Gaps were treated as a fifth character state, and *Arthrobacter globiformis* DSM 20124^{T} was defined as the outgroup. Using the same settings, bootstrap values were obtained from a search with 1000 replicates.

5.4 RESULTS

5.4.1 Localization of endosymbionts

Scanning electron micrographs of the antennal surface of *Philanthus triangulum*, *P. loefflingi*, and *P. fuscipennis* females revealed that the bacteria are present at the openings of the antennal

glands from which they are secreted into the brood cell (Kaltenpoth *et al.* 2005) (Fig. 5.1). The appearance of symbiotic bacteria on the outer surface of the antennae is probably due to accidental compressions of the antennae prior to or during preservation; under natural conditions they are unlikely to be found on the antennal surface, except during the secretion process in the brood cell.

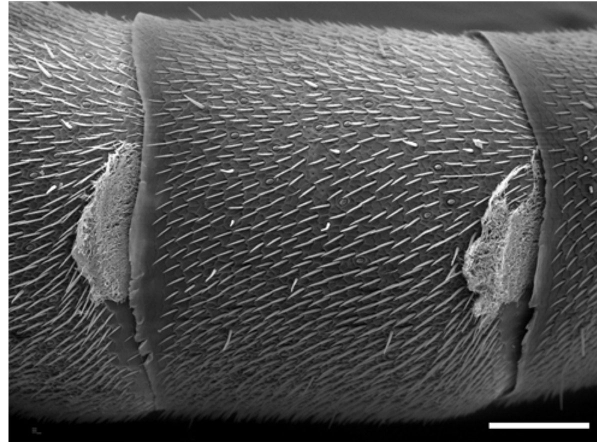

Figure 5.1: SEM image of an antenna of a female European beewolf (*P. triangulum*) with symbiotic *Streptomyces* bacteria being secreted from the antennal glands. Bar, 100 µm.

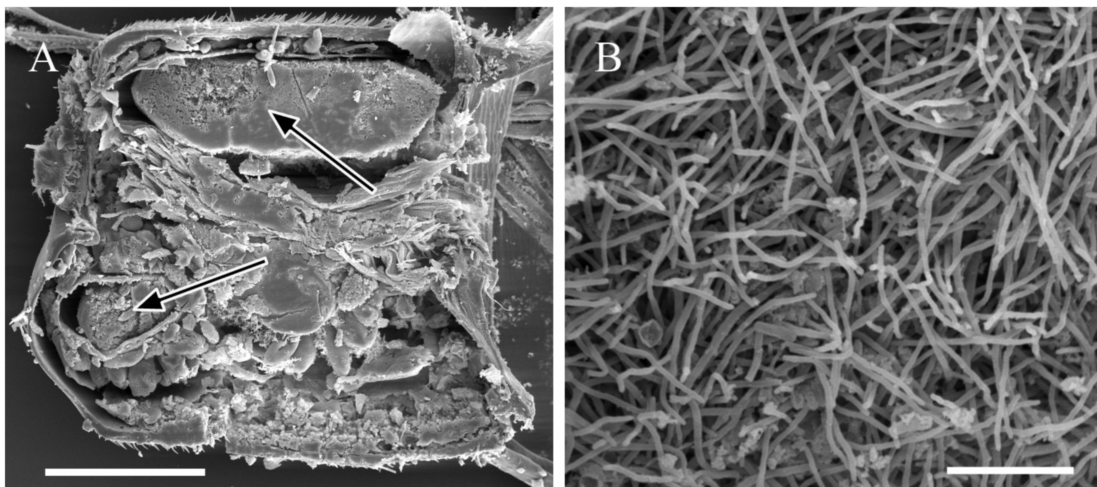

Figure 5.2: SEM image of the interior of an antennal segment of a female *P. loefflingi*. (A) Longitudinal section of a flagellomer. The reservoir of the antennal gland is indicated by arrows. (B) Symbiotic *Streptomyces* bacteria forming a dense cluster within the antennal gland. Bars, 200 µm (A) and 10 µm (B).

When a flagellomer was cut open, filamentous bacteria were clearly visible in large numbers within the gland reservoir (Fig. 5.2a), where they formed a dense cluster of cells (Fig. 5.2b). Transmission electron micrographs confirmed the presence of endosymbiotic bacteria within the antennal gland reservoir and suggest that the endosymbionts constitute the main component of the antennal gland content in female beewolves (Fig. 5.3). The bacteria showed a filamentous

morphology with long and sometimes branched cells and were embedded in a matrix containing numerous vesicles in the gland reservoir. Bacterial cells were 0.38 – 0.62 µm wide and highly variable in length (5 – 20 µm).

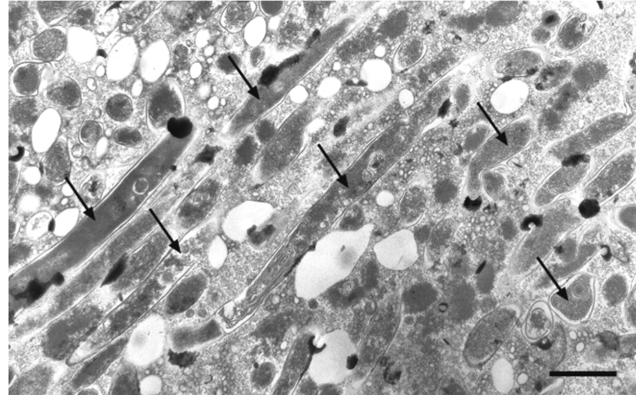

Figure 5.3: TEM image of a cross-section through the antennal gland of a female *P. triangulum*. Some endosymbiotic *Streptomyces* are indicated by arrows. Bar, 1 µm.

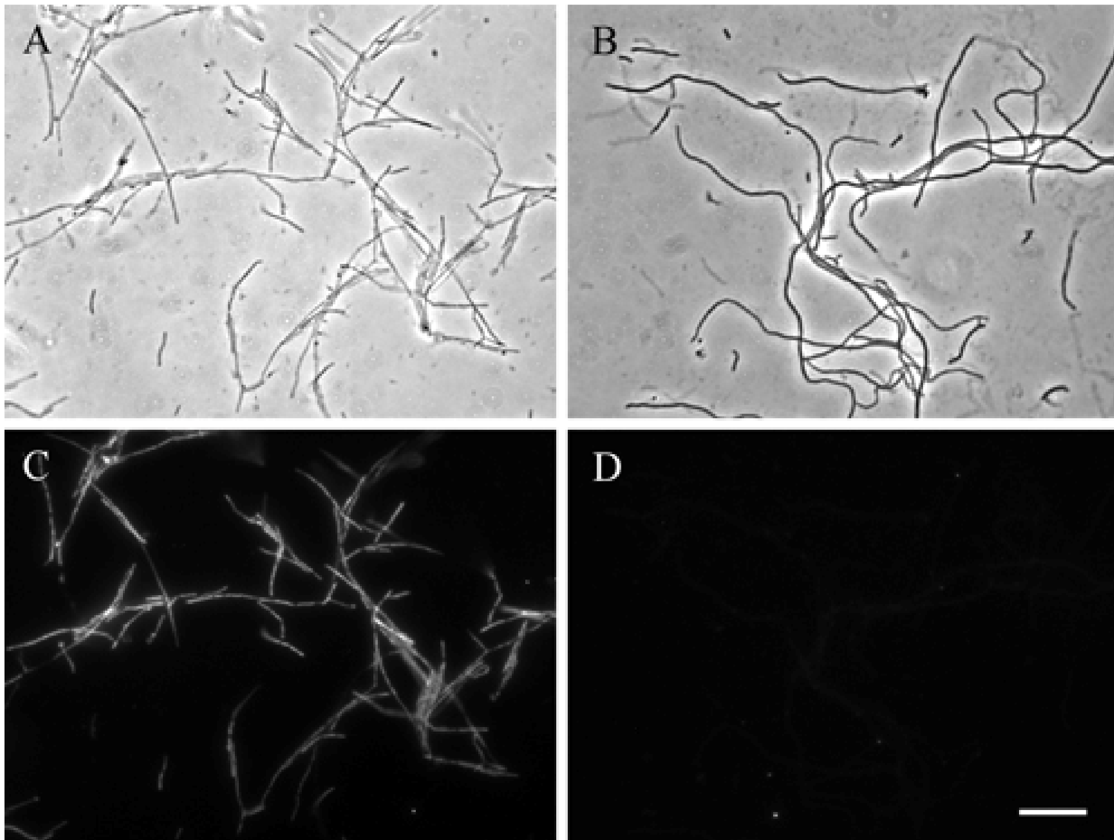

Figure 5.4: FISH of antennal *Streptomyces* endosymbionts. Phase-contrast micrograph of symbiotic bacteria in the antennal gland secretion of a female beewolf (A) and of a negative control strain of *Streptomyces rimosus* DSM 40260^T (B). (C, D) Epifluorescence micrographs of the same areas after staining with the specific Cy3-labeled probe SPT177. Bar, 10 µm.

The bacteria were clearly stained by the specific fluorescent probe SPT 177 both within female beewolf antennae and in the antennal gland secretion after it had been applied to the brood cell (Fig. 5.4). Reference strains of *S. aureofaciens, S. venezuelae, S. rimosus* and *B. subtilis* were not stained by the probe. The general eubacterial probe EUB 338 gave positive results in all cases.

5.4.2 Distribution of symbionts among philanthine wasps

Table 5.1: Occurrence of endosymbiotic *Streptomyces* bacteria in antennae of philanthine wasps (Hymenoptera, Crabronidae, Philanthinae) and amplification with the specific primers Strep_phil_fwd1-4 in combination with the general actinomycete primer Act-A19. To assess the specificity of the primers, the DNA of three cultivated *Streptomyces* species was included in the PCRs. ++, Strong amplification; +, weak amplification; -, no amplification; Y, symbionts present; N, symbionts not present; NA, not applicable; SA=South Africa, KZN=KwaZulu Natal, WCP=Western Cape Province, ECP=Eastern Cape Province. Standard two-letter abbreviations are used for US states.

Species	Specimens (n)	Geographical origin	Symbionts	Strep_phil amplicons				16s rRNA gene GenBank accession no.
				fwd1	fwd2	fwd3	fwd4	
***Philanthus* species**								
Philanthus barbiger	5	UT (USA)	Y	++	++	++	++	DQ375779
Philanthus basilaris	4	UT (USA)	Y	++	++	++	++	DQ375780
Philanthus bicinctus	3	WY (USA)	Y	++	++	++	++	DQ375781
Philanthus capensis	1	WCP (SA)	Y	++	++	++	++	DQ375782
Philanthus coarctatus	1	Oman	Y	++	++	++	++	DQ375783
Philanthus coronatus	1	Germany	Y	+	++	++	++	DQ375784
Philanthus crabroniformis	1	WY (USA)	Y	-	++	++	++	DQ375785
Philanthus crotoniphilus	2	UT (USA)	Y	++	++	++	++	DQ375786
Philanthus fuscipennis	4	ECP, WCP (SA)	Y	++	++	++	++	DQ375787
Philanthus gibbosus	4	UT (USA)	Y	+	++	++	++	DQ375788
Philanthus gloriosus	5	UT (USA)	Y	++	++	++	++	DQ375789
Philanthus histrio	1	WCP (SA)	Y	++	++	++	++	DQ375790
Philanthus inversus	2	UT (USA)	Y	+	++	++	++	DQ375791
Philanthus lepidus	3	MA (USA)	Y	++	++	++	-	DQ375792
Philanthus loefflingi	4	ECP, WCP (SA)	Y	++	++	++	++	DQ375793
Philanthus multimaculatus	7	UT (USA)	Y	++	++	++	++	DQ375794
Philanthus pacificus	4	UT (USA)	Y	++	++	++	++	DQ375795
Philanthus parkeri	6	UT (USA)	Y	++	++	++	++	DQ375796
Philanthus politus	1	MA (USA)	Y	+	++	++	+	DQ375797
Philanthus psyche	1	UT (USA)	Y	+	+	+	-	DQ375798
Philanthus pulcher	4	WY (USA)	Y	++	++	++	++	DQ375799
Philanthus rugosus	1	ECP (SA)	Y	+	++	++	++	DQ375800
Philanthus tarsatus	1	NE (USA)	Y	+	++	++	++	DQ375801
Philanthus triangulum triangulum	38	Germany, Greece, Ukraine	Y	++	++	++	++	DQ375802
Philanthus triangulum diadema	7	KZN, ECP, WCP (SA)	Y	++	++	++	++	DQ375803
Philanthus ventilabris	1	UT (USA)	Y	++	++	++	++	DQ375804
Philanthus venustus	3	Greece	Y	++	++	++	++	DQ375805
Philanthus zebratus	3	WY, CA (USA)	Y	++	++	++	++	DQ375806
Other wasp species								
Aphilanthops frigidus	1	MA (USA)	N	-	-	-	-	-
Cerceris arenaria	1	Germany	N	-	-	-	-	-
Cerceris rybyensis	3	Germany	N	-	-	-	-	-
Clypeadon haigi	1	UT (USA)	N	-	-	-	-	-
Clypeadon laticinctus	5	UT (USA)	N	-	-	-	-	-
Control bacterial species								
Streptomyces aureofaciens	NA	NA	NA	-	-	-	-	NA
Streptomyces rimosus	NA	NA	NA	-	+	-	-	NA
Streptomyces venezuelae	NA	NA	NA	-	-	-	-	NA

All 28 *Philanthus* species including the two subspecies of *P. triangulum* yielded amplicons of the expected length in at least three of the four PCR reactions with the specific 16S rRNA primers Strep_phil_fwd1-4 in combination with the general actinomycete primer Act-A19 (Stach *et al.* 2003) (Table 5.1). One species, *Philanthus psyche,* generally yielded only weak amplicons and failed to amplify altogether in one of the four specific PCRs. *Philanthus crabroniformis* and *Philanthus lepidus* also yielded no amplicons in one of the PCR reactions, but gave strong amplicons in all other PCRs.

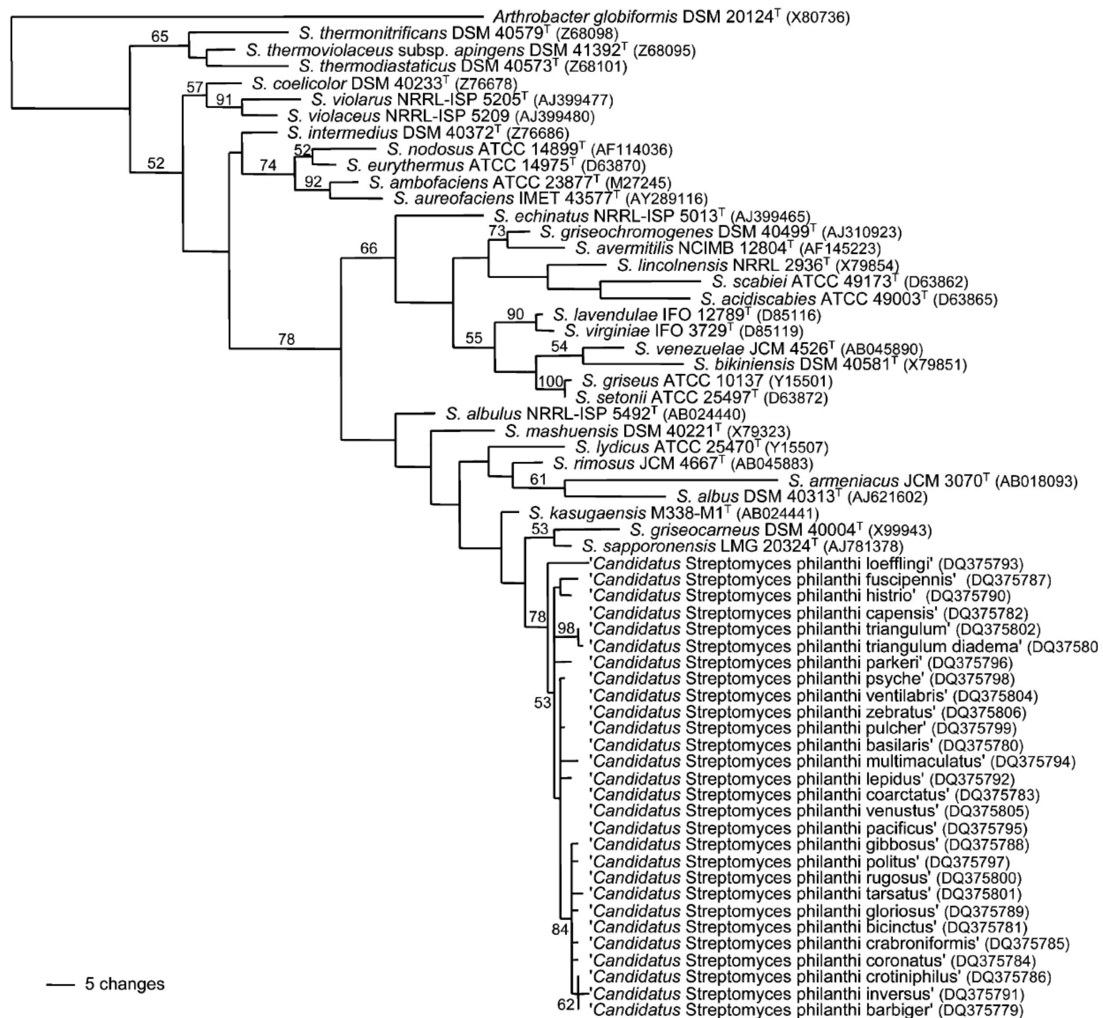

Figure 5.5: Phylogenetic position of *Philanthus* endosymbionts within the genus *Streptomyces* based on 1324 bp of 16S rRNA gene sequence (104 parsimony-informative characters). First of 600 equally parsimonious trees from a full heuristic search with random addition sequence (10 replicates) and TBR branch swapping. *Arthrobacter globiformis* was defined as the outgroup. Bootstrap values at nodes are percentages of 1000 replicates. GenBank accession numbers are given in parentheses. Bar, 5 changes.

Specimens of the other wasp genera of the subfamily Philantinae (*Aphilanthops, Clypeadon* and *Cerceris*) yielded no amplicons in any of the specific PCR reactions. In PCRs with general

actinomycete primers (Act-S20 and Act-A19), antennal DNA from *Aphilanthops, Clypeadon,* and *Cerceris* yielded no, or very weak, amplicons. The sequences obtained from the weak amplicons were not closely related to the *Philanthus* endosymbionts and were probably due to contamination of the antennae from surrounding soil during the life of the digger wasps within subterranean nests (data not shown). Thus, the symbiosis with bacteria of the genus *Streptomyces* seems to be widespread among wasps of the genus *Philanthus*, but appears to be absent in other genera of the subfamily.

The *Streptomyces* control strains yielded no amplicons in most of the PCRs, demonstrating specificity of the primers for the *Philanthus* endosymbionts. However, Strep_phil_fwd2/Act-A19 did amplify the 16S rRNA gene of *S. rimosus* DSM 40260^T, a close relative of the *Philanthus* symbionts (Fig. 5.5) which shares around 98.0 to 98.5% of its 16s rRNA gene sequence. Control PCRs with general actinomycete 16S rRNA primers (Act-S20/Act-A19) resulted in strong amplicons for all of the *Streptomyces* strains, showing that the lack of amplification in the specific PCRs was not due to general problems with the template DNA.

5.4.3 Phylogenetic position of '*Candidatus* Streptomyces philanthi'

The partial 16S rRNA gene sequences from the endosymbionts of 27 *Philanthus* species and one subspecies grouped together in a monophyletic clade within the genus *Streptomyces* (Fig. 5.5). The phylogenetic analysis indicates that the symbionts belong to the *Streptomyces armeniacus* group, the closest relatives being *Streptomyces kasugaensis* and *Streptomyces sapporonensis*, with a mean sequence divergence of about 1.1% and 1.2%, respectively. The similarity among the endosymbionts of the 28 different *Philanthus* taxa was relatively high, ranging from 98.9% to 100.0% 16S rRNA gene sequence similarity.

Almost complete 16S rRNA gene sequences for the 28 ecotypes of '*Candidatus* Streptomyces philanthi' have been deposited in the GenBank database with accession numbers DQ375779-DQ375806. The accession numbers for specific ecotypes are shown in Fig. 5.5 and Table 5.1.

5.5 DISCUSSION

Endosymbiotic bacteria of insects are usually localized in the gut or reside within specialized host cells, so-called mycetocytes or bacteriocytes, which often form dedicated organ-like structures or are associated with the mid-gut epithelium (Buchner 1921; Baumann & Moran 1997; Moran & Telang 1998; Ishikawa 2003). The *Philanthus-Streptomyces* association represents the first case of endosymbiotic bacteria being localized in insect antennae.

Correspondingly, the specialized antennal glands harbouring the symbionts have so far only been found in species of the genus *Philanthus* and appear to be absent even in closely related genera of philanthine wasps (Strohm, unpubl. data). As is the case with many other endosymbiotic bacteria, attempts to cultivate the *Philanthus* symbionts using standard cultivation techniques and media were not successful (see Chapter 5.6).

The endosymbionts are present in the antennal gland reservoir of *Philanthus* females in large numbers and they can be detected by SEM, TEM, FISH (with a specific oligonucleotide probe) and by PCRs with specific primers. Genetic analyses of the 16S rRNA gene sequences of endosymbionts from the antennae of different beewolf species revealed that all species investigated so far harbour *Streptomyces* bacteria, and that the *Philanthus* endosymbionts appear to represent a monophyletic clade within the genus *Streptomyces*. The antennal endosymbionts share on average 98.8-98.9% 16S rRNA gene sequence with their closest relatives, *S. kasugaensis* and *S. sapporonensis*. Despite this high sequence similarity, we propose the name '*Candidatus* Streptomyces philanthi' for the endosymbionts of *Philanthus* species because they are clearly separated from other species by their unique ecological niche. Several studies have shown that 16S rRNA gene sequence similarity alone is often inappropriate for the distinction of two species, and the general rule of 3% 16S rRNA gene sequence divergence between species tends to greatly underestimate the number of species (Cohan 2002; Konstantinidis & Tiedje 2005), as has been recently demonstrated for a number of *Streptomyces* groups (Sembiring *et al.* 2000; Manfio *et al.* 2003; Liu *et al.* 2005). Therefore, it is desirable to include ecological characteristics in the description of new species (Cohan 2002; Konstantinidis & Tiedje 2005). Among *Philanthus* endosymbionts, the 16S rRNA gene sequence similarity is relatively high (98.9% to 100.0%). We propose that the endosymbionts represent a single species with different ecotypes that are separated by their ecological niches (i.e. their host species).

The high degree of similarity among *Philanthus* endosymbionts suggests that they are transmitted vertically from mother to offspring, as has been described for many other endosymbiotic bacteria (Aksoy *et al.* 1997; Clark *et al.* 2000; Moran & Baumann 2000; Sauer *et al.* 2000; Clark *et al.* 2001; Ishikawa 2003). Alternatively, the bacteria may be taken up from the environment with certain mechanisms preventing the uptake of non-symbiotic bacteria, a transmission route that has been demonstrated for the symbionts of the squid *Euprymna scolopes* (McFall-Ngai & Ruby 1991; Nyholm *et al.* 2000; Nishiguchi 2002; Nyholm & McFall-Ngai 2004). The following evidence points to vertical transmission of the bacteria from mother to offspring in *Philanthus*: (i) the bacteria are secreted into the brood cell and later taken up by the larva and (ii) a female larva that was reared in the absence of the white substance in its brood cell apparently lacked the symbiotic bacteria as an adult (Kaltenpoth *et al.* 2005). However, further studies on the phylogenetic relationships of beewolves and their

endosymbionts are needed to confirm vertical transmission and to determine whether horizontal transfer of symbionts between *Philanthus* species (e.g. via chrysidid parasitoids, interspecific nest usurpation or nest reuse) may have played a role in the evolution of the symbiosis.

Moran *et al.* (1993) estimated an evolutionary age of 160-280 million years for the symbiosis between aphids and their endosymbiont *Buchnera aphidicola*, and Bandi *et al.* (1995) dated the origin of the association of cockroaches and termites with bacteria of the *Flavobacterium-Bacteroides* group to about 135 to 250 million years ago. Under the assumption of strictly vertical transmission of the symbionts, the low 16S rRNA gene sequence divergence among the endosymbionts of *Philanthus* wasps suggests that the symbiosis is of relatively recent origin. Assuming a mean rate of 0.008 to 0.02 substitutions per site per 50 million years (Ochman & Wilson 1987; Moran *et al.* 1993; Bandi *et al.* 1994), the maximum sequence divergence of 1.07% indicates that the origin of the symbiosis between beewolves and streptomycetes dates back about 26-67 million years. Taking into account that all *Philanthus* species investigated so far harbour the symbiotic bacteria, the association with bacteria probably evolved at around the time of origin of the genus *Philanthus*.

The evolution of specialized antennal glands in *Philanthus* females may have represented a key invention and evolutionary preadaptation for a symbiosis with *Streptomyces* bacteria. Strohm & Linsenmair (1995) demonstrated that the antennal gland secretion serves a second function by providing directional information to the beewolf larva that is necessary later for successful emergence. Thus, we hypothesize that the antennal glands originally evolved in the context of directing cocoon-spinning and emergence and that they might have been secondarily invaded by *Streptomyces* bacteria from the surrounding soil. In the beginning, the bacteria may have been commensals, or even parasites, in the antennal glands. In a sequence of evolutionary steps, including the uptake of the bacteria by the larva and their application to the cocoon, the antimicrobial activity of the streptomycetes might have been subsequently exploited by the beewolf hosts to protect their offspring against pathogen infection. Further studies are needed to investigate how related genera of ground-nesting digger wasps cope with the threat of pathogenic soil microorganisms infecting their progeny.

5.5.1 Description of '*Candidatus* Streptomyces philanthi'

'*Candidatus* Streptomyces philanthi' [phi.lan'thi. N.L. n. *Philanthus* (Hymenoptera, Crabronidae), the generic name of the host organism; N.L. gen. n. *philanthi* of *Philanthus*, referring to the association with digger wasps of the genus *Philanthus*].
The reference strain is '*Candidatus* Streptomyces philanthi triangulum'.

Uncultured, Gram-positive, non-motile, possibly sporulating, filamentous bacteria with sometimes branched cells that can be assigned to the genus *Streptomyces* on the basis of their 16S rRNA gene sequence. A detailed description of the methods used in an attempt to cultivate the endosymbionts can be found in the Appendix (Chapter 5.6). Cells are 0.38 – 0.62 µm wide and of highly variable length (5 – 20 µm). The bacteria live as symbionts within specialized antennal glands of female digger wasps of the genus *Philanthus*. They are secreted into the brood cells, taken up by the larva and applied to the cocoon, where they appear to protect the beewolf offspring against fungal infection (Kaltenpoth *et al.* 2005). Bacteria of different *Philanthus* species differ in their 16S rRNA gene sequence, but sequence divergence is relatively low (0-1.1%). We propose that endosymbionts of different *Philanthus* species should be treated as ecotypes of '*Candidatus* Streptomyces philanthi' and named according to the host species. The 16S rRNA gene sequences of all ecotypes found so far can be amplified selectively by the specific forward primer Strep_phil_fwd3 (5'-CATGGTTRGTGGTGGAAAGC-3') in combination with the general actinomycete reverse primer Act-A19 (Stach *et al.* 2003). The ecotype '*Candidatus* Streptomyces philanthi triangulum' can be stained with the fluorescent probe SPT 177: 5`-Cy3-CACCAACCATGCGATCGGTA-3` (Kaltenpoth *et al.* 2005).

[(Streptomyces) NC; G+; F; NAS (GenBank accession number DQ375802), oligonucleotide sequence of unique region of the 16S rRNA gene is 5'-TACCGATCGCATGGTTGGTG-3'; S (*Philanthus*, antennal glands); M]. Kaltenpoth *et al.*, this study.

5.6 APPENDIX

5.6.1 Attempts to cultivate '*Candidatus* Streptomyces philanthi'

In a first attempt to cultivate the *Philanthus* antennal symbionts, secretions from *Philanthus triangulum* female antennal glands were harvested from the brood cells and suspended in 100 µl of sterile water. 10-100 µl of the suspensions were spread onto a range of different solid media. Additionally, whole antennae of freshly killed female *P. triangulum* were plated out on the same media.

The following media were tested: LB agar (DSM Medium 381), Streptomyces Medium (DSM Medium 65), Streptomyces Medium supplemented with streptomycin (100 µg/ml) and kanamycin (50 µg/ml), Streptomyces Medium supplemented with homogenized bees from beewolf brood cells (12 bees per 500 ml medium), Streptomyces Medium supplemented with homogenized *P. triangulum* females (eight females per 20 ml medium), Powdered Chitin Agar (Hsu & Lockwood 1975), Powdered Chitin Agar supplemented with cycloheximide (100

µg/ml), and beewolf cocoon agar (a medium containing 30 empty *P. triangulum* cocoons per 250 ml agar medium). Plates were incubated at 25°C and 30°C under aerobic conditions for six to eight weeks.

Bacteria from culture plates were spread onto six-field microscope slides for fluorescence in-situ hybridization (FISH). The specific probe SPT 177 (Kaltenpoth *et al.* 2005) was used to screen for '*Candidatus* Streptomyces philanthi', and the general eubacterial probe EUB 338 (Amann *et al.* 1990) served as a positive control. Although bacterial colonies grew on all media tested and several of the colonies showed actinomycete morphology, none of the colonies was stained by the specific probe SPT 177. Amplification and sequencing of partial 16s rDNA sequences from some of the colonies with general eubacterial primers fD1 and rP2 (Weisburg *et al.* 1991) revealed the presence of *Acinetobacter* sp. and *Streptomyces* sp.

In a second cultivation attempt, female beewolf antennae were surface sterilized before cultivation. Therefore, four antennae were removed from live adult wasps and rinsed for 5 minutes in 1 ml of a sterile solution of 0.5% Triton X-100 to remove surface debris. The antennae were then surface sterilized by immersion in 1 ml of a freshly made sodium hypochlorite solution with 0.6 % available chlorine for 2 minutes. The antennae were then rinsed five times in 1 ml sterile water and transferred aseptically to a Dounce ground glass subcellular homogenizer (Kontes Scientific Glassware, Vineland, NJ) along with 1 ml sterile Mitsuhashi-Maramorosch (MM) basal medium (ICN Biomedicals). The antennae were then homogenized for 2 min to release bacteria and the homogenate was used as inoculum in a range of culture attempts.

Culture attempts were made using a range of solid media formulations under aerobic, anaerobic and microaerobic conditions. The media formulations tested included Streptomyces Medium (Sigma), supplemented with 0.2% (w/v) casamino acids (Difco), Potato Dextrose agar (Difco), MM agar (Dale *et al.* 2005), and Medium 199 (Gibco), solidified by addition of molten low-melt agarose (1% w/v final concentration) at 55 °C.

Cultures were initiated on solid phase media by streaking 20 µl of the antennal homogenate onto plates. Plates were incubated at 25 °C under an air atmosphere (to provide aerobic conditions) or in sealed gas jars flushed with at least 20 volumes of either nitrogen (for anaerobic conditions) or a mixture of 5 % oxygen, 10 % carbon dioxide and 85 % nitrogen (for microaerophilic conditions). Plates were maintained for 7 days and then removed and inspected under a stereo microscope. Bacterial colonies were removed and inoculated directly into PCR tubes. PCR was performed using universal bacterial 16S rDNA primers (Hugenholtz *et al.* 1998). The 16S rDNA amplicons were cloned into TOPO vectors, and sequenced using vector specific primers. The resulting sequences were then submitted to BLAST at the NCBI database.

Unfortunately, no *Philanthus* endosymbiont 16S rDNA sequences were detected; the 16S rDNA sequences obtained were all closely related to the genus *Serratia*.

5.6.2 Media Formulations

LB Agar

Trypone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Agar	15.0 g
Distilled water	1000.0 ml

pH adjusted to 7.0 with KOH before addition of agar and autoclaving.

Powdered Chitin Agar

Colloidal Chitin	4.0 g
K_2HPO_4	0.7 g
KH_2PO_4	0.3 g
$MgSO_4 \cdot 5\ H_2O$	0.5 g
$FeSO_4 \cdot 7\ H_2O$	0.01 g
$ZnSO_4$	0.001 g
$MnCl_2$	0.001 g
Agar	20.0 g
Distilled water	1000.0 ml

***Streptomyces* medium**

Glucose	4.0 g
Yeast extract	4.0 g
Malt extract	10.0 g
$CaCO_3$	2.0 g
Agar	12.0 g
Distilled water	1000.0 ml

pH adjusted to 7.2 with KOH before addition of agar and autoclaving.

MM agar

Sodium Chloride	7.0 g
Lactalbumin hydrolysate	6.5 g
Yeast extract	5.0 g
Glucose	4.0 g

$CaCl_2$	0.15 g
$MgCl_2$	0.05 g
KCl	0.2 g
$NaHPO_4$	0.17 g

Make up in 800 ml of water, add 0.12 g sodium bicarbonate, adjust pH to 6.9 and filter sterilize. Equilibrate the sterile media in a 55 °C water bath and then add 200 ml of autoclaved (and still molten) 5 % agarose. Pour plates and pre-equilibrate in a gas jar, if necessary.

Acknowledgements

We would like to thank Jon Seger, Michael Ohl, Protsenko Yura, Johannes Kroiss and Thomas Schmitt for providing *Philanthus* specimens. We are grateful to Fred and Sarah Gess and Simon van Noort for their help in collecting *Philanthus* in South Africa. The required collecting permits for South Africa were kindly issued by the nature conservation boards of KwaZulu Natal (Permit No. 4362/2004), the Eastern Cape Province (Permit No. 001-202-00026) and the Western Cape Province (Permit No. 001-506-00001). We want to thank the Department of Microbiology at the University of Würzburg for providing *Streptomyces* reference strains, and we thank Roy Gross for valuable comments on the manuscript. We gratefully acknowledge financial support from the German National Academic Foundation, the Arthur-von-Gwinner Foundation, the Unibund Würzburg, and the Deutsche Forschungsgemeinschaft (DFG STR 532/2-1 and DFG STR 532/1-2).

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Chapter 6

Chemical composition of the antennal gland secretion of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae)

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6.1 Summary

Many insects are associated with symbiotic microorganisms that supply the host with nutrients or serve as a defense against pathogens or parasitoids. European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae) cultivate bacteria of the genus *Streptomyces* within specialized antennal glands and secrete them to the brood cell. The bacteria are taken up by the larvae and used as a protection of the cocoon against fungi. We analyzed the chemical composition of the bacteria-containing antennal gland secretion (AGS) by SPME-GC-MS. We detected 28 substances, the majority of which were saturated and unsaturated hydrocarbons, with minor amounts of some branched alkanes, two ketones, and three unknown substances. Most of these are also found on the cuticle, in the hemolymph and in the postpharyngeal gland (PPG) of female beewolves in similar relative amounts. This suggests that hydrocarbons are sequestered into the gland from the hemolymph. There was a strong dimorphism in the chemical composition of the AGS among beewolf females. The two morphs had (*Z*)-9-pentacosene (C_{25}-type) and (*Z*)-9-heptacosene (C_{27}-type) as the main components, respectively, and they differed significantly in the relative amounts of most AGS substances. In addition to the chemical dimorphism, there were slight but significant interindividual differences in the chemical composition of the AGS among beewolf females, suggesting that beewolf females may differ in their ability to synthesize hydrocarbons and maybe nutrients for the bacteria.

6.2 INTRODUCTION

Many insects harbor endosymbiotic bacteria that are essential for reproduction or survival of the host (Moran & Baumann 1994; Bourtzis & Miller 2003). Most of these bacteria are intracellular symbionts in specialist feeders, e.g. phloem-feeding, blood-sucking, or wood-feeding insects (Baumann & Moran 1997). Because the diet of these specialists lacks essential nutrients, they depend on bacteria that are able to synthesize the limiting compounds (e.g. Nogge 1981; Sasaki *et al.* 1991; Sasaki & Ishikawa 1995; Schafer *et al.* 1996; e.g. Douglas 1998; Bourtzis & Miller 2003). In other cases, bacterial symbionts serve as a defense for the host by providing protection against pathogens (Charnley *et al.* 1985; Currie *et al.* 1999; Takatsuka & Kunimi 2000; Dillon *et al.* 2005), parasitoids (Oliver *et al.* 2003; Ferrari *et al.* 2004; Moran *et al.* 2005) or predators (Kellner 1999, 2001, 2002; Piel 2004). The bacteria benefit from symbiotic interactions with eukaryotes, because the hosts provide an unoccupied and long lasting ecological niche, supply them with nutrients, and ensure their transmission to the next generation (Currie 2001).

The European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) engages in a unique and highly specific symbiosis with bacteria of the recently described species '*Candidatus* Streptomyces philanthi' (Kaltenpoth *et al.* 2005; Kaltenpoth *et al.* 2006). Female beewolves construct nest burrows in sandy soil, hunt honeybees (*Apis mellifera*), paralyze them by stinging and carry the prey to the nest in flight (Strohm 1995; Strohm & Linsenmair 1995). One to five honeybees are provisioned as larval food in each brood cell. The larva feeds on the prey and spins a cocoon that is attached with its basal part to the wall of the brood cell. Larvae mostly overwinter and emerge next summer (Strohm & Linsenmair 1995; Strohm & Linsenmair 1999). Since the conditions in the brood cells are humid and warm, there is a continuous threat that the female's investment is wasted due to fungal or bacterial infestation of the provisions or the immature wasp (Strohm & Linsenmair 2001). Recent studies have shown that symbiotic bacteria protect the beewolf offspring against fungal infestation in the brood cell (Kaltenpoth *et al.* 2005).

The symbiotic bacteria are cultivated in specialized antennal glands of the beewolf female and are secreted to the brood cell prior to oviposition (Kaltenpoth *et al.* 2005; Kaltenpoth *et al.* 2006; Goettler *et al.* submitted). Later, they are taken up by the larva and applied to the outside of the cocoon, where they seem to provide protection against fungal infestation, presumably by producing antifungal secondary metabolites (Kaltenpoth *et al.* 2005). The bacteria certainly benefit from the association by obtaining an unoccupied and competition-free niche and a reliable route of transmission into the next generation. Furthermore, because the bacteria supposedly grow inside the antennal glands to replenish the stock for further brood cells, they probably receive nutrients from the host (Goettler *et al.* submitted). However, nutrient transfer from the beewolf to the bacteria has not yet been demonstrated.

Interestingly, the antennal gland secretion (AGS in the following) additionally serves a second purpose for the beewolf that has been described earlier (Strohm & Linsenmair 1995). The beewolf female always applies the secretion to the distal side of the brood cell, i.e. the side pointing away from the main nest burrow, thereby providing directional information to the larva (Strohm & Linsenmair 1995). The larva uses this information and attaches its cocoon to the distal side. Thus, when the adult beewolf emerges from the cocoon, it digs straight through the wall it is facing and reaches the still open main burrow, thereby finding the easiest way out of its brood cell deep in the soil (Strohm & Linsenmair 1995).

Both functions suggest that some chemicals are transported into the antennal reservoir of beewolf females, first to nourish the bacteria and to provide a detectable chemical marking for the orientation of the larva. Therefore, we analyzed the chemical composition of the AGS of female European beewolves using combined gas chromatography-mass spectrometry (GC-MS). Based on the ultrastructure of the walls of the reservoir, we furthermore hypothesized that some chemicals are sequestered from the hemolymph. Since these compounds are nearly identical to the content of the postpharyngeal gland (PPG, Strohm *et al.* in prep.), we compared the content of the antennal reservoir with that of the female PPG. We analyzed the antennal gland secretions of several brood cells from different females to assess the interindividual variability of the secretion, which allows us to draw conclusions on differences in the females' ability to mobilize chemicals for both the directional function and the bacteria in the AGS.

6.3 Methods and materials

6.3.1 Animals

Female European beewolves from populations in Würzburg and Schweinfurt, Germany, were reared in the laboratory at the University of Würzburg. Their daughters were kept individually in observation cages as described earlier (Strohm & Linsenmair 1995). They were supplied with honeybee workers (*Apis mellifera*) and honey *ad libitum*. The cages were checked daily for new brood cells, and brood cells in which the larva had not yet spun its cocoon (1-6 days old) were used for the chemical analysis of the AGS. The chemical composition of the AGS seemed to remain constant during the time in the brood cell (M.K., unpubl. data). The AGS of 37 brood cells from nine different beewolf females was analyzed by coupled gas chromatography – mass spectrometry.

6.3.2 Gas chromatography – mass spectrometry

Particles of AGS were touched with an SPME (solid phase micro-extraction) fiber coated with 100 µm polydimethylsiloxane (Supelco, Bellefonte, PA, USA) and analyzed by coupled capillary gas chromatography-mass spectrometry (GC-MS) 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 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. Peaks were identified by their retention times and mass spectra according to Strohm *et al.* (in prep.).

6.3.3 Statistical analysis

Some of the components in the AGS were present only in trace amounts and were below the detection limit in some of the samples. These peaks were excluded from further analysis. The remaining 19 components were manually integrated with MSD ChemStation software (Agilent Technologies) to calculate the relative amounts of these components in the white substance. Since there appeared to be two chemically distinct morphs of AGS with either pentacosene or heptacosene as their major component (C_{25}-type and C_{27}-type in the following), the relative amounts of the components were compared between these morphs in Mann-Whitney-U-tests using SPSS 12.0 software. A sequential Bonferroni correction was applied to correct for type I errors due to multiple testing (Rice 1989).

To assess the variation in the chemical composition of the AGS within and between beewolf individuals, the data from 28 secretions of four different females were subjected to a multivariate analysis using SPSS 12.0 software. Because the relative amounts constitute compositional data, they were transformed to logcontrasts prior to analysis (Aitchison 1986; Reyment 1989). The number of variables was reduced by a principle component analysis. The PCA factors were then subjected to a discriminant analysis (DA) to assess whether the white substance from brood cells of different females can be separated on the basis of their chemical composition.

For comparison of the chemical composition of the AGS with that of the postpharyngeal gland (PPG in the following) of female European beewolves, the PPGs of four females were extracted

as described elsewhere (Strohm *et al.* in prep.). For the comparison, 18 peaks were included that were detected in sufficient amounts in both the AGS and the PPG content. Although the position and configuration of double bonds was not determined for all of the unsaturated hydrocarbons in the AGS, the identity of the respective peaks in the AGS and in the PPG could be confirmed by comparisons of retention times and mass spectra. The relative peak areas were transformed to logcontrasts (Aitchison 1986; Reyment 1989) and then normalized by log_{10}-transformation prior to the analysis. Only the more frequent C_{25}-type females were included in the analysis to avoid systematic errors due to different proportions of the two chemical morphs in both datasets. Since both variables are GC-MS measurements and thus have the same measurement error, we performed a reduced major axis (RMA) regression to describe their relationship (Legendre and Legendre, 1998) using 'RMA Software for Reduced Major Axis Regression v.1.17' (A. J. Bohonak, San Diego University, U.S.A; freely available at http://bio.sdsu.edu/pub/andy/RMA.html). To assess the chemical similarity between the AGS and the PPG content, we tested for direct proportionality: i.e. the slope of the resulting regression line should not deviate significantly from 1 and the y-intercept should not deviate significantly from 0.

6.4 RESULTS

6.4.1 Chemical composition of the AGS

In the AGS of female European beewolves, 28 substances were identified by coupled GC-MS (Table 6.1, Fig. 6.1). The majority of these substances were saturated and unsaturated hydrocarbons, with minor amounts of branched alkanes, two ketones, and three unknown substances.

6.4.2 Interindividual differences in the chemical composition of the AGS

There was a distinct dimorphism in the chemical composition among beewolf females: The AGS of most females contained (*Z*)-9-pentacosene as the main component (C_{25}-type), whereas some females had (*Z*)-9-heptacosene as the dominant peak (C_{27}-type) (Fig. 6.1). The relative amounts of 15 out of 19 analyzed peaks of the AGS differed significantly between C_{25}- and C_{27}-type females (Table 6.1).

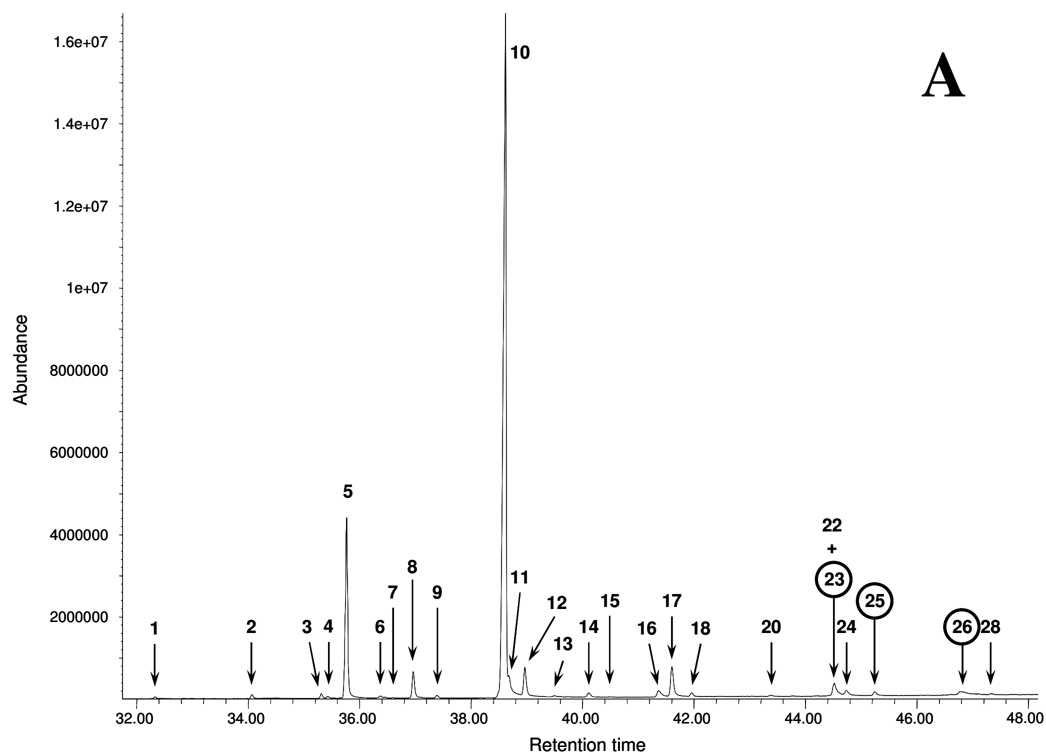
In the PCA, four principal components explaining 89% of the total variance were extracted. The discriminant analysis revealed significant differences in the composition of the AGS among

beewolf individuals (Fig. 6.2; Wilk's Lambda = 0.024, X^2 = 89.9, df = 6, P < 0.001). The two calculated discriminant functions resulted in 71.4% correct classifications (25% were expected by chance). The interindividual differences were mainly due to the difference between C_{25}- (females #10, 13, 15) and the one C_{27}-type female (female #14). However, when the C_{27}-type female was excluded from the analysis, the difference was still significant (Wilk's Lambda = 0.307, X^2 = 17.1, df = 8, P = 0.029), and 68.4% of the cases were classified correctly (33% were expected by chance).

Table 6.1: Chemical composition of the AGS of female European beewolves, *Philanthus triangulum*. Of the 28 components, nine were present in such low amounts that they were below the detection limit in most of the samples. These components were excluded from the statistical analysis. The mean relative amounts of these components were compared between C_{25}- and C_{27}-type females in Mann-Whitney-U-tests. Significant differences after sequential Bonferroni correction (P < 0.05) are flagged with asterisks (*).

Peak no.	Substance	Retention time (min.)	Mean relative amount (in %) C25-type	C27-type	P
1	Heneicosane	32,33	0,18	0,37	<0.001 *
2	Docosane	34,07	0,33	0,35	0.170
3	Tricosene (1)[1]	35,31	0,57	0,18	<0.001 *
4	Tricosene (2)[1]	35,43	0,17	0,03	<0.001 *
5	Tricosane	35,77	15,90	16,91	0.009 *
6	Methyltricosane (11-, 9-, 7-)	36,38	0,34	0,23	0.005 *
7	5-Methyltricosane	36,60	-	-	-
8	Δx-Tetracosene[1]	36,96	2,65	0,37	<0.001 *
9	Tetracosane	37,39	0,31	0,40	<0.001 *
10	Z-9-Pentacosene	38,62	68,35	8,84	<0.001 *
11	Z-7-Pentacosene	38,71	-	-	-
12	Pentacosane	38,97	4,34	5,39	0,001 *
13	Methylpentacosane (11-, 9-, 7-, 5-)	39,48	-	-	-
14	Δx-Hexacosene[1]	40,10	0,43	3,07	<0.001 *
15	Hexacosane	40,48	-	-	-
16	Δ-16-Pentacosen-8-one	41,36	0,65	0,15	<0.001 *
17	Z-9-Heptacosene	41,61	3,10	56,74	<0.001 *
18	Heptacosane	41,96	0,60	1,36	<0.001 *
19	Δx-Octacosene[1]	43,03	-	-	-
20	Octacosane	43,36	0,09	0,12	0.612
21	Δ-18-Heptacosen-10-one	44,21	0,03	1,18	<0.001 *
22	Δx-Nonacosene[1]	44,52	0,87	3,08	<0.001 *
23	Unknown compound 1	44,56	-	-	-
24	Nonacosane	44,73	0,82	1,07	0.036
25	Unknown compound 2	45,24	-	-	-
26	Unknown compound 3	46,76	-	-	-
27	Δx-Hentriacontene[1]	47,07	-	-	-
28	Hentriacontane	47,34	0,24	0,18	0.384

[1]position and configuration of the double bond were not determined



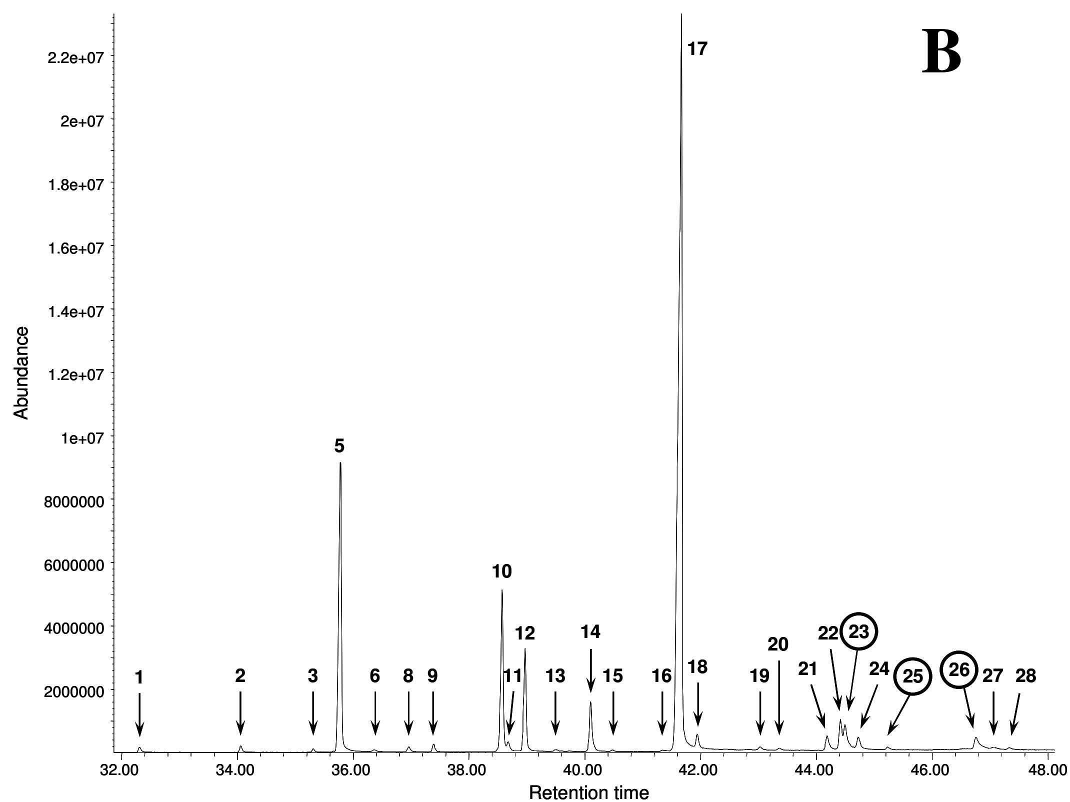


Figure 6.1: Chemical composition of the AGS of female European beewolves, *Philanthus triangulum* as revealed by SPME-GC-MS. Peaks only found in the AGS and not in the PPG or in the hemolymph are highlighted by circles. Peak numbers correspond to the components listed in Table 6.1. **A** C_{25}-type female. **B** C_{27}-type female.

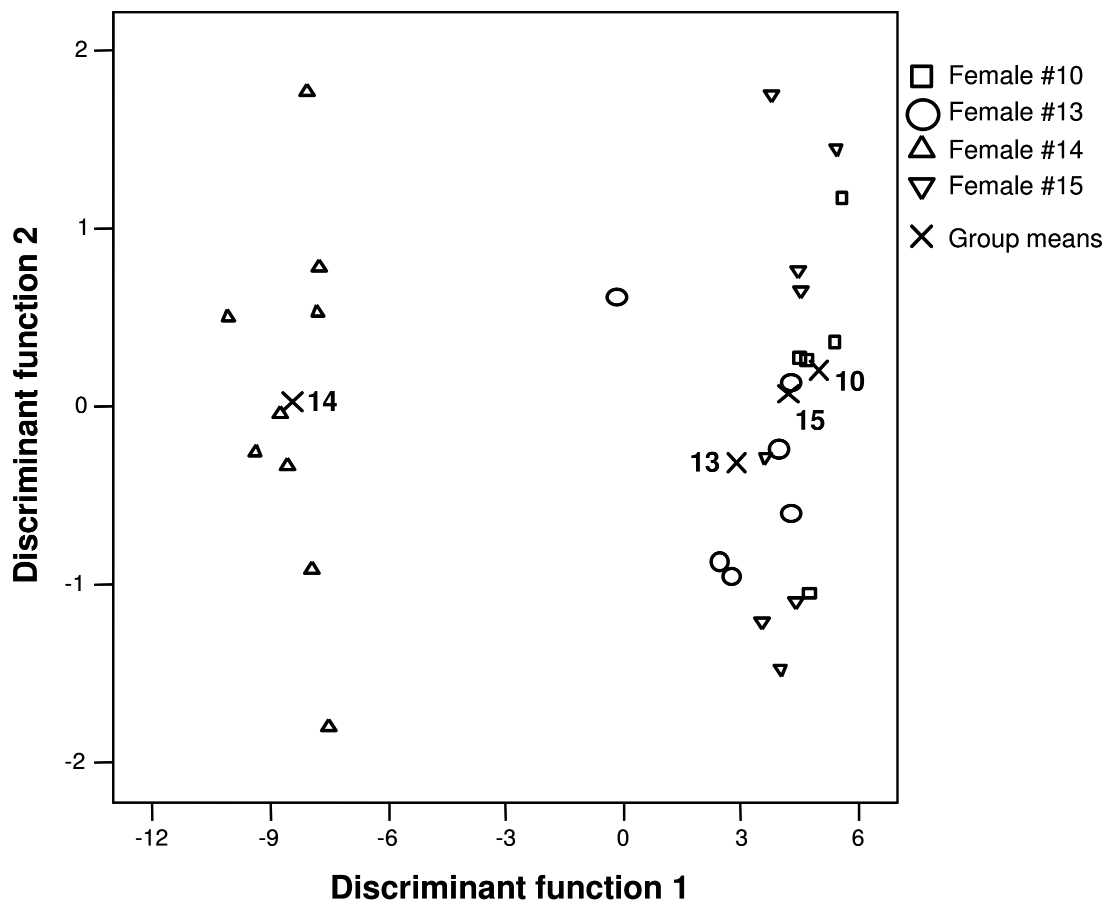


Figure 6.2: Differences in the chemical composition of the AGS among beewolf females. Each data point represents the composition of the AGS from a different brood cell. The discriminant analysis was performed based on four principal components extracted from the Aitchison-transformed data of 19 peaks. The chemical composition of the secretion differed significantly among beewolf individuals (Wilk's Lamba = 0.024, $P < 0.001$). Female #14 exhibits the C_{27}-type, all other females the C_{25}-type. Note the considerable variation among brood cells of the same individual.

6.4.3 Comparison of the chemical compositions of AGS and PPG

In the PPG extracts, 43 components could be detected (data not shown, see Strohm *et al.* in prep.) as compared to 28 substances in the AGS. The additional components were present in very small amounts and were probably detectable in the PPG but not in the AGS due to the much higher concentration of hydrocarbons in the PPG. Of the 28 compounds in the AGS, all except the three unknown substances were also found in the PPG.

For the comparison, 18 peaks were included that were detected in sufficient amounts in both the AGS and the PPG content. The relative amounts of the corresponding compounds in the AGS and in the PPG showed a strong linear correlation (R^2 = 0.913 after Aitchison- and $\log_{10}$-transformation; Fig. 6.3). The slope of the RMA-regression line was 1.073 (95% confidence intervals: 0.905 and 1.241), the y-intercept was 0.034 (95% confidence intervals: -0.099 and 0.167). Thus, there was no significant deviation from direct proportionality. The only substance deviating conspicuously from the regression line was Δ-18-heptacosen-10-one (peak no.21), which was more abundant in the PPG than in the AGS. Heneicosane (peak no.1) and Δ-16-Pentacosen-8-one (peak no.16) were present in slightly higher amounts in the PPG than in the AGS, while Δx-Nonacosene (peak no.22) was slightly more abundant in the AGS than in the PPG.

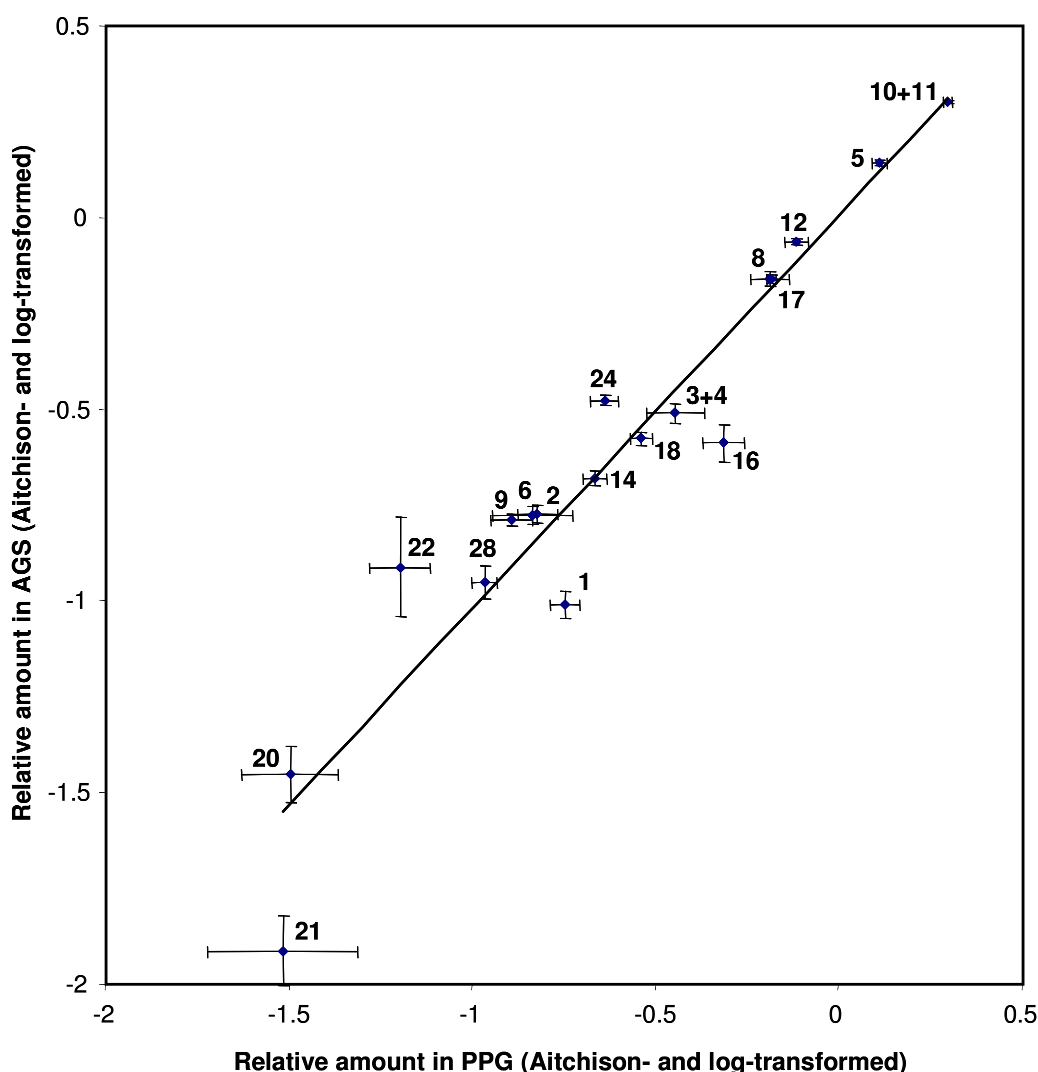


Figure 6.3: Correlation between relative amounts of hydrocarbons in the AGS and in the PPG based on C_{25}-type females only (after Aitchison- and $\log_{10}$-transformation). The bars represent the standard errors of the means. The numbers correspond to the peak numbers in Table 6.1. The reduced major axis (RMA) regression line follows the equation y = 1.073x + 0.034.

6.5 DISCUSSION

GC-MS analyses of the AGS of female European beewolves revealed large amounts of long-chain saturated and unsaturated hydrocarbons, two ketones and three unknown substances. The chemical composition is very similar to that of the postpharyngeal gland (PPG) content, the hemolymph and the cuticular hydrocarbon profile of female beewolves (Fig. 6.3; Strohm *et al.* in prep.). In fact, all but three components of the AGS are also detectable in the PPG, in the hemolymph and on the cuticle in similar relative amounts. Furthermore, all of these compartments exhibit the same distinct chemical dimorphism with the more common morph having (*Z*)-9-pentacosene as the main component, while the rarer type possesses (*Z*)-9-heptacosene as the major peak.

Based on the chemical similarity of hemolymph and PPG content, Strohm *et al.* (in prep.) suggested that the hydrocarbons may be produced in the oenocytes, as has been demonstrated for other insects (Soroker & Hefetz 2000; Fan *et al.* 2003), and then transported to the PPG. The same might be true for the AGS, with the hydrocarbons being transported into the antennal gland reservoir via the hemolymph. Ultrastructural investigations of the antennal gland reservoir have shown that parts of the surrounding walls show a net-like structure with a very thin cuticle as well as vesicles and microvilli that are indicative of a transport of chemicals across the epithelium that forms the walls of the reservoir (Goettler *et al.* submitted). Thus, transport of substances into the reservoir seems morphologically feasible, and it is possible that nutrients for the symbiotic bacteria are transported into the gland reservoir from the hemolymph (Goettler *et al.* submitted).

Our results show that the chemical composition of the AGS from different beewolf females differ significantly. This interindividual variability may reflect differences in the females' capability to mobilize hydrocarbons for the incorporation into the AGS , which may suggest that females differ in their ability to provide the symbiotic bacteria with nutrients. However, the composition of the secretion also showed considerable variation among brood cells of the same beewolf female, and the differences between individuals were in fact surprisingly small. The chemical composition of the PPG content is likewise subject to intra-individual variation during the lifetime of a female beewolf (Herzner *et al.*, unpubl. data). As mentioned above, both the AGS and the PPG content probably covary with the hydrocarbon composition of the hemolymph. In female German cockroaches, the hydrocarbon content of the hemolymph has been shown to fluctuate with the reproductive state, presumably due to the elevated demand for hydrocarbons in the developing oocytes (Sevala *et al.* 1999).

The function of the hydrocarbons in the AGS is not completely clear yet. Although most hydrocarbons (especially *n*-alkanes) exhibit low chemical reactivity, many microorganisms

have evolved pathways to utilize saturated and unsaturated hydrocarbons as a growth substrate (e.g. Zobell 1946; Berthe-Corti & Fetzner 2002). However, if the beewolf symbionts use the hydrocarbons in the AGS as carbon sources, they would require additional nutrients for nitrogen supply. Since hydrocarbons are often employed as olfactory signals in insects (e.g. Ayasse *et al.* 2001; Jurenka 2004; Keeling *et al.* 2004; Howard & Blomquist 2005), it seems more likely that the hydrocarbons in the AGS function as an olfactory or gustatory cue for the larva to localize the secretion. Localization of the AGS is essential for the larva for two reasons: (1) The AGS provides the directional information that is necessary for correct cocoon-spinning and emergence (Strohm & Linsenmair 1995), and (2) it contains the bacteria that the larva needs to take up and apply to its cocoon to protect it against fungal infestation (Kaltenpoth *et al.* 2005). Further experiments are necessary to investigate whether the larva indeed locates the AGS by the hydrocarbons and whether they are sufficient to provide the spatial information that is necessary for cocoon-spinning and emergence. The functional duality of the AGS with the hydrocarbons for directing cocoon-spinning and the bacteria for cocoon protection is consistent with the hypothesis proposed by Kaltenpoth *et al.* (2006) that the specialized antennal glands originally evolved for providing the directional information, and that they have been secondarily invaded by the symbiotic bacteria.

The three substances from the AGS that are not present in the hemolymph of female beewolves may either be secreted from the class 3 gland cells surrounding the reservoir or constitute secondary metabolites from the symbiotic bacteria in the AGS. The hypothesis of a bacterial origin of the three unknown compounds seems more likely, since the same substances were also detected on beewolf cocoons (M. Kaltenpoth, unpubl. data), where the bacteria occur in large numbers (Kaltenpoth *et al.* 2005). On the cocoon, two of these compounds are present in much larger amounts than in the AGS (M. Kaltenpoth, unpubl. data), so they may play a role in the protection of the cocoon against fungal infestation (see Kaltenpoth *et al.* 2005). Streptomycetes in general are known to produce a wide variety of secondary metabolites, many of which have potent antibiotic properties (Behal 2000; Watve *et al.* 2001). Identification of the three unknown compounds and analyses of the non-volatile fraction of the AGS (e.g. proteins, amino acids, lipids, carbohydrates) may provide valuable insights into metabolic interactions between hosts and symbionts and might lead to the discovery of novel antimicrobial substances that may prove useful for human medicine.

Acknowledgements

We would like to thank Gudrun Herzner for the PPG data and Johannes Kroiss for help with the statistical analysis. We gratefully acknowledge financial support from the German National Academic Foundation through a fellowship for M.K. This study was partly supported by the DFG (SFB 554 TP B3, DFG STR 532/2-1 and DFG STR 532/1-2).

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CHAPTER 7

THE CHEMISTRY OF THE POSTPHARYNGEAL GLAND OF FEMALE EUROPEAN BEEWOLVES (HYMENOPTERA, CRABRONIDAE) SUPPORTS A HOMOLOGY WITH THIS GLAND IN ANTS

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7.1 SUMMARY

Female European beewolves possess large glove-like head glands that morphologically resemble a gland in ants that was thought to be restricted to the formicidae, the postpharyngeal gland (PPG in the following). Beewolf females apply the content of the PPG on their prey, paralysed honeybees, where it delays fungus infestation. Here we test the hypothesis that the chemical characteristics of the putative PPG of beewolves are similar to those in ants. We tested three predictions based on the characteristics of the PPG in ants and obtained results in support of the hypothesis that the PPGs of both taxa are homologous. First, the PPG of beewolves contains mainly long chain hydrocarbons and few compounds with functional groups. Surprisingly, there was a dimorphism in the major component of the PPG with some females having pentacosene, others heptacosene as their predominant component. Second, the content of the PPG of beewolf females is similar to that of the cuticle. Third, the content of hydrocarbons in the PPG is similar to that in the hemolymph. Together with the structure of the gland epithelium and the huge requirements of gland secretion, the latter result suggests that the content of the putative PPG of beewolf females is sequestered from the hemolymph. Although there are some differences in the chemical nature of the PPGs of ants and beewolves, we conclude that our results are consistent with a homology between the PPG of ants and beewolves.

7.2 Introduction

Hymenoptera possess a huge variety of exocrine glands (e.g. Hölldobler & Wilson 1990). The chemistry and function of different types of these glands has been studied for a number of social species, whereas comparatively little is known from solitary bees and wasps. One type of gland that has recently received much attention because of its significance for social interactions is the postpharyngeal gland (PPG) in ants. It is a paired glove-like evagination of the pharynx and contains mainly long chain straight and branched hydrocarbons. The chemicals in the gland reservoir are partly sequestered from the hemolymph that typically contains the hydrocarbons that are found on the cuticle (Soroker *et al.* 1998; Soroker & Hefetz 2000). However, the content of the PPG is also in continuous exchange with the cuticles of nestmates since the cuticular hydrocarbons are constantly taken up from and delivered to nestmates during allogrooming and trophallaxis (Vienne *et al.* 1995; Lenoir *et al.* 2001). This mechanism leads to a mixing of the cuticular hydrocarbons of individuals (Hefetz *et al.* 1992; Soroker *et al.* 1994, 1995, 1998) and, thus, generates a colony's idiosyntratic "Gestalt" odour that differs from other colonies (Soroker *et al.* 1994; Hefetz *et al.* 1996; Dahbi *et al.* 1998; Lenoir *et al.* 1999; Oldham *et al.* 1999). This specific colony odour is crucial for the maintenance of the integrity of an ant society (VanderMeer *et al.* 1989). Furthermore, the PPG of ant queens may contain pheromones that signal her identity or fertility (Vargo & Hulsey 2000; Dietemann *et al.* 2003).

The PPG was believed to occur only in ants (Hölldobler & Wilson 1990; Schoeters & Billen 1997; Lenoir *et al.* 1999). This, at least implicitly, suggested that the PPG evolved in response to the requirements of a social group to establish a colony odour. Recently, however, a PPG has been described from a species of digger wasps, the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae, formerly Sphecidae, Melo 1999), that is not closely related to ants (Brothers 1999). The overall appearance and location as well as details of its morphology suggest that the PPGs of beewolves and ants are homologous (Strohm *et al.* in press). In this study, we analyse the chemistry of the beewolf PPG to establish whether its chemical composition also supports the hypothesis that the beewolf PPG is homologous to that in ants.

Since European Beewolves are solitary, the function of their PPG necessarily differs from that in ants. Strohm and Linsenmair (2001) hypothesised that the secretion of the beewolf PPG serves to protect the larval provisions from microbial attack. Female European beewolves hunt and paralyse honeybees, bring them to their nest burrow, and provision one to six bees in a brood cell as food resource for a larva. Due to the humid and warm conditions in the brood cell, the highly nutritive provisions are prone to detrimental microbial attack (Strohm & Linsenmair 2001). An early fungus infestation inevitably destroys the food resources and larvae would be killed by fungal toxins or starve to death. Observations in special cages revealed that beewolf females intensively lick the bodies of the paralysed bees and apply the secretion from the PPG

onto the bees' surface (Strohm & Linsenmair 2001; Herzner *et al.* in prep.). This treatment has the effect of delaying fungus growth for 2-3 days, which is a highly relevant effect given the very short larval feeding period of only 8-11 days. The primary mechanism of this delay seems not to be a chemical effect of the secretion on fungi but due to the prevention of water condensation on the bees that impairs the germination of fungus spores (Herzner *et al.* in prep.).

Assuming a homology between the PPGs of ants and beewolves, the chemical nature of the content and the acquisition of the chemicals and their distribution in the body should be similar. Thus, we first predict that the beewolf PPG contains mainly long chain hydrocarbons with few functional groups. In ants, the chemical composition of the PPG and the cuticle are apparently identical. A similar congruency in beewolves would further support the homology hypothesis. Consequently, the second prediction is that PPG and cuticle should have a nearly identical chemical composition in beewolves. Since there is no grooming of conspecifics in beewolves, the content of the beewolf PPG has to be either synthesised in gland cells that are associated to the reservoir or sequestered from the hemolymph. The latter (plus uptake from conspecifics) is the case in ants. Therefore, the homology hypothesis would likewise require sequestration from the hemolymph. Thus, the third prediction is that the hydrocarbons of the PPG should be similar to those found in the hemolymph. To evaluate these predictions, we analysed the chemical composition of the PPG, the hemolymph, and the cuticle of female European Beewolves using combined GC-MS analyses.

7.3 Material and Methods

To identify the volatile chemicals of the PPG, freshly killed females were decapitated and their PPGs were removed from the heads by grasping the hypopharynx with tweezers and gently pulling the attached gland out through the mouth (Strohm *et al.* in press). The glands were immediately transferred to 0.25 ml n-Hexane (Fluka) that was distilled prior to use. The gland is a glove-like reservoir that only consists of a thin epithelium. Therefore, they remained in the solvent until analysis. For the identification of the components in the PPG, the glands of several females were pooled. This enabled us to identify also the minor components that were not reliably detectable in the extracts of individual PPGs or abdomens. Preliminary investigations showed that due to the huge amount of hydrocarbons in the PPG, extracts of entire heads are identical to extracts of the dissected glands. Thus, as an easier alternative to the dissection of the PPG, entire heads can be extracted. To obtain data on the variability of the PPG content, we extracted the hydrocarbons from the heads of 37 females individually. The heads were cut off and soaked in distilled hexane for 4 hours.

Cuticular hydrocarbons were obtained by soaking the cuticle of the same 37 beewolf females whose heads had been extracted individually in 500µl distilled hexane for 10 min. To ensure that these extracts were not contaminated with the content of the head gland, only the abdomen of each female was used. To test for a relationship between the content of the head gland and the hydrocarbons of the cuticle we conducted a correlation and regression analysis between the mean proportions of components in the head and abdomen extracts. Since both variables have the same error we used reduced major axis regression to describe the relationship (Legendre & Legendre 1998).

Since preliminary analyses showed that the amount of volatiles in the hemolymph is too small to yield reasonable chromatograms with hexane extracts, we used SPME fibres to sample the hydrocarbons of the hemolymph. We dissected the abdomen of freshly killed females, removed the hemolymph with a pipette and transferred it to an eppendorf vial. An SPME (solid phase micro-extraction) fibre coated with 100 µm polydimethylsiloxane (Supelco, Bellefonte, PA, USA) was dragged through the hemolymph and immediately analysed in the GC-MS.

Identification of the chemicals was accomplished by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-Fourier transform infrared analyses (GC-FTIR). Dimethyl disulfide (DMDS) derivatization was carried out to determine the position of double bonds according to the method of Dunkelblum *et al.* (1985). Hydrogenation of double bonds with H_2 and palladium (Attygalle 1998) was used to obtain sufficient information by mass spectrometry.

For the identification of the hydrocarbons in the PPG, we used a Hewlett Packard HP 6890 Series GC System coupled to a Hewlett Packard HP 5973 Mass Selective Detector (Agilent Technologies, Böblingen, Germany). The GC was equipped with a DB-1 fused silica capillary column (30m x 0.25 mm ID; df = 0.25 µm; J&W, Folsom, CA, USA) Temperature was programmed from 100°C to 300°C with 6°C/min heating rate and held for 20 min at 300°C. Helium was used as carrier gas with a constant flow of 1 ml/min. Injection was carried out at 300°C in the splitless mode for 2 min. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV and a source temperature of 230°C. The software ChemStation (Agilent Technologies, Böblingen, Germany) for windows was used for data acquisition.

HRGC-FTIR spectra were obtained using an HP 5890 GC (Agilent Technologies, B¨oblingen, 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 D 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.

For the analysis of a larger sample of individual head extracts, of cuticular hydrocarbons of the abdomens of the same females, and of female hemolymph, 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 (head extracts and abdomen cuticle) or an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector (hemolymph). The columns and temperature programs were the same. We used a DB-5 MS fused silica capillary column (30 m x 0.25 mm i.d.; df = 0.25µm; J&W, Folsom, CA, USA). The GC temperature was held at 60° C for 1 min, raised to 300°C at a rate of 5°C/min and then held constant at 300°C for 10 min. Helium was used a the carrier gas with a constant flow of 1 ml/min. Wed chose a splitless injection mode (1µl) at an injector temperature of 250° C and a splitless period of 60 sec. The mass spectrometer was operated in EI mode at 70 eV. The software Xcalibur for Windows (head extracts and abdomens) and MSD ChemStation for Windows (hemolymph) were used for data acquisition.

7.4 RESULTS

7.4.1 Hydrocarbons in the PPG

The PPGs of females contained a total of 51 peaks representing 62 different compounds (Table 7.1). Three components could not yet be identified at all and some other compounds could not be completely characterised due to the very small amounts. The identified components comprise straight chain as well as methyl branched alkanes, alkenes, unique long chain unsaturated ketones and very few compounds with functional groups. By far the largest proportion of the hydrocarbons in the PPG is made up of one alkene. Surprisingly, this predominant compound differed among individuals. In some females the major alkene was *Z*-9-pentacosene in others it was *Z*-9-heptacosene (Table 7.2). Among the 44 females whose PPG content was analysed, 35 had pentacosene and 9 had heptacosene as their major peak. There was only one more or less intermediate individual that had similar amounts of both compounds. The frequency of the two types differs significantly from equality ($X^2 = 15.4$, df = 1, $p < 0.001$).

Table 7.1: List of compounds in the postpharyngeal gland of females of the European Beewolf. N.I. = not identified.

Peak Nr.	Compound name
1	Tetradecane
2	Pentadecane
3	Hexadecane
4	Heptadecane
5	x-Methylheptadecane
6	Octadecane
7	N.I.
8	x-Methyloctadecane
9	N.I.
10	Nonadecane
11	N.I. (Terpene?)
12	N.I.
13	Eicosane
14	9-Octadecenylmethylester
15	Heneicosane
16	Docosane
17	*Z*-9-Tricosene
18	*Z*-7-Tricosene
19	Tricosane
20	11-Methyltricosane, 9-Methyltricosane, 7-Methyltricosane
21	5-Methyltricosane
22	x-Tetracosene
23	Tetracosane
24	Δ-14-Tricosene-6-one
25	Δ-x-Pentacosadiene
26	*Z*-9-Pentacosene
27	*Z*-7-Pentacosene
28	Pentacosane
29	Δ-15-Tetracosene-7-one
30	11-Methylpentacosane, 9-Methylpentacosane, 7-Methylpentacosane, 5-Methylpentacosane
31	Δ-16-Pentacosen-8-ol
32	*Z*-9-hexacosene
33	Hexacosane
34	Δ-16-Pentacosene-8-one
35	8-Pentocosanone
36	*Z*-9-Heptacosene
37	Heptacosane
38	13-Methylheptacosane, 11-Methylheptacosane, 9-Methylheptacosane
39	Δ-17-Hexacosene-9-one
40	Octacosane
41	Δ-18-Heptacosene-10-one
42	Δ-10-Heptacosanone (?)
43	*Z*-9-Nonacosene (?)
44	Nonacosane
45	15-Methylnonacosane, 13-Methylnonacosane, 11-Methylnonacosane
46	Triacontane
47	*Z*-9-Hentriacontene (?)
48	Hentriacontane
49	15-Methyltriacontane, 13-Methyltriacontane, 11-Methyltriacontane
50	*Z*-9-Tritriacontene (?)
51	Tritriacontane

Table 7.2: Chemical composition of the postpharyngeal gland of females who had either pentacosene or heptacosene as their major component. Given are the retention times (RT) and the means and standard deviations (SD) of the percentages of different components for females of the pentacosene (N = 29) and of the heptacosene typ (N = 8) as well as the absolute difference in percentage.

		Pentacosene-type		Heptacosene-type		
Compound name	**RT**	**Mean**	**SD**	**Mean**	**SD**	**Difference**
Nonadecane	29.69	0.004	0.005	0.002	0.004	0.002
Heneicosane	33.75	0.210	0.149	0.185	0.132	0.026
Docosane	35.64	0.165	0.099	0.125	0.078	0.040
Z-9-Tricosene	37.00	0.297	0.234	0.072	0.057	0.225
Z-7-Tricosene	37.12	0.079	0.070	0.010	0.009	0.069
Tricosane	37.51	13.497	5.216	12.927	3.033	0.570
Δx-Tetracosene	38.78	1.226	0.686	0.137	0.084	1.089
Tetracosane	39.22	0.171	0.104	0.145	0.070	0.026
Z-7- & Z-9-Pentacosene	40.58	78.784	7.065	8.287	5.503	70.497
Pentacosane	40.92	2.092	0.942	2.824	1.197	0.732
Z-9-Hexacosene	42.16	0.130	0.054	1.624	0.724	1.493
Hexacosane	42.55	0.053	0.036	0.047	0.023	0.006
Δ-16-Pentacosene-8-one	43.48	1.149	0.733	0.247	0.146	0.902
Z-9-Heptacosene	43.73	1.013	0.392	69.785	8.813	68.772
Heptacosane	44.13	0.388	0.222	0.314	0.116	0.075
Octacosane	45.59	0.031	0.027	0.024	0.013	0.007
Δ-18-Heptacosene-10-one	46.49	0.060	0.144	1.726	0.753	1.667
Z-9-Nonacosene (?)	46.73	0.041	0.063	0.946	0.426	0.904
Nonacosane	47.06	0.304	0.300	0.321	0.105	0.016
Z-9-Hentriacontene (?)	49.50	0.045	0.040	0.089	0.046	0.044
Hentriacontane	49.80	0.076	0.081	0.063	0.021	0.013

7.4.2 Hydrocarbons on the cuticle

The extracts of the abdomens revealed a composition that was very similar to the PPG (Table 7.3, Fig. 7.1). The predominating peaks were identical. In particular, females with pentacosene (or heptacosene respectively) as their major peak in the PPG also had pentacosene (or heptacosene respectively) as their major peak on the cuticle. Moreover, the relative amounts of peaks on the abdomen showed a strong linear correlation with the corresponding peaks in the PPG ($r^2 = 0.75$, N = 24 compounds, $P < 0.001$). Thus, the chemical composition of the PPG and the cuticle are very similar.

7.4.3 Hydrocarbons in the hemolymph

The comparison of the content of the PPG and the hemolymph revealed a high similarity of the major peaks to both, the PPG and the cuticle (Table 7.3). However, due to the small amounts of hydrocarbons found in the hemolymph, minor peaks were not detectable with the methods used. Differences in the proportions of some components as compared to the PPG and the cuticle

(notably pentacosene and heptacosene) are probably due to the high proportion of heptacosene-type females in the small number of hemolymph samples (four out of six).

Table 7.3: Chemical composition of the postpharyngeal gland (PPG, N = 37 females), the cuticular hydrocarbons of the abdomen (N = 37 females), and the hemolymph (N = 6 females). Given are the retention times (RT), means and standard deviations (SD) of the percentages of different components.

		PPG		Abdomen		Hemolymph	
Compound name	**RT**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Nonadecane	29.69	0.003	0.004	0.000	0.000	0.000	0.000
Heneicosane	33.75	0.205	0.144	0.140	0.074	0.632	0.380
Docosane	35.64	0.157	0.095	0.182	0.106	0.477	0.215
Z-9-Tricosene	37.00	0.248	0.228	0.131	0.088	0.332	0.471
Z-7-Tricosene	37.12	0.064	0.068	0.067	0.141	0.986	1.103
Tricosane	37.51	13.374	4.796	16.202	8.507	17.497	1.050
Δx-Tetracosene	38.78	0.991	0.758	0.877	0.452	1.127	1.061
Tetracosane	39.22	0.165	0.097	0.461	0.288	0.363	0.198
Z-7- & Z-9-Pentacosene	40.58	63.541	30.172	53.236	27.227	27.549	28.147
Pentacosane	40.92	2.250	1.031	7.231	3.711	5.989	0.947
Z-9-Hexacosene	42.16	0.453	0.702	0.497	0.584	1.906	1.100
Hexacosane	42.55	0.052	0.033	0.220	0.264	0.015	0.036
Δ-16-Pentacosene-8-one	43.48	0.954	0.751	0.666	0.723	0.350	0.357
Z-9-Heptacosene	43.73	15.883	28.965	14.022	25.031	36.434	24.974
Heptacosane	44.13	0.372	0.205	1.658	1.122	1.835	0.936
Octacosane	45.59	0.030	0.024	0.157	0.204	0.022	0.035
Δ-18-Heptacosene-10-one	46.49	0.420	0.781	0.588	0.866	1.618	1.544
Z-9-Nonacosene (?)	46.73	0.237	0.425	0.290	0.540	1.344	1.063
Nonacosane	47.06	0.308	0.269	1.595	1.060	1.230	0.380
Z-9-Hentriacontene (?)	49.50	0.055	0.045	0.591	0.918	0.032	0.080
Hentriacontane	49.80	0.073	0.072	0.480	0.380	0.262	0.169

7.5 Discussion

The PPG of beewolf females contains predominantly unbranched unsaturated long chain hydrocarbons, smaller amounts of corresponding saturated hydrocarbons, and minor amounts of unsaturated ketones and methyl-alkanes. The PPGs of ants also contain mainly hydrocarbons but mostly saturated and branched hydrocarbons (mono-, di-, and trimethylalkanes) as well as minor proportions of esters, and acids (VanderMeer *et al.* 1982; Hefetz *et al.* 1992; Soroker *et al.* 1995). Thus, the chemical composition of the PPG of beewolves broadly resembles that of the PPGs of ants (e.g. Soroker *et al.* 1998; Soroker & Hefetz 2000), supporting the first prediction derived from the hypothesis that the PPG of beewolves and ants is homologous.

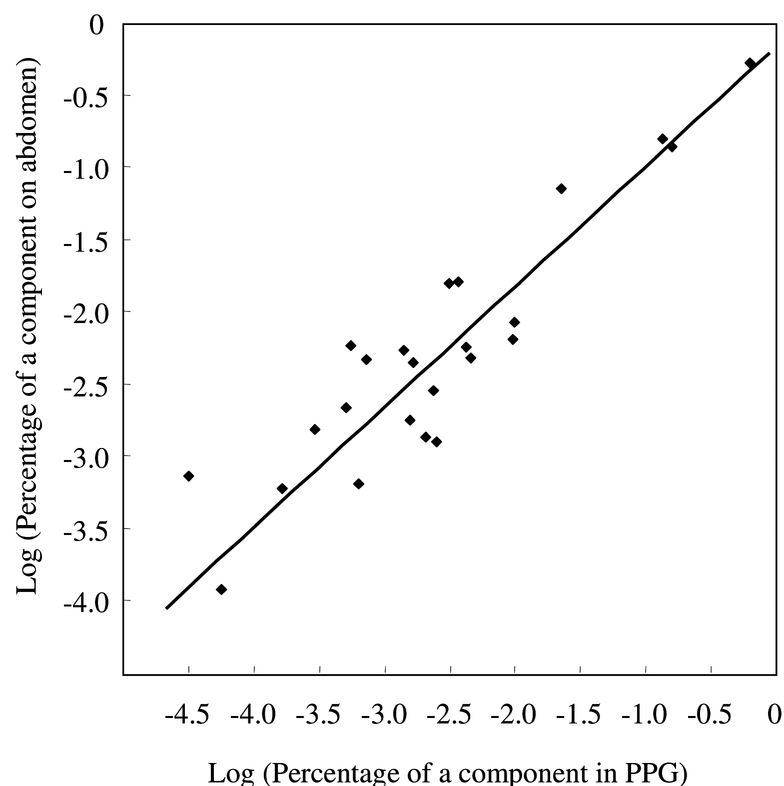

Figure 7.1: Correlation between the relative amount of a particular component in the postpharyngeal gland (PPG) and on the abdomens of beewolf females (r2 = 0.83, N = 24, P < 0.001). The trend line was generated using reduced major axis regression (y = -0,152 + 0,912*x). Only the 24 components that could be detected in the extracts of both, head and abdomen of individual females were included.

The hydrocarbon profiles of both the cuticle and the hemolymph of beewolf females appear to be nearly identical to their PPG. This might have two non-exclusive explanations. First, the hydrocarbons of the hemolymph are delivered to both the PPG and the cuticle. Second, females might spread the content of the PPG over their own bodies. Females frequently groom themselves which includes drawing their forelegs through their mouthparts and rubbing these on their bodies (E. Strohm, unpubl. observation). Thus, the content of the PPG might be delivered to the forelegs and then distributed on the body. The similarity of the chemical profiles between PPG and cuticle is consistent with the hypothesis of a homology between beewolves and ants.

The similarity between the hydrocarbon profiles of the hemolymph and the PPG suggests that the content of the latter is sequestered from the hemolymph. It can be calculated that a female has to produce a mean amount of 300 µg of PPG secretion per day (the average number of bees hunted per day is 3 [Strohm & Linsenmair 1997]; the average amount of PPG secretion on a bee is 100 µg [Herzner *et al.* in prep.]). The PPG is basically a glove-like reservoir. There are no distinct glandular cells associated with this reservoir. The walls of the PPG are formed by a thin

unicellular epithelium (Strohm *et al.* in press) that does not show any obvious signs of high metabolic activity (e.g. multiple nucleoli). Therefore, this epithelium hardly has the potential to synthesize these large amounts of hydrocarbons. Most probably, the compounds are synthesised in oenocytes as has been demonstrated for ants (Soroker & Hefetz 2000) and cockroaches (Fan *et al.* 2003). This strongly supports our third prediction of the homology hypothesis, that the content of the PPG is sequestered from the hemolymph.

The prevalence of long chain unsaturated hydrocarbons in the PPG of beewolf females probably coincides with their main function, the preservation of the paralysed honeybees in the brood cells. This preservation seems to be mainly accomplished by a physical mechanism (Herzner *et al.* in prep.): the secretion prevents the condensation of water on the bees, with the effect that the growth conditions for fungi are negatively affected. The molecular mechanism is as yet unclear. Possibly, the PPG secretion covers structures on the paralysed bee that would otherwise function as effective nuclei for water condensation. Scanning electron microscopy revealed that the PPG secretion forms an impervious layer over the whole surface of the prey (Herzner *et al.* in prep.). Alkenes might be an ideal compound to build up such layers.

Notably, there seems to be a polymorphism with regard to the major components in the beewolf PPG. It is either pentacosene or heptacosene and there are no or at least very few intermediate individuals. Both unsaturated hydrocarbons are widespread among aculeate Hymenoptera and other insects (e.g. Ayasse 1991; Sick 1993). All other components occur in similar amounts in the two types of females. Why females have either pentacosene or heptacosene is not clear. Possibly, conditions during development differed between the females and caused the activation of different genes (for example, the synthesis of heptacosene might be induced by high temperatures because of the higher melting point). However, also individuals that were bred under identical temperature conditions in the same climate chamber showed this polymorphism and beewolf females from different populations ranging from the Northern part of Germany to the Southern valleys of the alps not only showed both types of females but also in similar proportions (J. Kroiss *et al.* in prep). Together with the lack of intermediate individuals this might suggest that the polymorphism has a genetical basis. Such a polymorphism would have to be balanced since otherwise one allele would disappear either because it has a selective disadvantage or due to genetic drift. One possible explanation for a balanced polymorphism is a spatial difference in the suitability of the two alleles. Such spatial heterogeneity might either be generated by differences in abiotic or biotic conditions.

At the moment, possible abiotic factors are unclear, since the most plausible – varying temperature – does not seem to be responsible for the reasons explained above. Likewise, we can only speculate about biotic interactions that might maintain the observed polymorphism. Interactions with brood parasites provide a reasonable scenario. As shown above, the cuticles of

beewolf females show the same profile as their PPG and these compounds might also be transferred to the nest mounds and might be used for individual nest recognition. Several brood parasites attempt to oviposit on the bees while a female is entering the nest with a prey (Olberg 1953; Veenendaal 1987; Evans & O'Neill 1988; Strohm *et al.* 2001). Conspicuous differences in the odours of different nests might facilitate nest recognition and shorten the period a paralysed bee is exposed to the parasites. If a local aggregation has only one type of females, nest recognition might take longer with the consequence of an increased rate of parasitism. A female of the rarer type would then be favoured and the frequency of the rare allele would increase, despite a possible disadvantage with regard to other functions, eventually resulting in a balanced allele frequency.

Besides the ubiquitous alkanes and alkenes that we found in the PPG, there are some compounds that have not yet been described from nature: the long chain unsaturated ketones. The function of these compounds is not yet clear. Since the ketones are the only identified components in the PPG of beewolf females that have a functional group, they might be responsible for the slight chemical effect of the PPG secretion on the growth of fungi (Herzner *et al.* in prep.). Interestingly, male beewolves have a slightly shorter ketone in their marking secretion (Schmitt *et al.* 2003). Because of the rarity of such compounds in nature this is hardly explained by chance. Possibly, the ketone in the male head glands also has some antifungal function.

In conclusion, our results are consistent with the hypothesis that the PPGs of ants and beewolves are homologous. Besides the similarities in location, morphology and ultrastructure as well as the involvement in a grooming-like behaviour (Strohm *et al.* in press), the similar chemical compositions of the PPG and the cuticle as well as the probable sequestration of the chemicals from the hemolymph in both ants and beewolves provide strong evidence for a common origin. Thus, most probably the PPG in ants did not evolve *de novo* in response to the requirements of the social lifestyle. Instead, the PPG was already present in the ancestors of ants and beewolves but adopted a novel function in the complex social organisation of ants.

7.6 References

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CHAPTER 8

THE SCENT OF SENESCENCE: AGE-DEPENDENT CHANGES IN THE COMPOSITION OF THE CEPHALIC GLAND SECRETION OF THE MALE EUROPEAN BEEWOLF, *PHILANTHUS TRIANGULUM*

Journal of Insect Science, in press.

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8.1 SUMMARY

The process of aging inevitably leads to changes in physiology, performance and fertility of eukaryotic organisms and results in trade-offs in the resource allocation between current and future reproduction and longevity. Such constraints may also affect the production of complex and costly signals used for mate attraction and might therefore be important in the context of mate choice. We investigated age-related changes in the amount and composition of the cephalic gland secretion that the male European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae) use to mark their territories. The secretion mainly consists of eleven long-chain compounds with large proportions of a carbon acid, a ketone and two alcohols, and small proportions of several alkanes and alkenes. Both the total amount and the composition of the gland content varied with age. The four compounds with functional groups were present in much lower proportions in very young and very old males compared to middle-aged males, suggesting that these components may be more costly than the alkanes and alkenes. Thus, physiological constraints may cause the delayed onset and early decline of these substances in the cephalic gland. There were also minor but significant changes in four components among the middle-aged males. These age-related changes in the amount and composition of the male marking secretion might provide reliable indicators for female choice.

8.2 INTRODUCTION

Senescence is a process that inevitably affects all higher organisms. The continuous accumulation of deleterious mutations, changes in protein synthesis, oxidative damage in mitochondria, and the accumulation of harmful metabolic end-products all contribute to the aging process and result in a finite life span (Collatz *et al.* 1986). The restricted life time selects for an optimal allocation of limited resources to reproduction and survival in order to maximize their fitness (Roff 1992; Stearns 1992). Several studies have demonstrated the resulting trade-offs between current and future reproduction and longevity (Partridge & Farquhar 1981; Prowse & Partridge 1997).

Considering the trade-offs in resource allocation between reproduction and longevity, age is often regarded as an important factor for mate choice. Decreasing viability and fertility (quality and/or amount of sperm) and the accumulation of deleterious germ line mutations throughout the lifetime might contribute to a negative relationship between age and mate quality (Hansen & Price 1995; Brooks & Kemp 2001). As a consequence, females are often expected to prefer younger males (Hansen & Price 1995; Brooks & Kemp 2001). By contrast, many authors argue that females should prefer older mates because they have already demonstrated their viability and might therefore provide good genes for the offspring (Trivers 1972; Manning 1985). Thus, although it is generally accepted that age may be an important factor influencing mate choice, the direction of the preference is still a matter of debate and is likely to vary among organisms.

In animals where age plays a role for mate choice, indicator mechanisms must be available that allow the assessment of a potential mate's age. Many insects heavily rely on pheromones for inter- and intraspecific communication. Although in most insect taxa, sex pheromones are produced by females, male sex pheromones occur in a number of species. Several studies have demonstrated that male pheromones can communicate aspects of mate quality to females (Thornhill 1992; Droney & Hock 1998; Marco *et al.* 1998; Martin & Lopez 2000; Reusch *et al.* 2001; Spurgeon 2003), and there is some evidence for adaptive female choice on the basis of male sex pheromones (Jones & Hamilton 1998; Jones *et al.* 1998; Jones *et al.* 2000). However, studies investigating the effect of age on olfactory sexual communication are scarce. In mice, the amount and composition of urinary volatiles has been demonstrated to change with age, and this information is used by conspecifics to discriminate among age groups (Wilson & Harrison 1983; Osada *et al.* 2003). Age-related changes in the amount and composition of sex pheromone have also been found in male boll weevils (Coleoptera, Curculionidae) (Spurgeon 2003), but the potential importance for female choice has not been investigated.

Females of the European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae) hunt honeybees (*Apis mellifera*) with which they provision their offspring in underground nest

burrows (Strohm & Linsenmair 1997; Strohm & Linsenmair 1999; Strohm & Linsenmair 2000; Strohm & Marliani 2002). Male beewolves establish territories (about 0.25 m^2 in size), mostly in the vicinity of the females' nest aggregations, that do not contain any resources for females (Simon-Thomas & Poorter 1972; Strohm 1995). The males mark plants in their territories with the secretion of a cephalic gland and defend them against intruding males in combat flights without physical contact of the opponents (Simon-Thomas & Poorter 1972; Evans & O'Neill 1988; Strohm 1995; Strohm & Lechner 2000; Schmitt *et al.* 2003). In the field, males can survive for more than four weeks, although the apparent median life span is much shorter since emigrations from a site cannot be detected (Strohm & Lechner 2000). An individual male can occupy the same territory for several days and up to two weeks (Simon-Thomas & Poorter 1972; Strohm & Lechner 2000). The cephalic gland secretion is likely to serve as a pheromone that attracts receptive females to the male's territory (Evans & O'Neill 1988). Females of several *Philanthus* species including our study species have been observed to approach territories of conspecific males in a zigzagging flight pattern from the downwind side, probably orienting towards the windborne cephalic gland components (Evans & O'Neill 1988). Copulations usually occur within the males' territories (Simon-Thomas & Poorter 1972; Strohm 1995) and seem to be under the control of the females since they can easily repel unwanted mates by virtue of their larger body size (Evans & O'Neill 1988) or refuse copulations by bending their abdomen tip downwards (E.Strohm, pers. obs.). Territories of different males are often found close together, thereby constituting a lek situation in which the females have an ideal opportunity to choose among males (Simon-Thomas & Poorter 1972; Evans & O'Neill 1988). Since the copulation is not preceded by any kind of visual display, female choice appears to be, at least predominately, based on information obtained from the male marking compounds (E. Strohm and M. Kaltenpoth, unpubl. obs.).

Analyses of head extracts from male European beewolves revealed a complex blend of at least 11 compounds, with (*Z*)-11-eicosen-1-ol as the main component (Schmidt *et al.* 1990; Schmitt *et al.* 2003). All of these components are also found in samples of pure cephalic glands in the same relative amounts (Kroiss *et al.*, in prep.) and in extracts from male territories (E. Strohm, T. Schmitt, G. Herzner, J. Kroiss and M. Kaltenpoth, unpubl. data). Although behavioral studies on the biological activity of the components are lacking, these compounds might be important cues for females to assess male quality and choose among potential mates.

Mate choice can be assumed to be of particular importance in the European beewolf. Females most probably mate only once (Evans & O'Neill 1988). Thus, choosing a low-quality male will affect all daughters (due to the haplo-diploid sex determination mechanism, male offspring are not affected, because they do not inherit genes from their mother's mate). Due to the extraordinary physiological requirements for reproduction including the fact that females have to carry the comparatively heavy prey to their nest in flight, a daughter's reproductive success

heavily depends on her “quality” (Strohm & Linsenmair 1997; Strohm & Daniels 2003). Therefore, “bad” genes from the father might affect a daughter's ability to hunt honeybees and carry the prey in flight as well as her life span. Thus, female choice for males with “good genes” could strongly influence female fitness. Choosing males who signal either their youth or their high age by virtue of their cephalic gland secretion may be one important factor in this context.

In this study, we analyzed the cephalic gland content of male European beewolves of different age classes to assess the variation of the amount and composition with age. The results are discussed with regard to possible physiological constraints in the production of the compounds and the potential of the cephalic gland secretion as an indicator of male age for female choice.

8.3 Materials and Methods

8.3.1 Insects and sampling

European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae) were kept in the laboratory at the University of Würzburg. Cocoons with larvae were placed individually in Eppendorf® tubes and kept in boxes with moist sand at 10°C for about eight to nine months of overwintering. Cocoons were then transferred to warm conditions (cycles of 12 hours at 25°C and 12 hours at 22°C) and adult beewolves emerged four to six weeks later. Emerging males were marked individually and were allowed to fly in a climate chamber (2.5 x 1.8 x 2.1 m in size) with 12h light/dark cycles at 25°C/20°C and provided with honey *ad libitum*. Males were caught at different ages and kept overnight in small polystyrol vials (height: 80 mm; diameter: 35 mm) with moist sand and a drop of honey to allow the cephalic glands to be replenished. After anesthetizing the beewolf males with CO_2, they were killed by freezing and kept frozen (at –20°C) for up to six weeks until extraction of the cephalic gland content and GC-MS analysis. Overall, 107 males were randomly assigned to 13 different age groups, of which the first eleven were spaced four days apart. Groups 1, 5, 9, 13, 21, and 25 comprise only animals of the exact designated age (i.e. 1, 5, 9, 13, 21, and 25 days old at the day when they were frozen, respectively), while groups 17, 29, 33, 37, and 41 include individuals of the designated age and one to three individuals that are up to two days older. Due to the scarcity of very old males, group 47 includes all males between 45 and 49 days, and group 55 includes all males between 50 and 60 days old.

8.3.2 Gas chromatography - mass spectrometry

Frozen males were decapitated and the heads were incised at both sides to open up the glands. Heads were placed individually in glass vials (4 ml), and 20 µl of a 1g/l solution of octadecane in hexane (equivalent to a final amount of 20 µg of octadecane) was added as an internal standard to each vial to allow quantification of the cephalic gland content. The heads were then submerged in approximately 400 µl distilled hexane and chemicals were extracted for four hours at room temperature. Then each head was removed and the hexane was reduced to about 200 µl by a gentle constant flow of nitrogen. Samples were analyzed by coupled capillary gas chromatography-mass spectrometry (GC-MS) with an Agilent 6890N Series gas chromatograph (Agilent Technologies, www.agilent.com) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with a RH-5ms+ fused silica capillary column (J&W, 30 m x 0.25 mm ID; df = 0.25µm; temperature programme: from 60°C to 300°C at 5°C/min, held constant 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 in the splitless mode for 60 sec. The electron impact mass spectra 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 components of the cephalic gland content had already been characterised (Schmitt *et al.* 2003) and could be unambiguously identified by their retention times and mass spectra.

After GC-MS analysis, the width of the head capsule at the widest point was measured for all males under a dissecting scope (magnification: 40x) with an ocular micrometer scale to control for size differences among the age groups that might account for differences in the amount or composition of the cephalic gland content.

8.3.3 Statistical analysis

Ten components were included in the analysis and their peaks were integrated with MSD ChemStation software (Agilent Technologies) (Table 8.1). Using the octadecane peak as an internal standard, the total amount of cephalic gland secretion was calculated and then $\log_{10}$-transformed to obtain normally distributed data for statistical analysis. The relative amounts of the ten components were calculated. Two peaks (eicosenol and tricosene) had to be combined for the analysis, since they were not always clearly separated by the GC-MS. With regard to the detection of age-related changes this procedure is conservative. Since the amount of eicosenol by far exceeds that of tricosene (about 10 fold, see Schmitt *et al.*, 2003), the combined peak is labelled as "eicosenol" in the following. Because the relative amounts constitute compositional data, they were transformed to logcontrasts prior to analysis (Aitchison 1986). The $\log_{10}$-

transformed absolute amounts of the cephalic gland content and the Aitchison-transformed relative amounts of the components were compared among age groups by one-way ANOVAs with Tukey post hoc tests. Changes in relative amounts of the ten components with age were additionally analysed using Pearson's correlation analyses for the males between 5 and 49 days old (see results for reasons to exclude very young and very old males). SPSS 12.0 software was used for the calculations.

8.4 RESULTS

8.4.1 Amount of cephalic gland content

The total extracted amount of compounds in the cephalic gland ranged from 3 to 1404 µg. These values constituted 0.01 to 2.32 % of the respective male's total body weight. There were significant differences in the total amount of the gland content among the different age groups (ANOVA, $F_{12, 92} = 10.1$, $p < 0.001$, Fig. 8.1). The mean amount increased 13fold from day one (mean = 17 µg) to day five (mean = 221 µg), then again 1.8fold to day nine (mean = 408 µg) and afterwards remained more or less constant until the age of 47 days (Fig. 8.1). The oldest males (50-60 days) showed a significant decrease in the amount of compounds extracted from their head glands (mean = 112 µg).

There were no significant size differences (measured as head capsule width) among the age groups that might account for differences in the amount or composition of the gland content (ANOVA, $F_{12, 94} = 1.18$, $p = 0.311$).

8.4.2 Chemical composition of the cephalic gland content

The cephalic secretion of beewolf males of our study population is composed of 11 main components (Schmitt *et al.* 2003): (*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, and nonacosane.

The chemical composition of the cephalic gland content differed significantly among age groups (Fig. 8.2). Very young (one day old) males differed from middle-aged males in the relative amounts of most components. Generally, the compounds with functional groups (dihydrofarnesoic acid, nonadecenone, octadecanol, and the peak including eicosenol and tricosene) were present in lower relative quantities in very young males, whereas the alkanes

and alkenes (tricosane, pentacosene, pentacosane, heptacosane, heptacosene and nonacosane) were found in larger relative amounts in young males than in the middle-aged males (Fig. 8.2). The mean relative amounts of dihydrofarnesoic acid, nonadecenone, octadecanol, and eicosenol increased to 254%, 185%, 528%, and 206% from day 1 to day 5, respectively, whereas the mean relative amounts of tricosene, pentacosene, pentacosane, heptacosene, heptacosane, and nonacosane decreased to 26%, 21%, 23%, 19%, 42%, and 45%, respectively, from day 1 to day 5. Although the youngest males showed higher interindividual variation in most of the components than older males, this is unlikely to seriously affect the results of the ANOVAs, because (1) the sample sizes in the groups are roughly equal (especially in the first eight groups), thus reducing effects of unequal variances (Box 1954), and (2) the direction of the effect is the same for all compounds with functional groups. Thus, the low proportion of compounds with functional groups in very young males is consistent for the components with functional groups as opposed to those without functional groups. There was a non-significant trend that the relative amount of compounds with functional groups decreased in very old males (age 50-60 days), whereas the amount of alkanes and alkenes increased (Fig. 8.2).

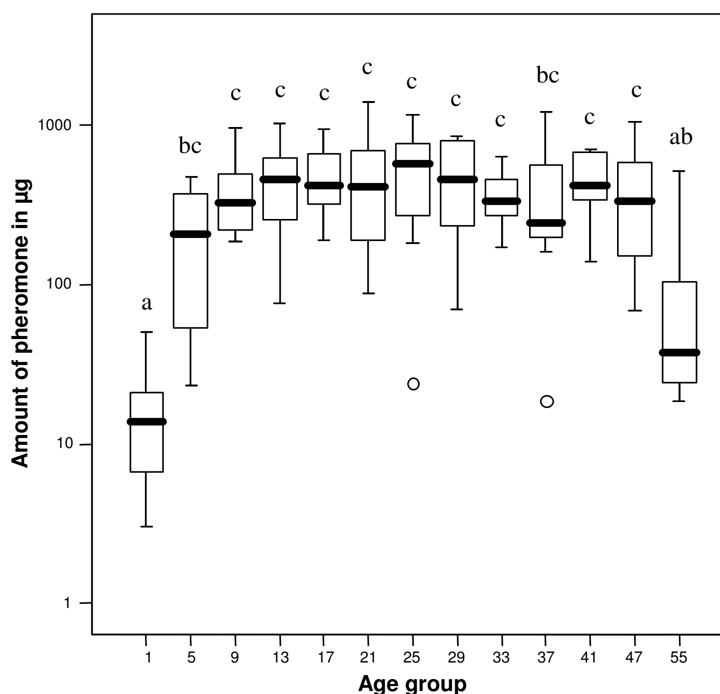

Figure 8.1: Amount of cephalic gland content extracted from male beewolves of different ages. Bold lines represent medians, boxes comprise the interquartile range, and bars indicate minimum and maximum values, except extreme values, these are represented by circles. Quantities are given in µg on a $\log_{10}$-scale. The age groups contain exclusively or predominantly males of the designated age (in days) (exceptions see "Materials and methods" section). Different letters above boxes indicate significant differences between groups ($p<0.05$). Sample sizes of age groups (from left to right) are: 8, 8, 8, 8, 9, 8, 8, 7, 8, 7, 5, 10, and 11, respectively.

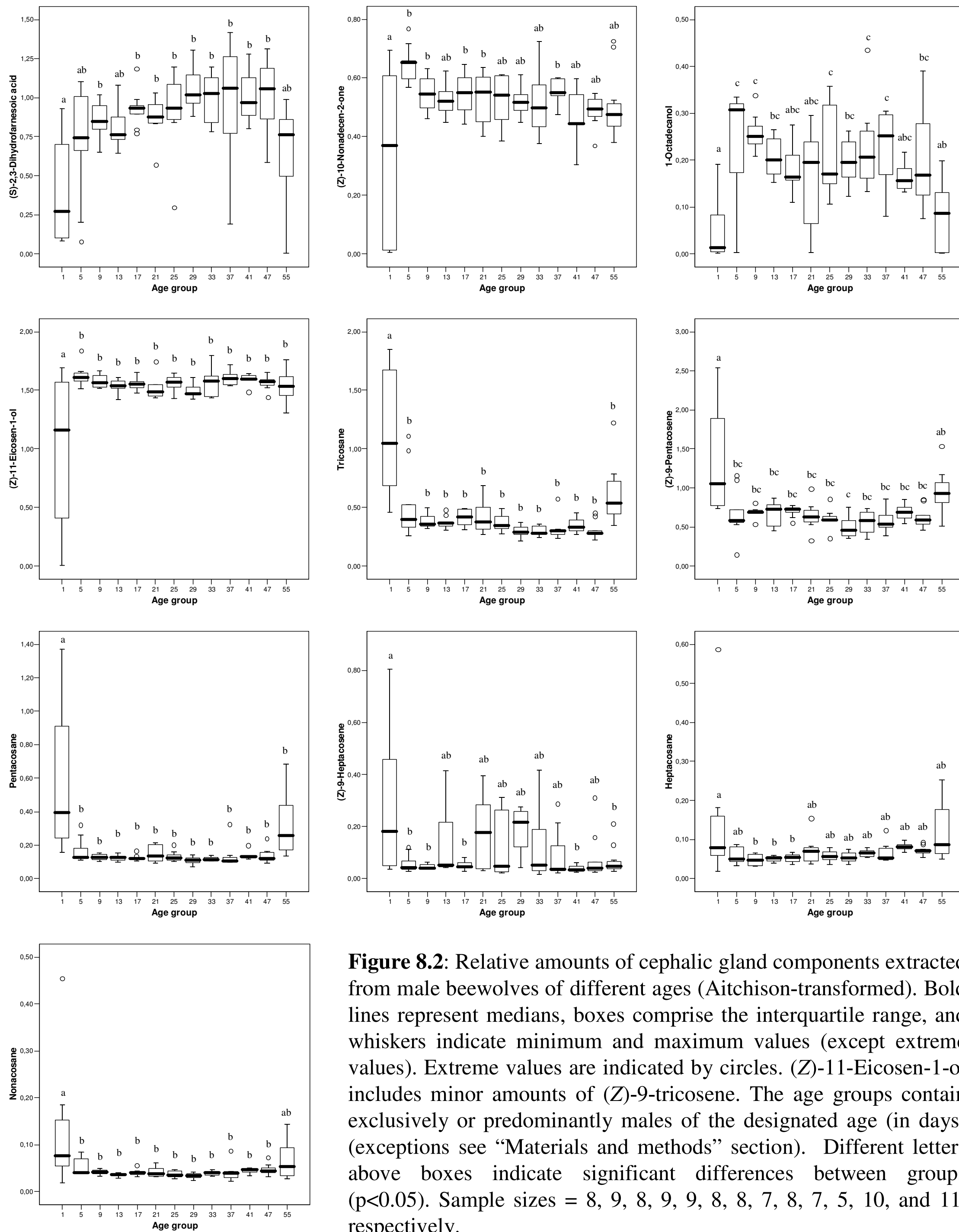

Figure 8.2: Relative amounts of cephalic gland components extracted from male beewolves of different ages (Aitchison-transformed). Bold lines represent medians, boxes comprise the interquartile range, and whiskers indicate minimum and maximum values (except extreme values). Extreme values are indicated by circles. (*Z*)-11-Eicosen-1-ol includes minor amounts of (*Z*)-9-tricosene. The age groups contain exclusively or predominantly males of the designated age (in days) (exceptions see "Materials and methods" section). Different letters above boxes indicate significant differences between groups ($p<0.05$). Sample sizes = 8, 9, 8, 9, 9, 8, 8, 7, 8, 7, 5, 10, and 11, respectively.

The majority of males that are active in the field might be neither very young, nor very old. Thus, to test whether there was an increase or decrease of components among the middle-aged males we conducted a correlation analysis excluding the groups containing very young and very old males. Only the two extreme groups (group 1 and 55) were excluded, because they differed significantly in the total amount (Fig. 8.1) as well as in the chemical composition (Fig. 8.2) of the cephalic gland secretion, thereby providing evidence for major physiological differences between these groups and the middle-aged males. Among the middle-aged males (5-49 days),

the relative amounts of four of the ten compounds were significantly correlated with age: dihydrofarnesoic acid and heptacosane increased with age, whereas nonadecenone and tricosane decreased with age (Table 8.1). However, these effects were rather small, explaining 12-15 % of the variance of the respective compound.

Table 8.1: Correlation between age and relative amount of the cephalic gland components extracted from male beewolves between 5 and 49 days of age (Aitchison-transformed). Significant correlations ($p<0.05$) are highlighted in bold.

	Correlation	
Cephalic gland component	**R^2**	**p**
(S)-2,3-Dihydrofarnesoic acid	**0.123**	**0.001**
(Z)-10-Nonadecen-2-one	**0.140**	**< 0.001**
1-Octadecanol	0.011	0.342
(Z)-11-Eicosen-1-ol*	0.002	0.687
Tricosane	**0.146**	**< 0.001**
(Z)-9-Pentacosene	0.031	0.100
Pentacosane	0.007	0.439
(Z)-9-Heptacosene	0.002	0.707
Heptacosane	**0.126**	**0.001**
Nonacosane	0.002	0.692

* including minor amounts of tricosene (see Materials and Methods section)

8.5 DISCUSSION

The results of this study demonstrate that both the total amount and the composition of the male cephalic gland content vary with age in the European beewolf *(Philanthus triangulum)*. This variation might convey information that could be used by females to assess a potential mate's age and to choose a mate based on its age.

The most dramatic changes in the composition of the males' cephalic gland content are due to the delay after emergence in the production of components with functional groups (dihydrofarnesoic acid, nonadecenone, octadecanol, and eicosenol) as compared to the alkane/alkene fraction. The onset of production might reflect the differences in the metabolic costs of these substances and/or their importance for other functions. The alkanes and alkenes also occur on the cuticle and in the hemolymph of beewolves (Strohm *et al.*, in prep.) and are probably constantly produced by basic metabolic pathways in the oenocytes, as has been shown for other insect taxa (Soroker & Hefetz 2000; Fan *et al.* 2003). Thus, the production of these compounds does not require specific enzymes and the production costs are therefore expected to be comparatively low.

Dihydrofarnesoic acid, nonadecenone, octadecanol, and eicosenol, however, are only present in the cephalic gland and, thus, specific enzymes are necessary for their production. Preliminary analyses suggest that these components are produced in specialized cells of the large mandibular glands (E. Strohm, G. Herzner, W. Goettler, unpubl. data), so the production additionally requires the formation and maintenance of specialized tissue. Therefore, dihydrofarnesoic acid, nonadecenone, octadecanol, and eicosenol probably inflict higher costs on the males than alkanes and alkenes, and the production of these substances could be limited in very young and very old males due to physiological constraints. This hypothesis is supported by the observation that very young and very old males have significantly smaller amounts of chemicals in the cephalic glands than middle-aged males. That pheromone production can be energetically costly and reduces the subsequent life span of the producer has recently been demonstrated in fruit flies (Johansson *et al.* 2005).

In very old males, the amount of the components with functional groups decreased, and their cephalic gland composition approached that of very young males. Although the discrimination of very young and very old males might theoretically pose a problem for female choice, this is unlikely to be the case in the field, because males will very rarely reach an age of 50 days in the field. In fact, the median estimated life span of males in the field was 9 days (lower and upper quartiles: 6 and 18.5), the oldest male was observed for 28 days (Strohm & Lechner 2000). However, these data probably underestimate the real life span of males in the field, because males that emigrated or escaped detection were counted as dead (Strohm & Lechner 2000). Observations in outdoor flight cages provide evidence that males older than 50 days do occur, but are rare even under semi-field conditions (median: 17 days, quartiles: 10 and 33 days; Strohm & Lechner 2000). Thus, the age of 50-60 days probably represents the physiological limit of the males' life span under optimal conditions. Field conditions and territorial activity, i.e. a probably limited availability of food resources and extensive scent marking as well as defense combats, might enhance the effect of age on the composition of the cephalic gland content that has been found under laboratory conditions in this study. Thus, our data probably provide only a lower boundary for the effect of age on the chemical composition of the cephalic gland content.

The changes in cephalic gland composition during the long and probably reproductively most important middle part of a male's life are less pronounced. However, there are four components that either significantly increase or decrease with age. This might be enough to provide females with some information on the age of a potential mate, especially because the sensitivity of female olfactory receptors and the central nervous processing might be much better than our analytical methods. Furthermore, it is unlikely that females mate only with males of a certain age; they probably rather choose either the youngest or the oldest of the available males, for example by applying a best-of-*n* (i.e. choosing the best mate from a sample of *n* individuals) or

a sequential sampling strategy (i.e. choosing the first encountered mate with a quality above a certain critical threshold) (Janetos 1980; Real 1990). Thus, by comparing the scents of a sample of different territories the variation might be sufficient to at least exclude the oldest or the youngest males.

The results of this study indicate that the production of some components of the male beewolf marking secretion vary with age. This might be due to physiological constraints and might therefore provide information on the age of the emitter that might be used for female choice. Behavioral observations and mate choice experiments are necessary to investigate whether females indeed choose mates based on their age, and if so, which components of the male cephalic gland secretion are important for female choice. The results will shed light on the importance of age as a factor for female choice in a species with male chemical signaling. Because of the close association between metabolism and the production of semiochemicals, mate choice based on olfactory cues might be much more common than is currently apparent from the few available studies.

Acknowledgements

We would like to thank the German National Academic Foundation for financial support through a fellowship for M.K. This study was partly supported by the DFG (SFB 554 TP B3, STR 532 1/2).

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CHAPTER 9

THE ODOR OF OPTIMAL OUTBREEDING? KINSHIP AND GEOGRAPHICAL ORIGIN ARE REFLECTED IN THE MARKING PHEROMONE OF MALE BEEWOLVES (*PHILANTHUS TRIANGULUM*, HYMENOPTERA, CRABRONIDAE)

Journal of Animal Ecology, submitted

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9.1 SUMMARY

Pheromones play an important role for mate finding and courtship in many insects. In species where males are the signaling sex, females are expected to choose among potential mates with regard to the emitter's quality and/or genetic compatibility. One important aspect is the balance between negative and positive effects of in- vs. outbreeding. We analyzed the sex pheromone composition of male European beewolves from eight different locations across Europe (six in Germany, one in England, and one in Italy). The pheromone constitutes a complex blend of various long-chain hydrocarbons (alkanes, alkenes, alcohols, ketones, and a carbon acid). We demonstrate that pheromone composition differs significantly among populations on a regional scale, among subpopulations on a local scale and between families within each (sub)population. The differences in the pheromone blend are positively correlated with geographical distances as might be expected according to an isolation-by-distance model. Concordantly, on a local scale, family membership has a larger effect on pheromone composition than (sub)population affiliation, while the reverse is true for the regional scale. Since inbreeding may be expected to be especially costly in this species due to its provisioning behavior and sex determination mechanism, females may be selected more strongly for the recognition of close kin than for the discrimination among local subpopulations. Our results show that male sex pheromones can contain information on both kinship and geographical origin that may be used by females to choose adaptively among potential mates on the basis of their genetic distance according to an optimal outbreeding strategy.

9.2 INTRODUCTION

Male sexual signals often enable females to choose adaptively among potential mates by providing information on species affiliation and mate quality (Droney & Hock 1998; Jones & Hamilton 1998; Lopez *et al.* 2003; O'Loghlen & Rothstein 2003; Slater 2003). By choosing a high-quality male, females could benefit directly, if males vary in their ability to provide essential resources to the females (Halliday 1983; Vahed 1998) or in the probability to transmit parasites or infections (Hamilton & Zuk 1982; Clayton 1991; Penn & Potts 1998). Females could also benefit indirectly, if offspring quality depends on the genetic background of the male. Several models have been proposed to explain female choice based on indirect benefits, the most prominent of these being the "good genes" model that assumes that a certain male with good genes is (at least currently) the best choice for all females (Andersson 1994; Johnstone 1995; Wilkinson *et al.* 1998; Møller & Alatalo 1999; Tomkins & Simmons 1999; Hine *et al.* 2002), and the model of the "best compatibility" presuming that one specific male is the best choice for a particular female (Halliday 1983; Johnsen *et al.* 2000; Tregenza & Wedell 2000; Colegrave *et al.* 2002; Reinhold 2002).

The genetic compatibility of a mate depends – among others – on the degree of kinship which ranges from strict inbreeding to intense outbreeding. Both in- and outbreeding have certain advantages (Partridge 1983) and disadvantages (Bateson 1983; Pusey & Wolf 1996). According to the model of optimal outbreeding, females should choose a mate of a certain genetic difference to balance the costs of in- and outbreeding (Bischof 1972; Alexander 1977; Bateson 1983), thus, simultaneously avoiding both inbreeding depression and the break-up of local adaptations.

In Hymenoptera, deleterious mutations, which have intense consequences in inbred diploid organisms, usually disappear quickly due to the haploidy of males (Goldstein 1994; Smith 2000; Henter 2003). However, in most Hymenoptera inbreeding should result in additional costs owing to the predominant mechanism of sex-determination, the single-locus complementary sex-determination (sl-CSD) (Beye *et al.* 2003).Typically, unfertilized (haploid) hymenopteran eggs develop into males, whereas fertilized (diploid) eggs develop into females. However, diploid animals that are homozygous at the sex-determination locus develop into diploid males, which are usually sterile (Cook 1993; Owen & Packer 1994; Cook & Crozier 1995). Since inbreeding increases the proportion of homozygosity and therefore the occurrence of diploid males, matings between close kin should be strongly selected against in hymenoptera with sl-CSD.

Sexual signals have been shown to vary with the degree of kinship as well as geographical distribution in many animal species, which could enable females to choose their mates

according to an optimal outbreeding model. Compared to the large literature on geographical variation in acoustical signals (e.g. Westcott & Kroon 2002; Nelson & Soha 2004; Packert & Martens 2004; e.g. Gammon *et al.* 2005), there are relatively few reports on the geographical variation in chemical sexual signals. This is rather surprising, since pheromones apparently play a dominant role for mate finding in the vast majority of species, especially in insects. Additionally, due to quantitative and qualitative variability in multicomponent semiochemicals (Hölldobler 1995; Ayasse 2001) and extreme sensitivity of olfactory systems (Kaissling 1971; Angioy *et al.* 2003), chemical signals have the potential for carrying a large amount of information for mate choice. Owing to their economic importance, studies on pheromone variation mostly deal with moth female sex-pheromones (Boo & Jung 1998; Huang *et al.* 1998; McElfresh & Millar 1999; Kawazu *et al.* 2000; Kawazu *et al.* 2002). Studies on other taxa, especially those in which males signal chemically, are scarce (e.g. male courtship peptide pheromones in certain newts and salamanders (Rollmann *et al.* 2000; Iwata *et al.* 2005).

Female European beewolves (*Philanthus triangulum*, Hymenoptera: Crabronidae) hunt honeybee workers (*Apis mellifera*) as provisions for their progeny (Strohm & Linsenmair 1997; Strohm & Linsenmair 1999; Strohm & Linsenmair 2000; Strohm & Marliani 2002). Male beewolves establish small territories in the vicinity of female nest aggregations, which do not contain any resources essential for beewolf females (Simon-Thomas & Poorter 1972; Strohm 1995). The males apply a marking pheromone from a cephalic gland (the postpharyngeal gland) onto plants within their territory and defend the territory against intruding males in combat flights without physical contact (Simon-Thomas & Poorter 1972; Evans & O'Neill 1988; Strohm 1995; Strohm & Lechner 2000; Schmitt *et al.* 2003; Kroiss *et al.* submitted). Behavioral observations provide evidence that the pheromone attracts receptive females to the males' territories: Females of several *Philanthus* species including *P. triangulum* have been observed to approach male territories in a zigzagging flight pattern from the downwind side, probably orienting towards the windborne pheromone (Evans & O'Neill 1988). Copulations usually occur within the males' territories (Simon-Thomas & Poorter 1972; Strohm 1995) and seem to be under the control of the females since they can easily repel unwanted males by virtue of their larger body size (Evans & O'Neill 1988) or refuse copulations by bending their abdomen tip downwards (E. Strohm, pers. obs.). Territories of different males are often aggregated, thereby constituting a lek situation in which the females have an ideal opportunity to choose among males (Simon-Thomas & Poorter 1972; Evans & O'Neill 1988). Since the copulation is not preceded by any kind of visual display, female choice appears to be, at least predominately, based on information obtained from the male sex pheromone (E. Strohm & M. Kaltenpoth, unpubl. obs.).

The marking-pheromone of male European beewolves comprises a complex blend of up to 55 compounds, with (*Z*)-11-eicosen-1-ol as the main component (Schmidt *et al.* 1990; Schmitt *et*

al. 2003; Kroiss *et al.* submitted). Both extracts from total heads and samples from pure postpharyngeal glands reveal these substances in the same relative amounts (Kroiss *et al.*, submitted), and the same compounds can also be found in extracts from male territories (E. Strohm, T. Schmitt, G. Herzner, J. Kroiss and M. Kaltenpoth, unpubl. data). The amount and composition of the cephalic gland content have been shown to differ between families and vary with the age of the males (Herzner *et al.* in press-a; Kaltenpoth & Strohm in press). The composition of this multicomponent signal might contain important cues for females to assess male quality and choose among potential mates.

Mate choice is likely to be very important in the European beewolf, because females probably mate only once (Evans & O'Neill 1988), thus, choosing a low-quality male will affect all daughters of a given female. Male offspring will not be influenced, since they do not inherit genes from their mother’s mate due to the haplo-diploid sex determination mechanism. A daughter's reproductive success heavily depends on her “quality” (Strohm & Linsenmair 1997; Strohm & Daniels 2003). Therefore, “bad” genes from the father might affect a daughter's life-span and her ability to hunt honeybees and transport the prey to her nest in flight. Thus, female choice for males with “good genes” could strongly influence female fitness. Since male beewolves do not seem to provide females with any resources for reproduction, female mate choice may be based on genetic benefits. Females can either choose a male with “good genes” or with genes of “best compatibility” with its own genes. Males with “good genes” could be chosen according to characters of the pheromone blend that are linked to attributes like size, age (Kaltenpoth & Strohm in press), good immunocompetence, or high resource holding power, as has been shown for the male sex-pheromone of a lizard species (Lopez *et al.* 2006).

Good compatibility of a male could be achieved by female choice based on pheromone characters that are correlated with the degree of relatedness. As mentioned above, inbreeding imposes exceptional costs on hymenoptera due to the nature of the sex-determination mechanism. The costs of sterile males developing from fertilized eggs are raised even more in mass-provisioning hunting wasps, where female larvae are provided with more resources than male larvae, which is the case in the European beewolf (Strohm & Linsenmair 2000). It has been shown recently that the marking-pheromone of male European beewolves varies with family affiliation (Herzner *et al.* in press-a). Females should be strongly selected for an avoidance of mating with close relatives. The variation of the marking-pheromone with family affiliation may enable the female to avoid close kin for mating and thus avoid the high costs of inbreeding. Extensive outbreeding, on the other hand, may be selected against if environmental conditions require local adaptations to soil composition and microclimate or the type and behaviour of brood parasites (Strohm *et al.* 2001). In this case, females would be expected to use information that may be contained in the male pheromone to choose a mate from the same (sub)population to avoid the break-up of local adaptations.

Here, we investigate whether the marking-pheromone of male European beewolves varies between populations, between subpopulations, and among families within subpopulations in a way that might allow females to choose mates adaptively with regard to both avoidance of inbreeding and avoidance of outbreeding. We compare the relative effects of population and family association on the composition of the pheromone and discuss consequences for optimal mate choice.

9.3 Materials and Methods

9.3.1 Insects and sampling

The analysis of geographical variation in the male beewolf sex pheromone was conducted with two data sets on two geographical scales. The first data set (data set 1) was implemented with focus on a local scale (subpopulation level), the second data set (data set 2) with emphasis on a regional scale (population level). For data set 1, seven (sub)populations with distances ranging from 3 to 274 km (median = 31 km) were sampled (populations: Würzburg, Biocenter, Germany, (49°46´47´´N, 09°58´11´´E), Würzburg, City, Germany, (49°47´56´´N, 09°55´38´´E), Veitshöchheim, Germany, (49°48´20´´N, 09°53´22´´E), Retzbach, Germany, (49°54´55´´N, 09°49´16´´E), Schweinfurt, Germany, (50°03´00´´N, 10°14´00´´E), and Düsseldorf, Germany, (51°11´13´´N, 6°48´09´´E)). In data set 2, five populations with distances ranging from 31 to 911 km (median = 490 km) were examined (populations: Würzburg, Germany, (49°46´47´´N, 09°58´11´´E), Schweinfurt, Germany, (50°03´00´´N, 10°14´00´´E), Düsseldorf, Germany, (51°11´13´´N, 6°48´09´´E), Vizzola Ticino near Milano, Italy, (45°37´35´´N, 08°42´14´´E), and Puttenham near London, UK, (51°13'20''N, 0°40'02''W)).

Female European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae) were collected at each of the locations given above. They were transferred to laboratory cages at the University of Würzburg and kept as described before (Strohm 1995). Cocoons with larvae of the F1 generation were placed individually in Eppendorf® tubes and kept in boxes with moist sand at 10°C for four to nine months of overwintering. Cocoons were then transferred to warm conditions (cycles of 12 hours at 25°C and 12 hours at 22°C) and adult beewolves emerged four to six weeks later. Emerging males were marked individually with up to three spots of acrylic paint on the dorsal side of the thorax and were allowed to fly in a climate chamber (2.5 x 1.8 x 2.1 m in size) with 12h light/dark cycles at 25°C/20°C and provided with honey *ad libitum*. Since very young males have been shown to considerably differ in amount and composition of the pheromone (Kaltenpoth & Strohm in press) males were all caught at an age of 12-17 days

and kept in small polystyrol vials (height: 80 mm; diameter: 35 mm) with moist sand and a drop of honey for two days to allow the pheromone glands to be replenished. After anesthetizing the males with CO_2, they were killed by freezing and kept frozen (at –20°C) until extraction of the pheromone and GC-MS analysis.

Overall, 393 males were used for the analysis (Data set 1: Würzburg, Biocenter, Germany: 54, Würzburg, City, Germany: 76, Veitshöchheim, Germany: 35, Retzbach, Germany: 26, Schweinfurt, Germany: 57, and Düsseldorf, Germany: 12; Data set 2: Würzburg, Germany: 46, Schweinfurt, Germany: 26, Düsseldorf, Germany: 8, Vizzola Ticino, Italy: 24, and Puttenham, UK: 29).

9.3.2 Gas chromatography - mass spectrometry

Frozen males were decapitated and their heads were incised at both sides to open up the postpharyngeal gland, which is the storage organ of the male sex pheromone (Herzner *et al.* in press-b; Kroiss *et al.* submitted). Heads were placed individually in glass vials (1.5 ml), and 20 µl of a 1g/l solution of octadecane in hexane (equivalent to a final amount of 20 µg of octadecane) was added as an internal standard to each vial to allow quantification of the pheromone. The heads were then submerged in approximately 1 ml distilled hexane and chemicals were extracted for four hours.

Samples were analyzed by coupled capillary gas chromatography-mass spectrometry (GC-MS) with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The two data sets were run on the same GC-MS device, but with different capillary columns and different temperature programs. GC-MS set-up 1 (data set 1): The GC was equipped with a HP-5 fused silica capillary column (J&W, 30 m x 0.32 mm ID; df = 0.25µm; temperature program: from 60°C to 300°C at 5°C/min, held constant for 1 min at 60°C and for 10 min at 300°C). GC-MS set-up 2 (data set 2): The GC was equipped with a RH-5ms+ fused silica capillary column (J&W, 30 m x 0.25 mm ID; df = 0.25µm; temperature program: from 120°C to 300°C at 3°C/min, held constant for 1 min at 120°C and for 1 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 in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. Since preliminary analyses had revealed that the total amount of chemicals in the sample has an effect on the detection and quantification of certain components, samples that were out of a predefined range of pheromone concentration were rerun after

adjusting the pheromone concentration by addition or evaporation of hexane. The software MSD ChemStation for Windows was used for data acquisition. Pheromone components had already been characterized (Schmitt *et al.* 2003; Kroiss *et al.* submitted) and could be unambiguously identified by their retention times and mass spectra.

9.3.3 Statistical analysis

Pheromone amount and composition

In the pheromone extracts, 25 components could be reliably detected in all samples, and their peaks were manually integrated with MSD ChemStation software (Agilent Technologies). The substances were identified by comparison of mass spectra and retention times with earlier analyses (Schmitt *et al.* 2003; Kroiss *et al.* submitted). Not all substances described as components of the pheromone by Kroiss *et al.* (submitted) could be detected due to the low concentrations of the pheromone extracted from single males. Using the octadecane peak as an internal standard, the total amount of pheromone was calculated and then $\log_{10}$-transformed to obtain normally distributed data for statistical analysis. The $\log_{10}$-transformed absolute amounts of pheromone were compared among populations by ANOVAs. SPSS 13.0 software was used for the calculations.

The relative amounts of the 25 pheromone components were calculated. Several peaks had to be combined for the analysis, because they were not always clearly separated by the GC-MS. This applies to (*Z*)-9-octadecen-1-ol and (Z)-10-nonadecen-2-one (C19enone in the following), 1-octadecanol and heneicosane (C18anol), "Unidentified substance 2" and docosane (C22ane), (*Z*)-11-eicosen-1-ol and tricosenes/tricosadiene (C20enol), and 1-eicosanol and tricosane (C23ane). Thus, the 25 detected substances were reduced to 20 variables that were included in the analysis. This procedure is conservative with regard to the hypotheses tested. Because the relative amounts constitute compositional data, they were transformed to logcontrasts prior to analysis (Aitchison 1986).

Differentiation and occurrence of C25- and C27-type

The pheromone of male European beewolves shows a distinct dimorphism (Kroiss *et al.*, submitted) that has to be taken into account. The two morphs differ mainly in the relative proportions of pentacosene (mixture of isomers with *Z*-(9)-pentacosene as the main component) and heptacosene (mixture of isomers with *Z*-(9)-heptacosene as the main component), and they can be distinguished unambiguously by the relative amount of heptacosene, which shows a clearly bimodal distribution (Fig. 9.1). The morph with a high proportion of pentacosene (C_{25}-type; Aitchison-transformed proportion of heptacosene < 0.55) is more abundant than the one having a high proportion of heptacosene instead (C_{27}-type; Aitchison-transformed proportion of

heptacosene > 0.55) (Fig. 9.1) (Kroiss *et al.* submitted). Since chance variations in the proportion of C_{25}- and C_{27}-type males among families and among populations can greatly influence the outcome of statistical analyses on the chemical differentiation, all of the following analyses were performed on C_{25}- and C_{27}-type individuals combined as well as on C_{25}-type individuals only. The sample size of C_{27}-type males was too small for a reasonable analysis excluding the C_{25}-type individuals.

Population differentiation

The number of describing variables was reduced by principal component analyses (PCA, Aitchison-transformed relative amounts of pheromone components as variables, varimax rotation, factor extraction: eigenvalues > 0.8). The extracted PCA factors were used for discriminant analyses (DA) to test whether males of different populations can be separated based on their pheromone profiles. A PCA and a DA was conducted for each of the two data sets, respectively. For PCA and DA, data set 1 was restricted to the local scale, including only subpopulations in close spatial vicinity (Würzburg, Biocenter, Würzburg, City, Veitshöchheim, Retzbach: maximum distance: 18km) to exclude regional effects on the outcome of the DA.

Family differentiation

To determine whether families within populations can be separated on the basis of the chemical profile, PCA and DA were conducted separately for each population, for which at least three families with five or more brothers were available. The number of PCA factors was restricted to a maximum of N/6 (N = total number of males in the analysis). SPSS 13.0 software was used for the principal component and discriminant analyses.

Relative effects of family and population affiliation on pheromone composition

To assess the relative effects of family and population affiliation on the pheromone composition of male European beewolves, we conducted a multivariate nested ANOVA on the Aitchison-transformed relative peak areas with family membership as a nested factor within populations. For each data set, two ANOVAs were computed, one including both C_{25}- and C_{27}-type males and the other one with C_{25}-type individuals only. For every pheromone peak, the proportion of variance explained by the two factors was estimated by partial η^2-values (Cohen 1973; Keppel 1991; Olejnik & Algina 2003). To assess the relative effects of family and population on the pheromone composition, the η^2-values for both effects were compared over all peaks in paired t-tests after checking for normal distributions using Kolmogorov-Smirnoff tests. All tests were computed using SPSS 13.0 software.

Association between geographical and chemical distance

The geographical distances between all sampled populations were calculated from the population coordinates with the DIVA-GIS software (freely available at http://www.diva-

gis.org/) and subsequently log-transformed. The chemical distances between the populations were calculated as follows: The mean for each of the 20 Aitchison-transformed pheromone components was calculated for all populations. The chemical distance between two given populations x and y was calculated as the Euclidian distance according to the formula $D_{chem}(x,y)=\sqrt{\sum_{i=1}^{n}(x_i-y_i)^2}$ with x_i as the mean of pheromone component i of population x. To be able to combine data sets 1 and 2, which slightly differed due to the differences in GC-MS set-ups, chemical distances were normalized. To this end, we assumed that the chemical distances between two populations that were sampled in both data sets were identical and served as a reference. Consequently, we were able to normalize the chemical distances of the two data sets with the distance between Würzburg and Schweinfurt, Würzburg and Düsseldorf, and Schweinfurt and Düsseldorf, respectively. The respective chemical distance was set to 1 in both data sets and all other values were converted to relative chemical distances. The normalizations based on the three different reference distances revealed qualitatively the same results in the following analyses, indicating that the procedure yielded valid results. The relationship between geographical and chemical distances was visualized using a scatter-plot and a linear regression line. We tested for a correlation between the matrices of geographical and chemical distances using a Mantel-test that can deal with missing values using the software R 2.3.0 (mantel.test from the ncf package) (Mantel 1967; Legendre & Legendre 1998). P-values were calculated based on 100,000 resamplings. Mantel-tests were performed with each dataset separately, and with the normalized combined dataset. All tests were conducted with both C_{25}- and C_{27}-type males and with C_{25}-type-males only to exclude effects of different relative frequencies of both chemo-types across populations.

9.4 Results

9.4.1 Pheromone amount and composition

In the GC-MS analysis of the male sex pheromone of the European beewolf *P. triangulum*, we found a total of 25 substances: (*S*)-2,3-dihydrofarnesoic acid (DHFS in the following); (*Z*)-9-octadecen-1-ol; (*Z*)-10-nonadecen-2-one; 1-octadecanol; heneicosane; "Unidentified substance 1" (unknown 1); "Unidentified substance 2"; docosane; (*Z*)-11-eicosen-1-ol; a mixture of (*Z*)-9-, (*Z*)-7-tricosene, and Δx,y,-tricosadiene; 1-eicosanol; tricosane; a mixture of (*Z*)-9-, (*Z*)-7-tetracosene, and Δx,y,-tetracosadiene (C24en); tetracosane (C24an); a mixture of (*Z*)-9-, (*Z*)-7-pentacosene, and Δx,y,-pentacosadiene (C25en); pentacosane (C25an); a mixture of 7-, 11-, and 13-methyl pentacosane (m-C25an); (*Z*)-9-hexacosene (C26en); hexacosane (C26an); Δ-16-

pentacosen-8-one (C25one); a mixture of (*Z*)-9-, (*Z*)-7-heptacosene, and Δx,y,-heptacosadiene (C27en); heptacosane (C27an); octacosane (C28an); nonacosane (C29an); hentriacontane (C31an) (for abbreviations of compounds that had to be combined for the analysis see "Statistical analysis" section in "Materials and methods"). The total amount of pheromone extracted from *P. triangulum* males varied between 101 and 2508µg (mean ± SD = 655 ± 377µg). In both data sets, the sampled populations differed significantly in their total amount of pheromone (data set 1: ANOVA, $F = 6.66$, $df = 5$, $p < 0.01$; data set 2: ANOVA, $F = 3.99$, $df = 4$, $p < 0.01$).

9.4.2 Differentiation and occurrence of C_{25}- and C_{27}-type

The histogram of the Aitchison-transformed proportion of heptacosene revealed a pronounced bimodal distribution (Fig. 9.1). The C_{25}-type (heptacosene in low proportion) was overall by far the more common type (79.1% of all males) compared to the C_{27}-type (20.9% of all males). The frequency of the C_{25}-type varied considerably between the sampled populations from 8.3 to 100.0% in the sampled populations (mean ± SD = 72.6 ± 26.3%).

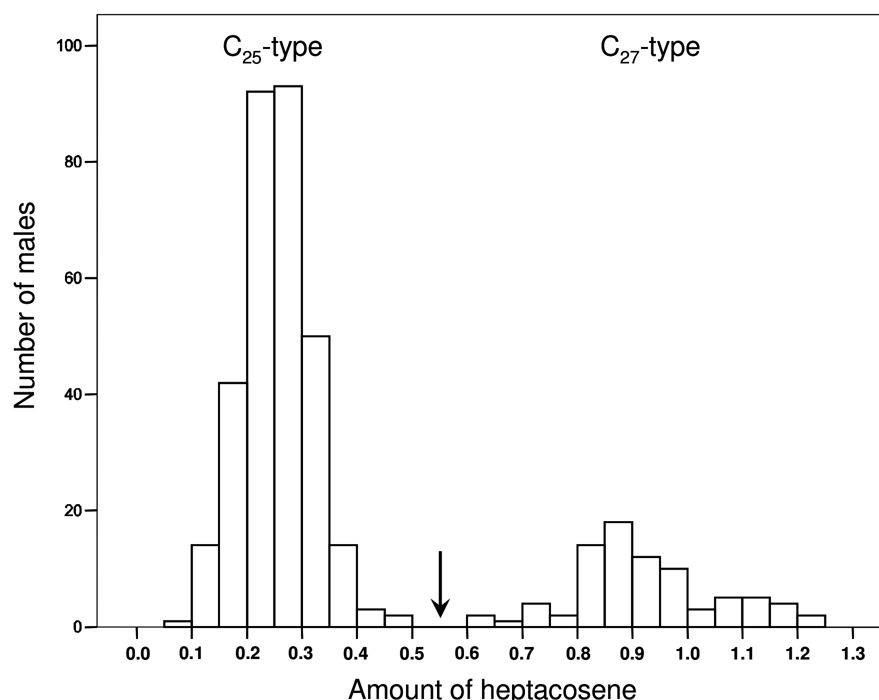

Figure 9.1: Frequency distribution of heptacosene in the sex-pheromone of 393 males (mixture of isomers, see text for explanation, Aitchison-transformed relative proportions). Note the clearly bimodal distribution with the C_{25}-type being the far more abundant type. The arrow denotes the limit of the relative proportion between the two pheromone types for type assignment (see text for explanation).

9.4.3 Population differentiation

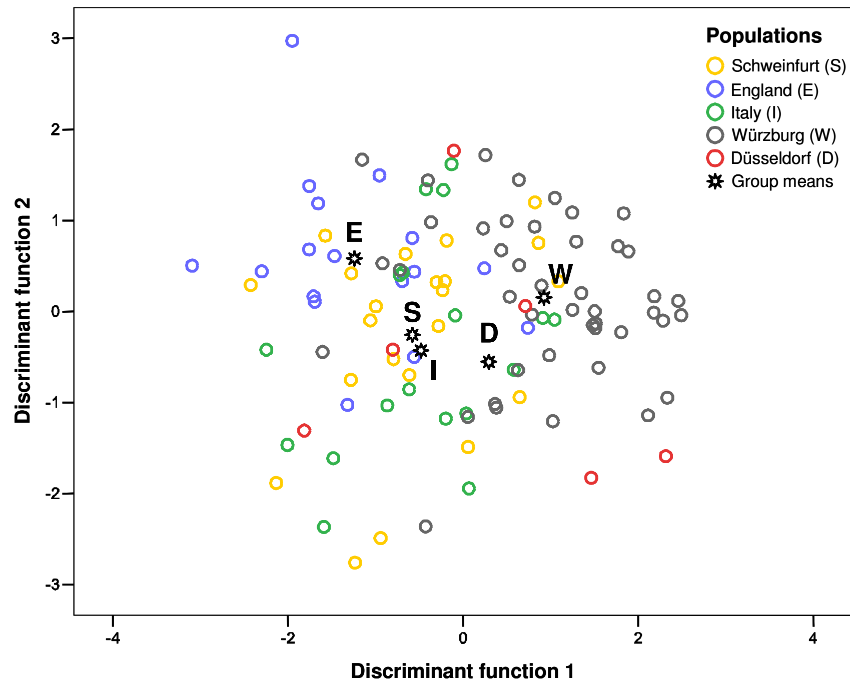

Figure 9.2: Discriminant analysis of geographical variation of the sex-pheromone on the regional scale (data set 2, five populations, C_{25}-type only). Despite some overlap, the populations are significantly separated (see Table 9.1 and text for details).

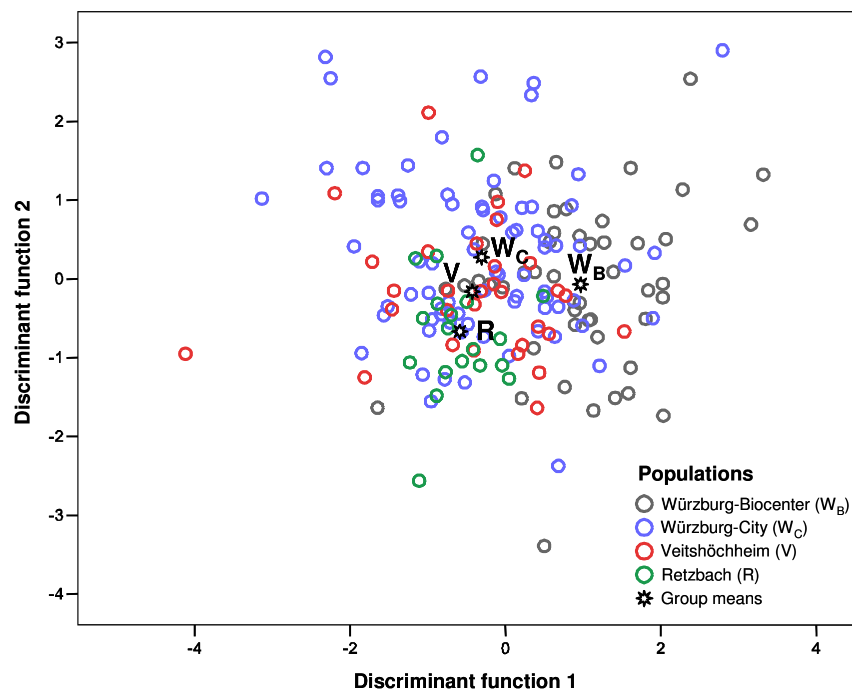

Figure 9.3: Discriminant analysis of geographical variation of the sex-pheromone on the local scale (data set 1, four subpopulations, C_{25}-type only). Despite broad overlap, the populations are significantly separated (see Table 9.1 and text for details).

Populations in both data sets and, thus, on two different spatial scales could be significantly separated by DAs (Table 9.1, Fig. 9.2 and 9.3). This was irrespective of the inclusion or omission of the C_{27}-type in the analysis. Classification of DA revealed that 45.0 to 56.1% of males were correctly assigned to the populations, depending on the data set and inclusion or omission of the C_{27}-type (20.0 or 25.0% correct classifications would have been expected by chance). Despite the higher number of groups in the DA, the classification results were generally more accurate for the populations on the regional than on the local scale, indicating that the chemical distances were positively correlated with the geographical scale.

Table 9.1: Population and family differentiation by Principal Component and Discriminant Analyses. “Type” indicates, whether only the C_{25}-type or both C_{25}- and C_{27}-type were included in the analysis. For PCAs, the number of factors as well as the cumulative explained variance is given. For DAs, the number of functions, Wilk's-λ, X^2, degrees of freedom, p-value, and the percentage of correct classifications by DA are given. (Population abbreviations: W: Würzburg, Germany; W_B: Würzburg, Biocenter, Germany; W_C: Würzburg, City, Germany; V: Veitshöchheim, Germany; R: Retzbach, Germany; D: Düsseldorf, Germany; I: Vizzola Ticino, Italy; E: Puttenham, UK).

						PCA		Discriminant analysis					
Scale	(Sub)Population(s)	Data set	Families	Type	n	Fact.	Expl.Var.(%)	Funct.	Wilk's-λ	X^2	df	p	Corr.class.(%)
Regional	W, S, D, I, E	2	-	C_{25}/C_{27}	133	7	84.07	4	0.531	79.65	28	**0.000**	52.6
Regional	W, S, D, I, E	2	-	C_{25}	107	7	83.79	4	0.485	72.40	28	**0.000**	56.1
Local	W_B, W_C, V, R	1	-	C_{25}/C_{27}	191	6	80.58	3	0.604	93.30	18	**0.000**	45.0
Local	W_B, W_C, V, R	1	-	C_{25}	175	6	80.41	3	0.611	83.14	18	**0.000**	49.7
Family	W_B	1	4	C_{25}/C_{27}	45	7	84.87	3	0.292	47.43	21	**0.001**	60.0
Family	W_B	1	4	C_{25}	44	7	86.09	3	0.240	53.44	21	**0.000**	75.0
Family	W_C	1	7	C_{25}	74	5	79.76	5	0.097	156.25	30	**0.000**	58.1
Family	W	2	4	C_{25}	36	6	84.62	3	0.206	47.44	18	**0.000**	66.7
Family	V	1	3	C_{25}/C_{27}	28	4	81.03	2	0.557	13.73	8	0.089	60.7
Family	V	1	3	C_{25}	24	4	77.46	2	0.614	9.51	8	0.301	62.5
Family	S	1	3	C_{25}/C_{27}	28	4	81.07	2	0.116	50.62	8	**0.000**	89.3
Family	S	2	3	C_{25}/C_{27}	20	3	66.36	2	0.358	16.45	6	**0.012**	60.0

9.4.4 Family differentiation

Families within populations could be significantly separated in six out of eight populations (Table 9.1, see Fig. 9.4 for a representative example). Individual males were correctly classified in 58.1 to 100.0% of cases (14.3 to 50.0% correct classifications would have been expected by chance). Although DAs were not always significant, the overall classifications of families within populations were more accurate than classifications between populations even if different numbers of groups in the DA are taken into account.

Thus, males belonging to different populations and males belonging to different families within a population can be separated from each other on the basis of quantitative differences in some of the pheromone compounds.

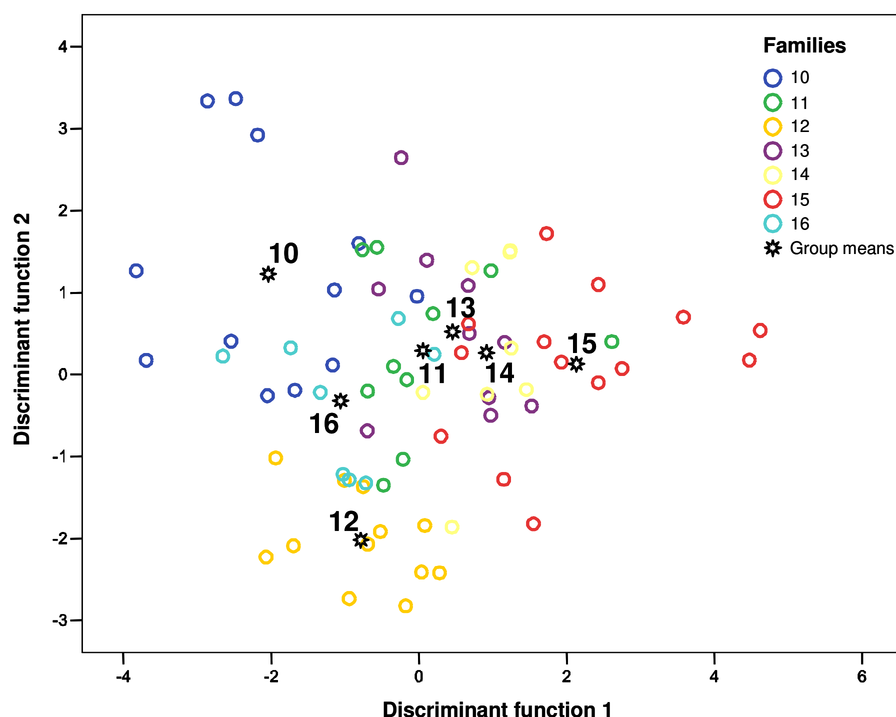

Figure 9.4: Discriminant analysis of the variation of the sex-pheromone on the family level (data set 1, one population: Würzburg City, C_{25}-type only). Despite some overlap, the families are significantly separated (see Table 9.1 and text for details).

9.4.5 Relative effects of family and population affiliation on pheromone composition

In the multivariate nested ANOVAs, both family and population affiliation had significant effects on the pheromone composition in each dataset, regardless of the omission or inclusion of C27-type males in the analysis (Table 9.2). Together, family and population affiliation explained between 11.4 and 90.8% of the variance in peak areas. The distribution of η^2-values did not deviate significantly from a normal distribution for any of the analyses (Kolmogorov-Smirnoff tests: $Z \leq 1.16$, $p \geq 0.135$ for all tests).

On a local scale (data set 1), family membership explained a significantly higher proportion of the variance in pheromone composition than subpopulation affiliation (paired t-tests, C_{25}-/C_{27}-type: df = 19, t = -6.22, p < 0.001; C_{25}-type only: df = 19, t = -6.17, p < 0.001). On a regional scale (data set 2), however, this effect was reversed, with population affiliation explaining more of the variance, although this effect was only significant when both C_{25}- and C_{27}-type males were included (paired t-tests, C_{25}-/C_{27}-type: df = 19, t = 2.11, p = 0.048; C_{25}-type only: df = 19, t = 1.84, p = 0.081).

Table 9.2: Relative effects of family and (sub)population affiliation on pheromone composition in male European beewolves. Proportions of variance explained by family and population membership in nested MANOVAs are estimated for each peak by partial η^2-values. MANOVA results are given for each data set, including and excluding C_{27}-type males, respectively (see text for peak abbreviations).

Data included in MANOVA	Data set 1: Local C_{25}- and C_{27}-type		Data set 1: Local C_{25}-type only		Data set 2: Regional C_{25}- and C_{27}-type		Data set 2: Regional C_{25}-type only	
Peak	η^2(Pop)	η^2(Fam)	η^2(Pop)	η^2(Fam)	η^2(Pop)	η^2(Fam)	η^2(Pop)	η^2(Fam)
DHFS	0.024	0.110	0.010	0.109	0.041	0.118	0.084	0.101
C19enone	0.089	0.243	0.086	0.250	0.439	0.060	0.462	0.105
C18anol	0.118	0.166	0.133	0.168	0.268	0.162	0.188	0.191
unknown1	0.055	0.074	0.033	0.081	0.273	0.070	0.207	0.076
C22ane	0.024	0.302	0.025	0.337	0.053	0.200	0.040	0.263
C20enol	0.088	0.063	0.083	0.072	0.137	0.085	0.152	0.079
C23ane	0.036	0.079	0.056	0.083	0.336	0.190	0.308	0.244
C24ene	0.044	0.354	0.012	0.376	0.069	0.185	0.162	0.139
C24ane	0.085	0.200	0.060	0.200	0.116	0.033	0.083	0.036
C25ene	0.001	0.190	0.016	0.217	0.062	0.156	0.126	0.154
C25ane	0.062	0.165	0.026	0.136	0.161	0.071	0.081	0.079
mC25ene	0.152	0.327	0.119	0.330	0.174	0.094	0.232	0.098
C26ene	0.001	0.182	0.107	0.301	0.284	0.072	0.307	0.096
C26ane	0.225	0.247	0.209	0.230	0.146	0.056	0.093	0.045
C25enone	0.012	0.239	0.012	0.243	0.222	0.289	0.397	0.511
C27ene	0.014	0.188	0.105	0.276	0.255	0.074	0.178	0.037
C27ane	0.205	0.388	0.191	0.397	0.133	0.141	0.091	0.083
C28ane	0.074	0.141	0.066	0.119	0.046	0.085	0.051	0.080
C29ane	0.080	0.166	0.045	0.165	0.219	0.140	0.168	0.080
C31ane	0.076	0.135	0.068	0.135	0.270	0.197	0.285	0.216
MANOVA results:								
Pillai's trace	**0.944**	**3.582**	**0.988**	**3.703**	**2.029**	**2.414**	**2.301**	**2.618**
F	**5.137**	**2.994**	**5.366**	**3.019**	**3.501**	**1.869**	**3.184**	**1.897**
p	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

Table 9.3: Geographical (white, in km) and chemical (grey, normalized to Würzburg - Düsseldorf) distances between the nine sampled (sub)populations of *P. triangulum*. For population abbreviations refer to Table 9.1.

	W	W_B	W_C	V	R	S	D	I	E
W	-	NA	NA	6	18	31	274	472	746
W_B	NA	-	4	6	18	31	274	472	746
W_C	NA	0.29	-	3	15	32	270	473	743
V	NA	0.58	0.47	-	13	33	267	474	740
R	NA	0.70	0.55	0.68	-	31	256	485	732
S	1.73	0.76	0.82	0.57	1.13	-	276	501	758
D	1.00	1.00	0.97	0.81	0.79	1.08	-	635	479
I	1.59	NA	NA	NA	NA	0.64	1.51	-	911
E	3.48	NA	NA	NA	NA	2.23	3.40	2.41	-

9.4.6 Correlation between geographical and chemical distance

To test for a correlation between the matrices of geographical and chemical distances of the populations we performed a Mantel-test that can deal with missing values. We detected a strong correlation between geographical and chemical distance for data set 1 and the normalized combination of both datasets irrespective of which combination of populations was used as a

reference for normalization and independent of the inclusion or omission of the C_{27}-type in the analysis (Tables 9.3 and 9.4, Fig. 9.5). A Mantel-test restricted to data set 2 revealed no significant correlation.

Table 9.4: Correlation between geographic and chemical distances of populations of *P. triangulum*. Given are regression coefficients (r) and p-values of Mantel tests. The column "type" indicates, whether only the C_{25}-type or both C_{25}- and C_{27}-type were included in the analysis. Data sets 1 and 2 were normalized to Würzburg – Schweinfurt (W–S), Würzburg – Düsseldorf (W–D), or Schweinfurt – Düsseldorf (S–D). For population abbreviations see Table 9.1. P-values < 0.05 are given in bold.

Data set	Scale	Populations	Type	Normalization	Mantel test r	p
1	Local	W_B, W_C, V, R, S, D	C25/C27	-	0.924	**0.003**
1	Local	W_B, W_C, V, R, S, D	C25	-	0.764	**0.004**
2	Regional	W, S, D, I, E	C25/C27	-	0.303	0.254
2	Regional	W, S, D, I, E	C25	-	0.272	0.319
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25/C27	S-W	0.684	**0.008**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25/C27	D-W	0.780	**0.003**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25/C27	S-D	0.762	**0.004**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25	S-W	0.503	**0.016**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25	D-W	0.630	**0.013**
1 and 2	Local+Regional	W, W_B, W_C, V, R, S, D, I, E	C25	S-D	0.602	**0.005**

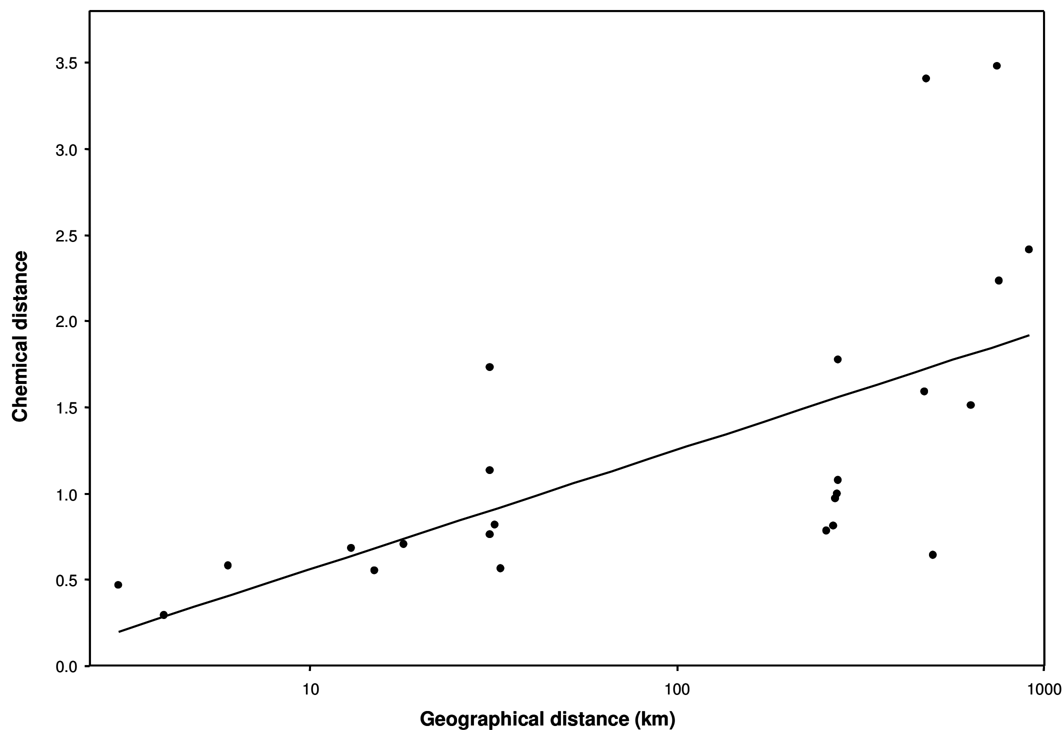

Figure 9.5: Correlation between pairwise geographic and chemical distances of populations of *P. triangulum* males (data sets 1 and 2; C_{25}-type only; normalization: Würzburg - Düsseldorf; Mantel-test: r = 0.630, p = 0.013; see also Table 9.4 and text for details). The trend line was obtained by linear regression in order to visualize the association.

9.5 DISCUSSION

In the present study, we investigated interindividual differences in the pheromone composition of male European beewolves on three different levels: between families, among subpopulations on a local scale, and among geographically distant populations. Our results show that there are significant differences in pheromone composition on all three levels and that the chemical distance between populations is correlated with the geographical distance.

Geographical variation in sex pheromone composition has been described for several insect taxa (Clearwater *et al.* 1991; Yatsynin *et al.* 1996; Grosman *et al.* 1997; Huang *et al.* 1998; McElfresh & Millar 1999; Gemeno *et al.* 2000; Kawazu *et al.* 2000; McElfresh & Millar 2001; Krokos *et al.* 2002; El-Sayed *et al.* 2003). Previous studies, however, have focused on pheromones produced by females; evidence for geographical variation in male sex pheromones is largely lacking (but see Hamilton *et al.* 2005; Watts *et al.* 2005). Since there is usually a reproductive conflict of interest between the sexes (Trivers 1972), male sex pheromones are expected to underlie completely different selective pressures than female pheromones (Phelan 1992, 1997). Females produce few large eggs, and they usually invest much time and resources in brood care, whereas males produce numerous small sperm and often do not contribute to brood care (Trivers 1972). This asymmetry generally leads to competition among males for limited females, and males are expected to maximize their fitness predominantly by attracting and mating with as many females as possible (Bateman 1948; Trivers 1972; Andersson 1994). Females, on the other hand, are usually limited by resources for offspring production and brood care, so they should be choosy and mate with the best available male (Trivers 1972; Gould & Gould 1997). Thus, females should be selected to obtain information on the suitability of a potential mate from his courtship signals.

Our results demonstrate that the sex pheromone composition of male European beewolves differs quantitatively among (sub)populations and that chemical and geographical distances are correlated. Since beewolves have good flying abilities and are pioneer species that frequently colonize new habitats (Evans 1974), females are likely to encounter males from other local subpopulations in the field. Thus, female beewolves may use the information contained in the male pheromone to choose adaptively among potential mates and thereby avoid outbreeding depression. Deleterious effects of extensive outbreeding have been demonstrated in many recent studies (Demeester 1993; Edmands 1999; Aspi 2000; Marshall & Spalton 2000; Andersen *et al.* 2002; Marr *et al.* 2002; Goldberg *et al.* 2005; Peer & Taborskyi 2005; Sagvik *et al.* 2005), and several hypotheses have been proposed to explain why outbreeding depression occurs (e.g. break-up of coadapted gene complexes, disruption of epistatic interactions, loss of local adaptions, dispersal hazards, and risk of parasite infection; see Bateson 1983; Pusey & Wolf 1996).

Although local subpopulations as well as geographically distant populations of beewolves could be separated on the basis of the male sex pheromone, only 45 - 56% of the individual males were classified correctly by the discriminant analyses, and chemical profiles of different populations overlapped considerably. However, the existing differences might be enough for females to reduce the incidence of outbreeding, especially because the sensitivity of female chemoreceptors may exceed that of our analytical methods by several orders of magnitude (Kaissling 1971; Angioy *et al.* 2003). Furthermore, our data provide only a lower boundary for the actual effect of geographical origin on the pheromone composition, since all the animals were reared under the same conditions in the laboratory. Differences in environmental factors during larval development have been shown to affect the pheromone composition of male beewolves (K. Roeser-Mueller, unpubl. data). Since developmental conditions are likely to vary between populations, actual differences between populations in the field may be much larger than those observed under controlled conditions in the laboratory.

Within populations, beewolf male pheromones differ significantly among families (this study and Herzner *et al.* in press-a). The family-related differences may enable females to reduce the chances of mating with close kin and thereby avoid inbreeding depression, which is likely to impose especially high costs on beewolves (see introductory paragraphs). Kin recognition in animals is generally mediated by phenotype matching, by the recognition of genetically compatible mates, or by imprinting or learning of the individuals that occur in the same nest or birth place (Hepper 1986; Fletcher & Michener 1987; Waldman *et al.* 1988; Brown & Eklund 1994; Pusey & Wolf 1996; Hauber & Sherman 2001). The mechanisms by which female beewolves could distinguish between kin and non-kin are unclear (for discussion see Herzner *et al.* in press-a). The discrimination of males from different populations may be possible for females by sampling pheromones from different males in a lek and avoiding individuals with a pheromone blend that differs markedly from the population mean. Further studies are necessary to elucidate the mechanisms of kin- and population-recognition in European beewolves.

The multivariate nested ANOVAs indicate that pheromone composition is affected more strongly by family than subpopulation affiliation on a local scale, whereas the effect of population affiliation is larger than the family effect on a regional level (Table 9.2). These results are consistent with the positive correlation between chemical and geographical distance (Fig. 9.5) and suggest that (1) the subpopulations sampled on the local scale may be connected by a relatively frequent interchange of individuals and might, thus, represent a single population, and (2) small differences in the chemical profile between local subpopulations may add up on a regional scale according to an isolation-by-distance model (Wright 1943). Interestingly, the family effect exceeds the local subpopulation effect, making family differentiation potentially easier for beewolf females than subpopulation discrimination.

If female beewolves use the information on kinship and geographical origin that is contained in the male sex pheromone, they may be able to choose adaptively among potential mates according to the model of optimal outbreeding, thus, avoiding the deleterious effects of both in- and outbreeding by choosing a mate of intermediate genetic distance (Bateson 1983; Shields 1993). Studies considering both in- and outbreeding avoidance in an integrated model of "optimal outbreeding" are scarce (but see Bateson 1978, 1980; Palmer & Edmands 2000). The European beewolf constitutes an interesting model system to test for optimal outbreeding in a species with a complex male sex pheromone, and further studies may show whether females indeed use the male pheromone to avoid in- and outbreeding.

Acknowledgements

We thank Jeremy Field and Thomas Eltz for providing specimens from the UK and Düsseldorf. Paolo Rosa is acknowledged for his help in finding beewolves in Italy. Collection of specimens in Italy was made possible by a travel grant from the Arthur von Gwinner-foundation to JK. We thank Thomas Hovestadt for valuable suggestions concerning the statistical analysis and Johannes Penner for assistance with the calculations of the geographical distances in DIVA-GIS. This study was supported by a grant of the German Science Foundation (STR 532/1-2) and by a PhD fellowship of the German National Academic Foundation to MK.

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CHAPTER 10

POLYMORPHIC MICROSATELLITE MARKERS FOR A SOLITARY DIGGER WASP, THE EUROPEAN BEEWOLF (*PHILANTHUS TRIANGULUM*; HYMENOPTERA, SPHECIDAE)

Molecular Ecology Notes 4 (4), December 2004: 589-592.

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10.1 SUMMARY

We present primer sequences for 14 polymorphic microsatellite loci in the European beewolf, *Philanthus triangulum*. Microsatellites were isolated using an efficient enrichment procedure. The number of alleles ranged from two to 28, with observed levels of heterozygosity between 0.090 and 0.828. The microsatellites were designed to determine paternity in female offspring of *P. triangulum* and to study female choice in this species. We also report the applicability of these markers for a congeneric North American species, *Philanthus gibbosus*.

10.2 INTRODUCTION

The European beewolf, *Philanthus triangulum*, is a solitary digger wasp that lives in warm and sandy habitats from Europe to Southern Africa. Female beewolves hunt honeybees (*Apis mellifera*), paralyse them by stinging and carry them to their nest in flight where the bees serve as larval food (Strohm 1995). Males defend mating territories in low vegetation against intruding competitors. They apply a pheromone from their mandibular glands to the vegetation

within their territory that is likely to serve as a sex attractant for females (e.g. Evans & O'Neill 1988; Schmitt *et al.* 2003).

Male pheromone composition differs among individuals, and there appears to be a genetic basis for this variability (Herzner *et al.* submitted). However, it is not known whether females use differences in the pheromone blend for mate choice. To study female choice in the European beewolf, mating success of males with different pheromone composition has to be determined. However, copulations are difficult to observe under field and laboratory conditions because they are not preceded by any apparent courtship behaviour, occur at no particular time between one and 14 days after female emergence and last only ten to fifteen minutes. Thus, genetic analysis of paternity is the more appropriate way to determine male reproductive success. Therefore, we established polymorphic microsatellite markers for paternity analysis to study female choice in *P. triangulum.*

10.3 METHODS, RESULTS AND DISCUSSION

Microsatellites were isolated from genomic DNA of *P. triangulum* according to an enrichment procedure introduced by Fischer & Bachmann (1998) and modified after Rütten *et al.* (2001) and Kronauer & Gadau (2002). Genomic DNA was extracted from the thorax of an adult female following a standard phenol chloroform extraction protocol. Approximately 2-3 µg of extracted DNA were digested with HinfI, and the resulting fragments were ligated to the adapter described in Rütten *et al.* (2001). Enrichment of fragments containing microsatellites was performed by hybridisation of a biotinylated $(CA)_{10}$-, $(GA)_{10}$-, or $(CT)_{10}$-oligonucleotide, subsequent binding of those hybrids to streptavidin-coated iron beads, and magnetic separation (Rutten *et al.* 2001). Five steps of washing with increasing stringency ensured the removement of unhybridized or imperfectly hybridized fragments (Fischer & Bachmann 1998). After denaturation at 95°C for 5 min the remaining fragments were eluted in 50µl sterile water.

Target DNA was amplified by polymerase chain reaction using the reverse adapter oligonucleotide as single primer (Rutten *et al.* 2001). PCR products were ligated into the pCR®2.1-TOPO® vector (InvitrogenTM) and transformed into OneShot® Chemically Competent Escherichia coli cells (InvitrogenTM). We picked white clones from plates, and amplified the inserts via PCR with the flanking primers T7-Promoter and M13-reverse primers. An additional screen for microsatellite-positive clones was achieved by a second PCR with one of the flanking primers and a repeat-specific primer that was used for the initial selection [$(TC)_{10}$ or $(AC)_{10}$, respectively]. Of the positive clones, 103 were sequenced on a Beckmann-

Coulter CEQ 2000 XL sequencer. We designed primer pairs for 26 loci of microsatellites, 14 of which turned out to be polymorphic in *P. triangulum* (Table 10.1).

To assess the number of allelels and the heterozygosity, 60 beewolf females were collected in the immediate proximity of the Biocenter of the University of Würzburg, Germany. For comparison with a distant population we also analysed 20 females from Düsseldorf, Germany. PCR amplification was performed on a T1 Thermocycler (Biometra®), or an Eppendorf® Mastercycler in a total reaction volume of 12.5 µl containing approximately 10 ng of template, 1X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 2.5 mM $MgCl_2$, 240 µM dNTPs, 10 pmol of each primer, and 0.5 U of Taq DNA polymerase (MBI Fermentas). Cycle parameters were as follows: 3 min. at 94°C, followed by 32 cycles of 94°C for 40 sec., 1 min. at the primer-specific annealing temperature (see Table 10.1), 72°C for 1 min., and a final extension time of 4 min. at 72°C. 2 µl of PCR products were run on Spreadex® EL 600 gels (Elchrom Scientific) at 55°C. Gels were stained with SYBRTM Green and alleles were scored by eye. For eight loci (Ptr-2, Ptr-8, Ptr-9, Ptr-16, Ptr-20, Ptr-22, Ptr-23, Ptr-25), primers labelled with infrared dyes DY-681 or DY-781 (Biomers) were used in PCR reactions (5 pmol per 12.5 µl reaction), and amplification products were run on 6% polyacrylamide gels on a LI-COR 4300 DNA Analyser. Alleles were analysed using Saga Generation 2 software.

Table 10.1: Characteristics of microsatellite loci isolated from Philanthus triangulum. N, number of individuals (females) genotyped; A, observed number of alleles; HO, observed heterozygosity; HE, expected heterozygosity.

Locus	Primer sequence (5´-3´)	Annealing Temp. (°C)	Repeat motif*	Size (bp)*	Population: Würzburg/Düsseldorf				GenBank accession no.
					N	A	H_O	H_E	
Ptr-2	ACTCGATGTACGTGTGTA TCCATGCTGTCGTACTTTCC	60	$(GA)_3AA(GA)_2GG(GA)_6$	174	29/5	3/2	0.241/0.600	0.444/0.420	AY591584
Ptr-8	GGAGACACTTAGAGCGTAAAACG TAACGCCTCTCGGCGATG	60	$(AG)_2G(AG)_2AA(AG)_7N_6(AG)_4$	217	15/5	3/3	0.533/0.600	0.420/0.620	AY591585
Ptr-9	ACCACGACCTATTCCAATGC GTCCCGCGGTACGTAAATG	60	$(GA)_2N_6(GA)_7TA(GA)_4AA(GA)_2$	162	55/20	12/10	0.745/0.900	0.832/0.863	AY591586
Ptr-10	AAAAGAAGCCTAGCGGAAGC GCGTGTTATGGGACAAGAGC	60	$(CAG)AAA(CAG)_3AAA(CAG)_3TAG(CAG)_3$	235	9/4	2/2	0.111/0.500	0.278/0.375	AY591587
Ptr-12	TACCCCCTCCATTTCCAAG CGTAGCTGGGGGAGGAAG	62	irregular CT-repeat	240	11/4	2/2	0.090/0.250	0.350/0.220	AY591588
Ptr-13	CCACCTCGTTCAATTTCCAG GAAAGGAAGGGTTCTACGTTTG	60	irregular CT-repeat	218	11/4	2/2	0.450/0.750	0.500/0.469	AY591589
Ptr-14	CTTCCTCCCCCAGCTACAAC GGAGAGTCACAAAGGGAGACG	63	irregular GA-repeat	164	9/4	2/2	0.440/0.250	0.350/0.220	AY591590
Ptr-16	CGTACAAAGGAATACAGAGGAAG TCTCTCTGCACATATTTTTCTCC	60	$A_8TA_3TAGA_4TTGA_7GA_4GGGAGA_5$	215	59/20	7/6	0.610/0.700	0.786/0.700	AY591591
Ptr-17	CCACTTCAAAAACCATATGAGAG CTCAACTTCTTGTCGGCATATAG	60	$(TG)(AG)_2(TG)_5$	229	8/4	3/2	0.250/0.250	0.461/0.220	AY591592
Ptr-20	ATGAGAGAGCGAGAACAAATG CTCGCACCTTCTTCGCTAC	60	$(AG)_{19}$	137	58/18	18/12	0.828/0.833	0.919/0.881	AY591593
Ptr-22	TCGTTATTCCTTTTCTTTG TTGGGTATGGGGCATATCTC	53	$(GA)_6N_7(GA)A(GA)AA(GA)_3GG(GA)_8$	161	56/20	8/4	0.554/0.650	0.760/0.704	AY591594
Ptr-23	CGACAATAATGTGCGTTTGC TCAGGGAGAGTGAGGAAAGG	60	$(CT)_{25}$	193	58/19	28/14	0.828/0.842	0.941/0.893	AY591595
Ptr-24	GATACGTCGCCTCTGTAATACG CCGTCCCCTGAGTTTACG	60	$(GA)_2G(GA)_7GG(GA)GG(GA)_2GG(GA)_3$	180	8/4	4/3	0.500/0.250	0.649/0.220	AY591596
Ptr-25	AATTTTGAACACCCCAAAACC TAGACGAGGAGACGGTTTGC	60	$(AG)_{26}$	220	58/20	25/14	0.517/0.550	0.928/0.913	AY591597

*Cloned allele

The number of alleles per locus ranged from two to 28, with an observed heterozygosity between 0.090 and 0.828 in the population from Würzburg (Table 10.1). Two to 14 alleles were observed in the population from Düsseldorf, with observed heterozygosities ranging from 0.250

to 0.900 (Table 10.1). Thus, there is ample polymorphism for paternity analysis in P. *triangulum* using these microsatellites.

We tested the six most variable loci (Ptr-9, Ptr-16, Ptr-20, Ptr-22, Ptr-23, and Ptr-25) for deviations from Hardy-Weinberg equilibrium and for linkage disequilibrium using Genepop 3.4. For the Würzburg population, allele distributions for all loci except Ptr-9 deviated significantly from Hardy-Weinberg equilibrium ($p < 0.05$), with strong heterozygote deficits, whereas only Ptr-25 deviated from HWE in the Düsseldorf population (Table 10.2). These results suggest that the Würzburg population experiences high levels of inbreeding that lead to a deficiency in heterozygotes across loci. Inbreeding may also explain the significant linkage disequilibrium we found for some loci in the Würzburg population, but not in the population from Düsseldorf (Table 10.2).

Table 10.2: Results of probability tests for deviations from Hardy-Weinberg-equilibrium and for linkage disequilibrium. P-values below 0.05 are printed in bold. For the linkage disequilirbium, the upper right part of the table represents the Würzburg population, the lower left part represents the Düsseldorf population.

	Deviations from HWE (p-values ± S.E.)		Linkage disequilibrium (p-values) Würzburg / Düsseldorf					
Locus	Würzburg	Düsseldorf	Ptr-9	Ptr-16	Ptr-20	Ptr-22	Ptr-23	Ptr-25
Ptr-9	0.07 ± 0.01	0.66 ± 0.02		**0.00**	**0.05**	0.22	0.35	0.07
Ptr-16	**0.00 ± 0.00**	0.66 ± 0.01	0.52		**0.01**	0.43	0.20	0.08
Ptr-20	**0.03 ± 0.01**	0.18 ± 0.02	1.00	1.00		**0.00**	**0.03**	**0.04**
Ptr-22	**0.00 ± 0.00**	0.57 ± 0.01	1.00	0.55	0.43		**0.00**	**0.01**
Ptr-23	**0.00 ± 0.00**	0.53 ± 0.04	1.00	0.24	1.00	1.00		0.32
Ptr-25	**0.00 ± 0.00**	**0.00 ± 0.00**	1.00	1.00	1.00	0.45	1.00	

We also tested the six most variable loci in four individuals of a congeneric species, *P. gibbosus* from North America. Amplification products were obtained for all six loci. The four loci Ptr-9, Ptr-16, Ptr-22, and Ptr-25 gave distinct bands on the LI-COR sequencer, with four, one, three, and five alleles, respectively. Scoring of alleles for Ptr-20 was difficult due to stutter bands and alleles of Ptr-23 were too weak to be defined. Both problems might be overcome by optimising the PCR conditions. Thus, loci presented in this study will also be useful for genetic analyses in closely related species of *P. triangulum*.

Acknowledgements

We would like to thank Thomas Eltz for providing us with specimens from Düsseldorf and Jon Seger for sending *Philanthus gibbosus* from North America. We thank Daniel Kronauer for help

with the microsatellite enrichment protocol. This work was supported by a grant of the Deutsche Forschungsgemeinschaft SFB 554 TB-B3.

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CHAPTER 11

GENERAL DISCUSSION

11.1 ANTENNAL ENDOSYMBIONTS OF THE EUROPEAN BEEWOLF

The first chapters (2-7) of this thesis demonstrate that the European beewolf engages in a highly specific interaction with bacteria of the genus *Streptomyces* that are cultivated in the antennal gland reservoirs of females. These studies provide the first case of symbiotic microorganisms in insect antennae and one of the few known interactions between insects and the important antibiotic-producing group of actinomycete bacteria. In the following, the ecology and evolution of the beewolf-*Streptomyces* interaction will be discussed in the light of other symbiotic interactions between insects and microorganisms.

11.1.1 Localization

The vast majority of symbiotic microorganisms in insects have been located in abdominal organs of the hosts, especially in the digestive tract tissue, in specialized bacteriomes associated with the digestive tract, in the ovaries, or in the fat body (Blochmann 1892; Buchner 1921; McLean & Houk 1973; Ishikawa 2003; Dillon & Dillon 2004). Some symbionts can also be found in the hemolymph and are, thus, distributed over the whole body (Hypsa & Dale 1997; Dale & Maudlin 1999; Fukatsu *et al.* 2001; Moran *et al.* 2005c; Tsuchida *et al.* 2005). In leaf-cutter ants, exosymbiotic bacteria are cultivated in elaborate cuticular crypts that are supported by exocrine glands (Currie *et al.* 1999; Currie *et al.* 2006).

Beewolves constitute the first known example of a symbiosis with microorganisms that are cultivated in insect antennae (chapters 2-5). '*Candidatus* Streptomyces philanthi' resides in huge reservoirs of specialized antennal glands that are so far only known from the genus *Philanthus* (chapters 4 & 5). The glands show structural similarity to the cuticular crypts of leaf-cutter ants (Currie *et al.* 2006), since they also constitute invaginations of the cuticle and are supported by exocrine glands. Antennal glands have been described for several other insect taxa, but they are generally much smaller than in beewolves (Bartlet *et al.* 1994; Isidoro & Bin 1995; Bin *et al.* 1999; Isidoro *et al.* 1999; Isidoro *et al.* 2000; Guerrieri *et al.* 2001; Battaglia *et al.* 2002; Romani *et al.* 2003; Romani *et al.* 2005). Furthermore, these glands are usually restricted to males and the secretions seem to be involved in courtship and mating (Isidoro &

Bin 1995; Bin *et al.* 1999; Isidoro *et al.* 1999; Guerrieri *et al.* 2001; Battaglia *et al.* 2002; Romani *et al.* 2003; Romani *et al.* 2005).

11.1.2 Phylogenetic position

Microbial endosymbionts of insects are phylogenetically diverse: Many species belong to the Proteobacteria (α-, β- and γ-subdivision) (e.g. Unterman *et al.* 1989; Campbell *et al.* 1992; Clark *et al.* 1992; Aksoy *et al.* 1995; Werren *et al.* 1995; Chen *et al.* 1996; Moran *et al.* 2005b; Gottlieb *et al.* 2006), but symbionts from the Flavobacteria/Bacteroidetes group (Bandi *et al.* 1994; Bandi *et al.* 1995; Moran *et al.* 2005a), the Mollicutes (Hurst *et al.* 1999; Williamson *et al.* 1999), the Spirochetes (Varma *et al.* 1994; Breznak 2000; Lilburn *et al.* 2001; Breznak 2002; Hongoh *et al.* 2003), the low-GC gram-positive bacteria (Ohkuma & Kudo 1996; Schafer *et al.* 1996; Ohkuma & Kudo 1998; Gebhardt *et al.* 2002b) and the Actinobacteria (Auden 1974; Hill *et al.* 1976; Currie *et al.* 1999; Beard *et al.* 2002; Watanabe *et al.* 2003) have also been reported (for review see also Baumann & Moran 1997; Bourtzis & Miller 2003). However, bacteria of the family Actinomycetaceae, and especially the genus *Streptomyces*, have rarely been found as symbionts of insects (but see Hill *et al.* 1976; Bignell *et al.* 1991; Schafer *et al.* 1996; Currie *et al.* 1999; Gebhardt *et al.* 2002a). This is insofar surprising, as this group comprises numerous species that produce secondary metabolites with potent antibiotic properties (Behal 2000; Watve *et al.* 2001) and, thus, could possibly provide benefits to insect hosts by protecting them against pathogens. Culture-independent PCR-based genetic analyses of the beewolf antennal bacteria revealed that they can be unambiguously assigned to the actinomycete genus *Streptomyces* on the basis of their 16S rDNA sequence (chapters 2 & 5) and the gene sequence encoding elongation factor Tu (M. Kaltenpoth, unpubl. data), a GTP binding protein that plays a central role in protein synthesis (Alberts *et al.* 2002). Additionally, the phylogenetic position within the genus *Streptomyces* was confirmed with fluorescence in-situ hybridization using specific oligonucleotide probes (chapters 2, 3 and 5). Thus, symbioses with actinomycete bacteria might be more common among insects than was previously apparent from the few available studies.

11.1.3 Distribution among Philanthinae

The beewolf antennal endosymbiont '*Candidatus* Streptomyces philanthi' could be detected in all 28 species and subspecies of the genus *Philanthus* that have been investigated so far (chapter 5). Thus, the symbiosis with '*Candidatus* Streptomyces philanthi' might be universal for digger wasps of the genus *Philanthus*. Due to their rarity, the most closely related genera, *Trachypus* and *Philanthinus* (Alexander 1992), have not been examined for the presence of '*Candidatus* Streptomyces philanthi' yet. However, three other genera within the subfamily Philanthinae (*Aphilanthops*, *Clypeadon* and *Cerceris*) apparently lack the antennal bacteria (chapter 5). Thus,

the association with '*Candidatus* Streptomyces philanthi' probably evolved around the origin of the genus *Philanthus*, possibly also comprising the genera *Trachypus* and *Philanthinus*. The genus *Trachypus* would be especially interesting to investigate for the presence of '*Candidatus* Streptomyces philanthi', since it apparently groups within the genus *Philanthus* (Alexander 1992).

Since there are no data available on the evolutionary age of the genus *Philanthus*, the origin of the symbiosis with *Streptomyces* can at present only be approximated with the sequence divergence of the endosymbionts. The maximum 16S rDNA divergence of 1.07% among different ecotypes of '*Candidatus* Streptomyces philanthi' suggests an evolutionary age of the symbiosis of about 26-67 million years (chapter 5), assuming that the nucleotide substitution rate is comparable to that of other bacterial 16S rDNA sequences (Ochman & Wilson 1987; Moran *et al.* 1993; Bandi *et al.* 1994). Moran *et al.* (1993) estimated an evolutionary age of 160-280 million years for the symbiosis between aphids and their endosymbiont *Buchnera aphidicola*, and Bandi *et al.* (1995) dated the origin of the association of cockroaches and termites with bacteria of the *Flavobacterium-Bacteroides* group to about 135 to 250 million years ago. Thus, compared to other insect-bacteria symbiosis, the beewolf-*Streptomyces* association appears to be of relatively recent origin. Further studies on the phylogenies of both the symbionts and their hosts are necessary to reconstruct the evolutionary history of the association more accurately.

11.1.4 Transmission route

Several pieces of evidence suggest that '*Candidatus* Streptomyces philanthi' is transmitted vertically from mother to offspring: First, although the bacteria are apparently absent from beewolf eggs and transovarial transmission can therefore be ruled out (M. Kaltenpoth, unpubl. data), vertical transfer from one generation to the next is possible, since the bacteria are secreted into the brood cell by the mother and later taken up by the larva (chapters 2 & 3). Second, female larvae that were reared in the absence of white substance in their brood cell lacked the symbiotic bacteria as adults (chapter 2 and M. Kaltenpoth, unpubl. data). Third, the identity of 16S rDNA sequences of '*Candidatus* Streptomyces philanthi triangulum' from distant populations and the high similarity of sequences from different ecotypes of the symbionts render vertical transmission very likely, although a highly specific uptake mechanism from the environment could also explain the high degree of sequence similarity (McFall-Ngai & Ruby 1991; Nyholm *et al.* 2000; Nishiguchi 2002; Nyholm & McFall-Ngai 2004). However, '*Candidatus* Streptomyces philanthi' has not been found outside of the beewolf host or brood cell, not even by fluorescence in-situ hybridization of the surrounding sand (M. Kaltenpoth, unpubl. data). Thus, taken together these data strongly suggest that the beewolf antennal

symbionts are transmitted vertically from mother to offspring, but comparative phylogenetic analyses of hosts and symbionts are necessary to demonstrate coevolution and cospeciation of both partners.

Assuming predominantly vertical transmission for '*Candidatus* Streptomyces philanthi', one step in the transmission process from the mother to the offspring is still unknown: the incorporation of the bacteria in the antennal gland reservoirs of the adult beewolf. It has been observed that the mother applies the streptomycetes to the brood cell, and the larva apparently takes them up and applies them to the cocoon silk during the spinning process (chapters 2 & 3). *A priori*, there are two different possible ways to incorporate the bacteria into the antennal gland reservoirs: After uptake of the bacteria by the larva, they remain within the larva's body and are later transported to the antennae of the adult beewolf via the hemolymph; or the bacteria are directly taken up from the cocoon with the antennae by the adult beewolf during eclosion. At present, theoretical considerations as well as preliminary data provide evidence for the second transmission route: scanning electron microscopic analyses located the *Streptomyces* bacteria on both sides of the cocoon (chapters 2 & 5; M. Kaltenpoth, unpubl. data), thus rendering uptake of the bacteria prior to or during eclosion possible. Furthermore, no antennal symbionts could be detected in three female beewolves that had been removed from the cocoon as larvae and transferred to autoclaved cocoons or sterile Eppendorf vials until eclosion (M. Kaltenpoth, unpubl. data). However, these results have to be considered with caution because of the small sample size and the lack of appropriate controls.

Vertical transmission is apparently the predominant mode of transfer for most symbiotic microorganisms. It has been demonstrated for obligate or primary endosymbionts (Aksoy *et al.* 1997; Clark *et al.* 2000; Thao *et al.* 2001; Moran *et al.* 2003; Baumann & Baumann 2005; Moran *et al.* 2005a; Gottlieb *et al.* 2006) as well as for several facultative or secondary symbionts (e.g. Fukatsu *et al.* 2000; Darby *et al.* 2001; Fukatsu *et al.* 2001; Tsuchida *et al.* 2005). Horizontal transfer events appear to be rare or absent for obligate intracellular endosymbionts, while they are commonly observed in facultative symbionts (Sandstrom *et al.* 2001; Russell *et al.* 2003; Russell & Moran 2005; Tsuchida *et al.* 2005). Comparative phylogenetic analyses of beewolves and their symbiotic bacteria are lacking, so at present it can only be speculated about the occurrence of horizontal transfer among hosts. Theoretically, horizontal transfer is possible in the beewolf-*Streptomyces* symbiosis, since the bacteria spend part of their life-cycle in the brood cell outside of the host, and transfer between different beewolf individuals or species could be established via Chrysidid parasitoids, burrow sharing, nest usurpation, nest reuse, and especially through interspecific predation (see Evans & O'Neill 1988). The possibility of horizontal transfer of bacteria among beewolves or from other taxa is further substantiated by genetic analyses of *Wolbachia* infections in beewolves: Several phylogenetically diverse strains of these bacteria were detected in species of the genus

Philanthus, and horizontal transfer has apparently played an important role for the distribution of *Wolbachia*, since the strains of different *Philanthus* species do not cluster together in the *Wolbachia* phylogeny (Fig. 11.1) (M. Kaltenpoth, unpubl. data).

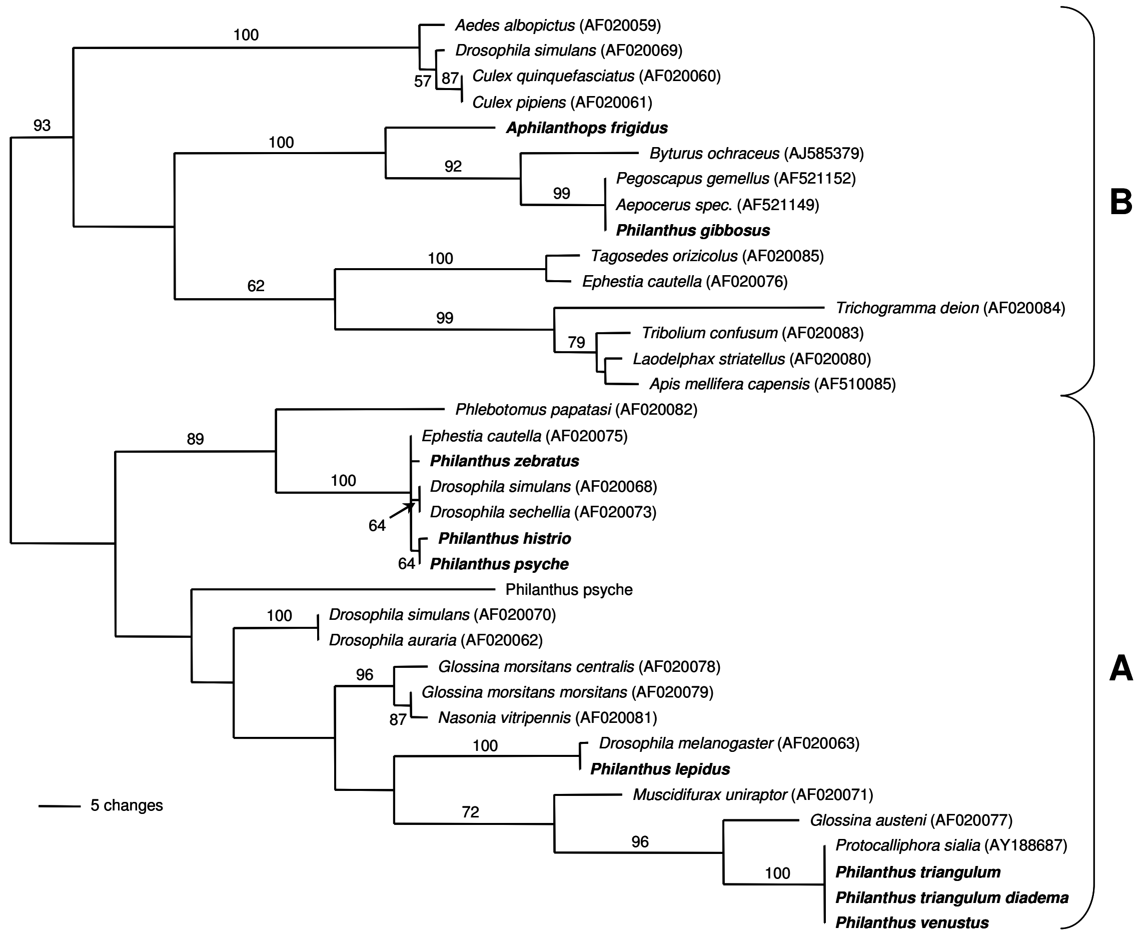

Figure 11.1: Phylogeny of *Wolbachia* (denoted by their respective host species) based on wsp sequences. First of two equally parsimonious trees from a full heuristic search with TBR branch swapping and 10 random addition sequence replicates based on 455 bp of wsp sequences (242 constant, 35 variable but parsimony-uninformative, 178 parsimony-informative). Numbers at the nodes represent bootstrap values from a search with 1000 replicates, using the same heuristic search settings. GenBank accession numbers of published sequences are given behind the species names. Sequences for philanthine wasps are highlighted in bold (M. Kaltenpoth, unpubl. data). The letters A and B denote the two major subclades of *Wolbachia* (Zhou *et al.* 1998).

11.1.5 Benefits for the host

Symbiotic actinomycete bacteria have been described from triatomine bugs, termites, leaf-cutter ants, several other arthropods, and from marine invertebrates. In *Rhodnius prolixus* (Hemiptera, Reduviidae), the actinomycete *Rhodococcus rhodnii* aids in the processing of B-complex

vitamins and in sexual maturation of the bug (Harington 1960; Lake & Friend 1968; Auden 1974; Hill *et al.* 1976; Benyakir 1987). Termites have been found to harbor *Streptomyces* and other actinomycete bacteria in the digestive tract, where they appear to aid in cellulose and hemicellulose degradation and thereby contribute to the termites' carbon metabolism (Bignell *et al.* 1991; Varma *et al.* 1994; Schafer *et al.* 1996). Antibiotic-producing *Streptomyces* strains have been isolated from the guts of several arthropod species (Gebhardt *et al.* 2002a; Krastel & Zeeck 2002) as well as from marine sponges (Imamura *et al.* 1993; Lee *et al.* 1998; Selvin *et al.* 2004; Lee *et al.* 2005), but their metabolic activity *in situ* and the benefits for the hosts have not been investigated. In leaf-cutter ants, antibiotic-producing exosymbiotic bacteria of the genus *Pseudonocardia* produce antibiotics and thereby protect the hosts' fungus gardens against a specialized fungal parasite (Currie *et al.* 1999; Currie 2001; Currie *et al.* 2003a; Currie *et al.* 2003b; Cafaro & Currie 2005; Currie *et al.* 2006). Thus, symbiotic actinomycete bacteria can be involved in the nutrition as well as in the defense of invertebrate hosts against pathogen attack.

In the European beewolf, the symbiotic actinomycete '*Candidatus* Streptomyces philanthi' is involved in the protection of the immature wasps against pathogen infestation (chapters 2 & 3). Removal of the bacteria from the beewolf brood cell resulted in drastically increased mortality rates of the larvae (chapter 2 & 3). Presumably, the symbiotic bacteria that had been applied to the cocoon by the larva during the spinning process (chapter 2 & 3) suppress the growth of other microorganisms on the cocoon and thereby inhibit the invasion of pathogenic fungi or bacteria into the cocoon. Since species of the genus *Streptomyces* are known to produce a wide variety of potent antibiotic substances (Behal 2000; Watve *et al.* 2001), it seems likely that this inhibitory effect is due to secondary metabolites from the symbionts. This hypothesis is supported by observations that fungal growth on wet paper towels showed clear inhibition zones around hibernating cocoons of *Philanthus sanbornii* (Jon Seger, pers. comm.). Alternatively, '*Candidatus* Streptomyces philanthi' may be competitively superior to other microorganisms on the cocoon, thus exploiting the substrate more efficiently and thereby suppressing the growth of potential pathogens, as has been demonstrated for other bacterial species (Godfray *et al.* 1999; Dillon & Dillon 2004).

11.1.6 Benefits for the symbionts

Symbiotic microorganisms generally benefit from the association with insects or other eukaryotic organisms by obtaining an unoccupied and usually competition-free niche and a reliable transmission route into the next generation (Currie 2001). Additionally, they are often supplied with limiting nutrients by the host (*Buchnera, Blochmannia*, etc.). Several pieces of evidence strongly suggest that '*Candidatus* Streptomyces philanthi' receives nutrients from the beewolf host: (1) Female beewolves often construct several brood cells per day and secrete

massive amounts of bacteria from the antennal gland reservoirs (Strohm & Linsenmair 1995); thus, to replenish the stock for further brood cells, the bacteria have to grow quickly within the reservoirs, which is only possibly with a continuous supply of nutrients from the host. (2) The reservoirs are surrounded by class 3 gland cells that show an extraordinarily high density of rough endoplasmatic reticulum (chapter 4). It is therefore likely that these cells produce large amounts of proteins that may be secreted into the gland reservoir and possibly serve as nutrient supply for the endosymbionts. (3) One of the walls of the antennal gland reservoir is of a net-like structure with a very thin epicuticle (chapter 4), thus possibly allowing nutrient transfer from the hemolymph into the gland reservoir. This possibility is further supported by chemical analyses of the hydrocarbon profile of the antennal gland secretion and beewolf hemolymph: Except for two substances that are probably produced by the bacteria themselves (chapter 6), the hydrocarbon composition of the white substance and the hemolymph is virtually identical (chapters 6 & 7).

During the time on the cocoon, the growth rate of the bacteria is probably very low, since the bacteria are limited in space and presumably also in resources. These resources might either consist of proteins from the cocoon silk, or they might be supplied by the larva from inside of the cocoon. Preliminary chemical analyses suggest that metabolic byproducts from the excretory pathways of the larva might be present on the cocoon (M. Kaltenpoth, unpubl. data). Like several other *Streptomyces* species (Nitsch & Kutzner 1968; Williams *et al.* 1983; Katsifas *et al.* 2000), '*Candidatus* Streptomyces philanthi' has a urease gene (M. Kaltenpoth, unpubl. data), thus, excretory byproducts from the larva might be recycled by the bacteria and used as carbon and nitrogen supply.

Over long evolutionary times, symbiotic interactions often lead to obligate mutual interdependence of hosts and symbionts (Zientz *et al.* 2004). On the symbionts' side, this is often reflected in extensive genome reductions and rearrangements (Shigenobu *et al.* 2000; Akman *et al.* 2001; Akman *et al.* 2002; Gil *et al.* 2002; Tamas *et al.* 2002; Gil *et al.* 2003; Rio *et al.* 2003; van Ham *et al.* 2003; Moran & Plague 2004; Degnan *et al.* 2005). However, '*Candidatus* Streptomyces philanthi' may not be able to dispense with as many genes as intracellular endosymbionts like *Buchnera* or *Blochmannia*, since it lives extracellularly and it has to retain the ability to survive both in the antennal gland reservoirs and on the cocoon. Therefore, it is unlikely that *S. philanthi* experienced reductions in the genome that are comparable to those of the obligate intracellular symbionts for which the whole genome sequences are currently available (Shigenobu *et al.* 2000; Akman *et al.* 2002; Tamas *et al.* 2002; Gil *et al.* 2003; van Ham *et al.* 2003; Degnan *et al.* 2005). Since all these genome sequences stem from intracellular symbionts of the Proteobacteriaceae, whole-genome analyses of '*Candidatus* Streptomyces philanthi' would be extremely interesting to elucidate the effect of the extracellular symbiotic life-style on the genome of a high-GC gram-positive bacterium.

Additionally, the genome sequence of *S. philanthi* would allow us to draw conclusions on the metabolic interactions between hosts and symbionts.

11.1.7 Evolutionary implications

During the development in subterranean brood cells, the larvae of mass-provisioning digger wasps generally face a high risk of infection by pathogenic microorganisms from the surrounding soil (Janzen 1977; Strohm & Linsenmair 2001). Beewolves evolved several mechanisms to reduce pathogen infestation of the provisioned honeybees (Rathmayer 1962; Strohm & Linsenmair 2001) and the larva itself (chapters 2 & 3). The specific symbiosis with '*Candidatus* Streptomyces philanthi' for the protection of the larva against pathogens might have constituted a key invention in the evolution of ground-nesting and mass-provisioning behavior in the genus *Philanthus*.

The initial steps in the evolution of symbiotic interactions are often very difficult to reconstruct. European beewolves may have been preadapted for the symbiosis with bacteria by the presence of specialized antennal glands. The original function of the glands might have been to provide directional information to the larva that is later necessary for emergence (Strohm & Linsenmair 1995). Subsequently, the gland reservoir may have been invaded by opportunistic soil-dwelling bacteria that were able to utilize a wide range of nutrient supplies. In this scenario, the incorporation of streptomycetes seems likely, since they are abundant in sandy soil (Kutzner 1981) and they can exploit an exceptional variety of carbon and nitrogen sources (Kutzner 1981; Williams *et al.* 1983; Holt *et al.* 1994). At this stage, the bacteria might have been commensals or even parasites. Later on, the beewolf may have benefited from the association by incorporating the bacteria into the cocoon for the protection of the larva.

The *Philanthus-Streptomyces* symbiosis provides the second example for a highly specific alliance of insects with protective actinomycete bacteria. As in the leaf-cutter ant-*Pseudonocardia* system, the development of the holometabolous hosts occurs in subterranean nests and involves the mass-storage of provisions for the larvae. Developmental conditions that are similarly susceptible to pathogen infestation can be found in several other hymenopteran taxa, e.g. in other crabronid and sphecid wasps and in ground-nesting bees and wasps, and also in other insect orders, e.g. in fungus-growing termites and beetles. The mechanisms of pathogen defense in these taxa remain largely unknown (but see Cane *et al.* 1983; Hefetz 1987; Gambino 1993; Rosengaus *et al.* 2000; Ayasse & Paxton 2002), and it is possible that actinomycete bacteria are much more common as protective symbionts than is currently apparent from the few available studies.

11.2 PHEROMONE VARIATION AND MATE CHOICE IN THE EUROPEAN BEEWOLF

In the second part of this thesis (chapters 8-10), we report on age-related changes and geographical variation in the amount and composition of the male beewolf marking pheromone, and we present polymorphic microsatellite markers that can be used for studying female choice by genetic paternity analysis. As most of the specific aspects have been discussed in the respective chapters, I will focus here on more general considerations concerning the information content of the male marking pheromone and its potential as an indicator for female choice, and I will discuss the implications of the results for the evolution of the beewolf marking pheromone.

11.2.1 Potential of the male marking pheromone as an indicator for female choice

Sexual selection theory generally expects females to be choosier than males, because they are usually the limiting sex (Trivers 1972; Andersson 1994; Gould & Gould 1997). The choice for a high-quality mate can significantly increase a female's fitness by providing direct or indirect benefits (Bateson 1983b; Andersson 1994; Kokko *et al.* 2003; Neff & Pitcher 2005). Since male European beewolves neither provide resources to females nor do they contribute to parental care, direct benefits are unlikely to play a role for mate choice in this species. Therefore, I will focus on indirect fitness benefits that a female might obtain for its offspring by choosing adaptively among potential mates.

Species recognition

Sexual advertisement and courtship signals often provide a set of information on the sender that can be used by the receiver for choosing adaptively among potential mates (e.g. Andersson 1994; Droney & Hock 1998; Jones & Hamilton 1998; Lopez *et al.* 2003; O'Loghlen & Rothstein 2003; Slater 2003). One of the most important factors for an adaptive mate choice is the unambiguous identification of conspecific individuals (Bateson 1983a). In beewolves, male pheromones differ markedly in composition among allopatric as well as sympatric species (McDaniel *et al.* 1987; Schmidt *et al.* 1990; McDaniel *et al.* 1992; Schmitt *et al.* 2003; M. Kaltenpoth, G. Herzner & E. Strohm, unpubl. data). The pheromone composition within each species, however, is qualitatively remarkably constant, even over large geographic areas; e.g. male European beewolves from distant localities (England, Germany, Italy, South Africa) contain the same substances within their cephalic glands (chapter 8 and M. Kaltenpoth, unpubl. data). Thus, the composition of the male marking pheromone probably allows for unambiguous species identification by receptive females.

Optimal outbreeding

The degree of kinship between potential mates has long been recognized as an important factor influencing mate choice decisions in animals (Bateson 1983b; Thornhill 1993). Numerous studies provide evidence that individuals of many species can recognize close kin (e.g. Porter & Moore 1981; Gadagkar 1985; Hepper 1986; Bull *et al.* 2001; Mateo 2003; Gamboa 2004; Nakagawa & Waas 2004; Vokey *et al.* 2004) and actively avoid inbreeding when given the choice between kin and non-kin as mates (see Pusey & Wolf 1996 for review). Inbreeding avoidance is generally assumed to be adaptive, and several studies have demonstrated deleterious consequences of matings between close kin (e.g. Packer 1979; Charlesworth & Charlesworth 1987; Dewsbury 1988; Simmons 1989; Bollinger *et al.* 1991; Krackow & Matuschak 1991; Chen 1993; Jimenez *et al.* 1994; Keller *et al.* 1994; Alberts & Altmann 1995; Crnokrak & Roff 1999; Kristensen & Sorensen 2005).

Although empirical evidence is largely lacking, several mechanisms have been proposed to explain the deleterious effects of inbreeding, all of which are directly or indirectly associated with the increase in homozygosity that is caused by inbreeding (Bateson 1983a; Partridge 1983; Barrett & Charlesworth 1991; Pusey & Wolf 1996): First, the reduction in heterozygosity through inbreeding entails a high risk of being homozygous for low-frequency deleterious recessive alleles that would rarely be expressed in outbred populations (Bateson 1983a; Partridge 1983; Pusey & Wolf 1996). Second, for the same reason, beneficial interactions between different alleles at the same locus (heterosis or heterozygous advantage) would be lost in inbred populations (Bateson 1983a; Partridge 1983; Pusey & Wolf 1996). Heterosis effects have been demonstrated for several gene loci (see e.g. Comings & MacMurray 2000; Penn 2002; Wegner *et al.* 2004), notably that of sickle-cell anaemia in humans (Allison 1954, 1964; Aidoo *et al.* 2002). And third, inbreeding leads to the loss of potential for genetic recombination, thereby imposing disadvantages in a variable environment, e.g. higher susceptibility to specialized pathogens (Bateson 1983a; Partridge 1983; Pusey & Wolf 1996). Due to the predominant mechanism of sex determination in hymenoptera, the single-locus complementary sex determination (sl-CSD) (Cook 1993; Haig 1998; Beye *et al.* 2003), inbreeding confers additional costs on hymenoptera, because it increases the incidence of diploid males, which are usually effectively sterile (Cook 1993; Cook & Crozier 1995; Haig 1998; Zayed & Packer 2005).

Although inbreeding can impose significant costs on mating partners, breeding with distantly related individuals ("outbreeding") might be as bad as or even worse than close inbreeding (Shields 1993). Choosing a mating partner of a different species would constitute an extreme case of outbreeding and usually reduce both partners' fitness significantly (Bateson 1983a). But also among different populations of the same species, outbreeding depression has been demonstrated in many recent studies (e.g. Demeester 1993; Edmands 1999; Aspi 2000;

Marshall & Spalton 2000; Andersen *et al.* 2002; Marr *et al.* 2002; Goldberg *et al.* 2005; Peer & Taborskyi 2005; Sagvik *et al.* 2005), and several hypotheses have been proposed to explain the deleterious effects of outbreeding: Mating with a partner from a distant locality may result in the break-up of coadapted gene complexes, disruption of epistatic interactions, and loss of local adaptation in the progeny (Bateson 1983a; Partridge 1983; Pusey & Wolf 1996; Aspi 2000; Andersen *et al.* 2002; Goldberg *et al.* 2005). Additionally, outbreeding individuals may face high direct costs due to dispersal hazards, a lack of site experience, high risk of parasite infection by a distantly related mate, and possibly disrupted parenting by a mismatch of habits acquired by mates in different environments (Bateson 1983a; Partridge 1983; Brown 1991; Pusey & Wolf 1996; Marr *et al.* 2002).

Thus, individuals are generally expected to choose mates of an intermediate genetic distance to balance the costs of in- and outbreeding (Bateson 1983a; Shields 1993). As mentioned above, inbreeding avoidance has been demonstrated for several species (Pusey & Wolf 1996), but studies considering both in- and outbreeding avoidance in an integrated model of "optimal outbreeding" are scarce (but see Bateson 1978; Bateson 1980; Palmer & Edmands 2000). The European beewolf constitutes an interesting model system to study mate choice based on optimal outbreeding, since inbreeding can be expected to impose exceptionally high costs on beewolves: Due to sl-CSD, inbreeding would probably result in a high proportion of diploid males, which are especially costly in beewolves, because diploid offspring are supplied with about twice as many honeybees as haploid offspring (Strohm 1995; Strohm & Linsenmair 2000). Outbreeding might confer costs due to hazards associated with dispersal and the possible break-up of local adaptations, e.g. with regard to specialized parasites or soil conditions (Strohm *et al.* 2001).

Studies on the composition of the male marking pheromone revealed differences among families (Herzner *et al.* in press) as well as between populations of European beewolves from different geographical locations (chapter 9). Thus, the pheromone might contain the necessary information to choose a mate of optimal genetic distance. The establishment of polymorphic microsatellite markers (chapter 10) now allows the assessment of male reproductive success without the time-consuming direct observations of mating activity and therefore renders mate choice studies under semi-field conditions possible. Preliminary choice experiments with European beewolves from two different geographical locations (Schweinfurt and Würzburg, Germany; distance approximately 35 km) revealed no strong female preference for males from the same population (J. Kroiß & M. Kaltenpoth, unpubl. data). However, the distance between these two populations may have been too small to expect serious effects of outbreeding depression, since on this geographical scale significant local adaptations are unlikely in a species with good dispersal abilities like the European beewolf. Further studies are necessary to

investigate the importance of in- and outbreeding avoidance for adaptive female choice in the European beewolf.

Good genes and sexy sons

In addition to the genetic distance, other traits may also be important for mate choice in providing indirect benefits to choosy females (Bateson 1983b; Andersson 1994; Kokko *et al.* 2003; Neff & Pitcher 2005). Attractive males, and the females that preferentially mate with them, will have offspring that inherit the genes for both attractiveness and the mating preference (Fisher 1930). The co-segregation of trait and preference triggers a runaway process that may lead to exaggerated male traits and elaboration of the female preference (Fisher 1930). This process is known as the "Fisherian runaway selection" or the "sexy son" hypothesis, and it is thought to be driven solely by the benefits of mating with "arbitrarily" attractive males to produce "sexy sons".

The "good genes" model has long been considered as an alternative to Fisher's runaway process, because it postulates that the male trait provides honest information on the genetic quality of the male, and, thus, enables females to choose high-quality mates and thereby enhance the fitness of their offspring (Hamilton & Zuk 1982; Andersson 1994). However, since in this scenario the female preference necessarily also becomes correlated with the male trait when both have a heritable genetic basis, the two models have recently been recognized as mutually compatible processes that represent different points on a continuum (Kirkpatrick & Ryan 1991; Kokko *et al.* 2002; Kokko *et al.* 2003; Mead & Arnold 2004).

Several studies have provided evidence for mate choice based on traits that constitute honest indicators of mate quality, and some of these also demonstrated positive fitness consequences of mate choice for the offspring as postulated by the "good genes" model (Partridge 1980; Houde & Torio 1992; Wilkinson & Reillo 1994; Sheldon *et al.* 1997; Promislow *et al.* 1998; Sauer *et al.* 1998; Barber *et al.* 2001; Doty & Welch 2001). There is also evidence that sexual selection can lead to a genetic correlation between male trait and female preference (Bakker 1993; Houde 1994; Pomiankowski & Sheridan 1994; Wilkinson & Reillo 1994; Brooks & Couldridge 1999), which is a key assumption of Fisher's "runaway process" (Fisher 1930). However, the vast majority of these studies focus on visual or acoustical indicators for male quality. Evidence for adaptive female choice on the basis of olfactory signals is scarce (but see Jones & Hamilton 1998; Jones *et al.* 1998; Jones *et al.* 2000), although male pheromones have been shown to communicate aspects of mate quality to females in several species of insects (Thornhill 1992; Moore 1997; Droney & Hock 1998; Beeler *et al.* 2002; Pai & Yan 2002; Spurgeon 2003; Kortet & Hedrick 2005), in sticklebacks (Reusch *et al.* 2001; Milinski 2003; Milinski *et al.* 2005), salamanders (Marco *et al.* 1998), lizards (Martin & Lopez 2000), rodents (Krackow &

Matuschak 1991; Willis & Poulin 2000) and in humans (Wedekind *et al.* 1995; Wedekind & Furi 1997; Havlicek *et al.* 2005).

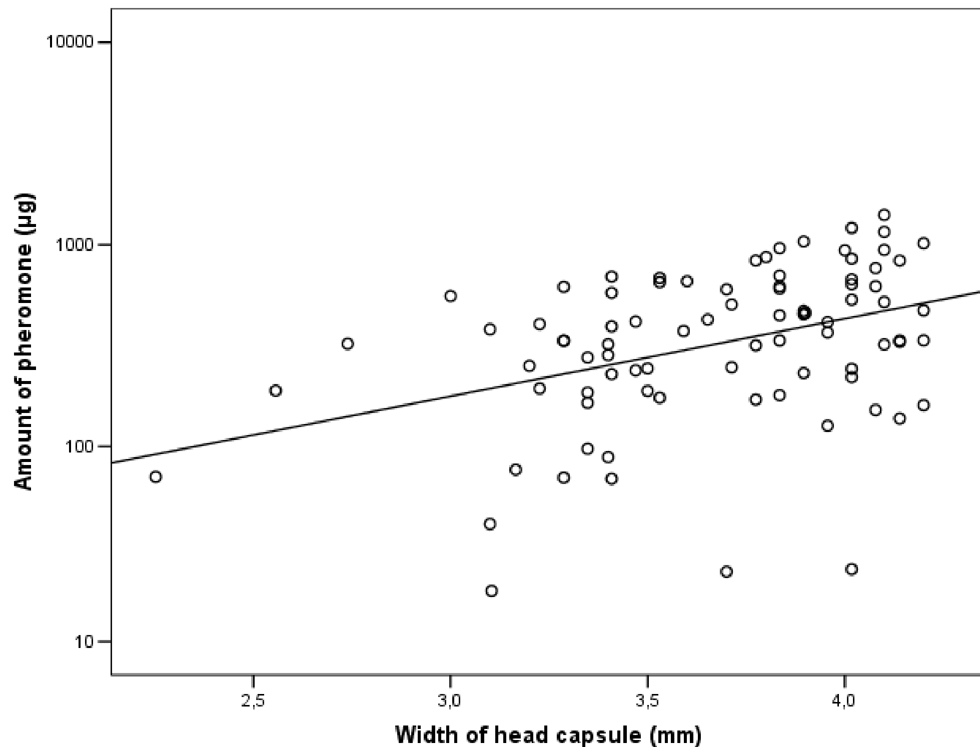

Figure 11.2: Correlation between male size (measured as head capsule width) and total amount of pheromone (on a logarithmic scale) in the cephalic gland (N=86; $F_{1,84}=15.3$; $p<0.001$; $r^2=0.154$) (M. Kaltenpoth, unpubl. data).

One important factor that is often found to affect female mate choice decisions is male body size (Andersson 1994), and it has been shown that chemical signals have the potential to provide information on the emitter's size (Beeler *et al.* 2002). In the European beewolf, differences in male size are reflected in significant changes in the maximum total amount of pheromone extracted from the cephalic glands (Fig. 11.2), while pheromone composition remains relatively constant (M. Kaltenpoth, unpubl. data). Although larger beewolf males are apparently not superior to smaller ones in terms of territory ownership, intensity of scent marking or longevity (Strohm & Lechner 2000), the higher amount of pheromone may provide them with a selective advantage, if they are able to apply more pheromone to their territory than smaller males and, thus, attract more receptive females (Droney & Hock 1998). Females may benefit from choosing larger males, because male size is an indicator of the mother's ability to hunt honeybees (Strohm & Linsenmair 2000), thus, female choice for large males may provide their daughters with "good genes" for hunting.

Age has an effect on both the amount and the composition of the male marking pheromone in European beewolves (chapter 8). These age-related differences may allow females to choose among potential mates on the basis of their age. That male olfactory signals may change with age has also been shown in weevils (Spurgeon 2003) and mice (Wilson & Harrison 1983; Osada *et al.* 2003). Mice are apparently able to distinguish between age groups on the basis of olfactory cues alone (Osada *et al.* 2003). However, little is known about the importance of age-related changes in pheromones for female choice. Generally, many authors agree that male age may be an important factor influencing female choice, but the direction of the female preference is a matter of debate and apparently varies among species (Trivers 1972; Manning 1985; Hansen & Price 1995; Brooks & Kemp 2001).

Recent studies have shown that the pheromone composition of male beewolves varies with the conditions during larval development in the brood cell (Roeser-Mueller 2006). The rearing temperature as well as the number of provisioned honeybees significantly affects the amount and composition of the adult male's pheromone (Roeser-Mueller 2006). In dense field populations, females sometimes compete for the nesting sites with optimal climatic conditions (Simon-Thomas & Simon-Thomas 1972; Evans & O'Neill 1988; Strohm 1995), thus, the temperature in the brood cell might be dependent on the competitive abilities of the mother. Along the same lines, the number of provisioned prey items (and subsequently also adult size) depends on the mother's quality, since hunting and provisioning honeybees is both time- and energy-consuming, and hunting success greatly varies among female beewolves (Strohm 1995; Strohm & Linsenmair 1997, 2000; Strohm & Daniels 2003). Thus, information on developmental conditions of a male may allow females to assess the quality of the potential mates' mother. Choosing a male on the basis of its mother's genetic quality would make sense in hymenoptera, since – due to haplo-diploidy – the male genes are only transmitted to and expressed in the daughters. Under these circumstances, a female preference for males that carry alleles beneficial for daughters is expected even if these alleles have neutral or slightly deleterious effects in sons (Trivers 1985; Seger & Trivers 1986; Albert & Otto 2005).

11.2.2 Evolution of the male beewolf marking pheromone

The evolution of the beewolf marking pheromone has probably been shaped by several complementing factors. Recent studies indicate that pre-existing female preferences that probably evolved to optimize prey hunting have subsequently been exploited by males to maximize their attractiveness for receptive females (Herzner 2004; Herzner *et al.* 2005). Notably, the presence of large amounts of the probably costly major pheromone component, (*Z*)-11-eicosen-1-ol, is likely to play a vital role for female attraction that could be explained by a sensory exploitation model (Herzner 2004; Herzner *et al.* 2005). The other components with

functional groups may also be costly and might therefore constitute honest indicators for male qualities (e.g. age, chapter 8) that could be important for female choice. The large numbers of alkanes and alkenes in the male marking secretion may also provide females with information on male characteristics, e.g. kinship (Herzner *et al.* in press; G. Herzner & M. Kaltenpoth, unpubl. data).

The same alkanes and alkenes are also present on the cuticle, in the hemolymph, and in the postpharyngeal gland secretion of female beewolves (chapter 7), where they apparently play an important role for the preservation of the honeybee prey in the subterranean brood cells (Strohm & Linsenmair 2001; Herzner *et al.* in prep.). Thus, if the metabolic pathways involved in the production of these substances are similar in males and females, the incorporation into the male pheromone blend may provide females with information on the genetic quality of males concerning genes involved in the synthesis of these hydrocarbons. Female choice for males with high metabolic potential may result in increased fitness of the daughters, because of an elevated production of substances for both prey preservation and the cuticular hydrocarbon profile that is important for the prevention of water loss and for pathogen resistance (e.g. Hadley 1980; Buckner 1993; Stanley-Samuelson & Nelson 1993; Nation 2002). Additionally, the grandsons of males with high metabolic potential may inherit the “good genes” from their grandfather and will, thus, be more attractive for females according to Fisher’s “sexy son” hypothesis (Fisher 1930).

Finally, the symbiotic bacteria may also be involved in shaping the male beewolf pheromone blend due to their influence on the developmental conditions in the brood cell (chapters 2 & 3). Gut bacteria have recently been shown to produce components of a cohesion pheromone in locusts (Dillon *et al.* 2000, 2002). Such a connection is unlikely in beewolves, since the symbionts are apparently absent in adult males (M. Kaltenpoth, unpubl. data). However, the presence of the bacteria has an effect on pathogen infestation of male larvae (chapters 2 & 3) and may therefore affect resource allocation in immune system and metabolic abilities, resulting in changes of pheromone production in the adult male beewolf. In *P. triangulum*, preliminary studies found no effect of the bacteria on the amount or composition of the male pheromone, but experimental drawbacks may be responsible for these results (Roeser-Mueller 2006). One piece of evidence supporting the hypothesis of a connection between symbionts and pheromone is provided by recent analyses of the North American beewolf species *Philanthus albopilosus*: This species appears to be the only *Philanthus* species in which males neither have clypeal brushes nor do they defend and mark territories (Evans & O'Neill 1988). Concordantly, chemical analyses revealed no pheromone in a male *P. albopilosus* head (M. Kaltenpoth, unpubl. data). Interestingly, symbiotic bacteria could not be detected in a female specimen of this species either (M. Kaltenpoth, unpubl. data). Thus, although more specimens are necessary

to confirm these results, a connection between bacteria and pheromone production cannot be ruled out at the moment.

The pheromone of the European beewolf constitutes an interesting model for an olfactory communication system with several evolutionary factors influencing the shape of the signal. Further studies may shed light on the relative importance of natural and sexual selection in the evolution of the male pheromone, and they might provide insights into the potential influence of symbiotic bacteria on the pheromone composition.

11.3 FINAL CONCLUSIONS

The studies presented in this PhD thesis demonstrate that beewolves of the genus *Philanthus* engage in a specialized symbiosis with actinomycete bacteria for the protection of their offspring against pathogenic microorganisms. The discovery of this association suggests that defensive mutualisms are more common in insects than was previously recognized and that the symbiosis with *Streptomyces* may have represented a key invention in the evolution of ground-nesting behavior in solitary hymenoptera. Actinomycete bacteria are apparently predisposed to function as protective symbionts, due to their high potential of producing secondary metabolites with antibiotic properties. Ultimately, the study of symbiotic actinomycetes may yield valuable knowledge on novel antimicrobial substances and might, thus, provide human medicine with new weapons in the ongoing arms race with increasingly resistant pathogens.

Our analyses of male beewolf pheromones revealed that the complex blend contains information on several aspects of male characteristics that may be used by females to discriminate and choose adaptively among potential mates. The establishment of polymorphic microsatellites provides the basis for mate choice experiments via genetic paternity analysis in this species. Detailed investigations of female choice in beewolves will yield valuable insights into the mechanisms of sexual selection in organisms with olfactory communication – an important field of research that has as yet received little attention despite its evolutionary as well as ecological significance.

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CHAPTER 12

SUMMARY

12.1 BACTERIAL ENDOSYMBIONTS OF BEEWOLVES

Symbiotic interactions between different species are ubiquitous and essential components of the natural world and have probably affected the evolution of every living organism. Insects are the most diverse metazoan class on earth, and they benefit from the extensive metabolic potential of microorganisms in a wide variety of symbiotic associations. The vast majority of well-studied insect-microbe symbioses to date are nutritional interactions in which the symbionts provide their hosts with essential nutrients. Some cases, however, have been described in which symbiotic bacteria play an important role in intraspecific olfactory communication or serve as a defense against pathogens or parasitoids.

This thesis reports on a unique and highly specialized association between a digger wasp, the European beewolf (*Philanthus triangulum,* Hymenoptera, Crabronidae), and actinomycete bacteria. In contrast to all other known symbioses, the beewolf bacteria are cultivated in the reservoirs of unique antennal glands in female beewolves. The female secretes the bacteria into its subterranean brood cells prior to oviposition. Several days later, when the beewolf larva has finished feeding on the paralyzed honeybees that had been provisioned by the mother, it takes up the bacteria and applies them to the cocoon silk during the spinning process. On the cocoon, the symbionts play an important role in reducing the incidence of fungal infestation and thereby significantly enhance the survival probability of the larva in the cocoon during the long and potentially very dangerous inactive phase of hibernation in the underground brood cell. Observations of beewolf larvae as well as experiments in which female beewolf larvae were reared in the absence of the bacteria suggest that the symbionts are transmitted vertically from mothers to daughters. Presumably, the bacteria are taken up from the cocoon during eclosion and incorporated into the antennal gland reservoirs. Phylogenetic analyses of hosts and symbionts as well as artificial transfer experiments are necessary to investigate whether horizontal transmission of bacteria between beewolf species may occasionally occur.

Genetic analyses revealed that the symbionts constitute an undescribed species of the genus *Streptomyces* within the eubacterial family Actinomycetaceae. 16S rDNA primers and an oligonucletide probe were designed for the specific detection of the *Philanthus* endosymbionts by PCR and fluorescence in-situ hybridization (FISH). By PCR-based screening, closely related endosymbionts were found in 28 *Philanthus* species and subspecies. By contrast, no symbionts

could be detected in closely related genera of the subfamily Philanthinae (*Aphilanthops, Clypeadon, Cerceris*), indicating that the symbiosis might be restricted to the genus *Philanthus*. Based on almost complete 16S rRNA gene sequence data, the symbionts of all analyzed *Philanthus* species formed a monophyletic clade within the genus *Streptomyces*, indicating that the symbiosis is highly specific and most likely the product of a long history of coevolution and cospeciation. Sequence divergences among symbionts suggest an origin of the *Philanthus-Streptomyces* association about 26-67 million years ago, which may have coincided with the origin of the genus *Philanthus*. On the basis of 16S rDNA sequences and ultrastructural data, the new taxon '*Candidatus* Streptomyces philanthi' is proposed for the antennal symbionts of *Philanthus* species, with symbionts from different host species being treated as ecotypes and named according to their hosts (e.g. '*Candidatus* Streptomyces philanthi triangulum').

It is not yet clear how the bacteria benefit from the association with *Philanthus* species. Certainly, they obtain an unoccupied and presumably competition-free niche in the beewolf antennae and a reliable transmission route to the next generation. Additionally, several pieces of evidence suggest that they may also receive nutrients from their host: (1) Females secrete massive amounts of bacteria into each brood cell and sometimes construct several brood cells per day; thus, the bacteria have to grow quickly inside the antennal gland reservoirs to replenish the stock for further brood cells. (2) The reservoirs are surrounded by class 3 gland cells that may supply the bacteria with nutrients (e.g. amino acids). (3) One of the walls bordering the antennal gland is of a net-like structure, thus, possibly allowing hemolymph to enter the reservoir lumen and provide nutrients to the symbionts. This possibility is further substantiated by chemical analyses of the hydrocarbon profile of the antennal gland secretion and female hemolymph, which revealed very similar compositions.

The beewolf-*Streptomyces* symbiosis constitutes the first known case of bacteria being cultivated in insect antennae and one of the few examples involving the pharmaceutically important group of actinomycete bacteria as insect endosymbionts. Further studies on ecological and evolutionary aspects of the symbiosis will provide valuable insights into the importance of actinomycete bacteria for pathogen defense in insects and may also identify novel secondary metabolites with antibiotic properties that might prove useful for human medicine.

12.2 Chemical communication and mate choice in the European beewolf

Chemical signals constitute both the most ancient and the most common form of communication among organisms. In insects, pheromones play an essential role in mediating intraspecific communication. Many recent studies have investigated the importance of insect olfactory signals in the context of courtship and mating. However, since most of these studies have focused on female pheromones, male sex pheromones have as yet received little attention despite their potential ecological as well as evolutionary importance for mate attraction and mate choice.

Male European beewolves establish and defend small territories that they mark with a secretion from cephalic glands. Presumably, the secretion acts as a sex pheromone and attracts receptive females to the territory. Since male territories are clumped around female nesting sites, females have the opportunity to choose among potential mates. The marking pheromone of male beewolves varies with kinship, and it is demonstrated here that geographic origin, age and size also affect the amount and/or composition of the pheromone. Thus, the marking secretion contains information on a variety of male characters that may be important in the context of female choice. Both genetic distance ("optimal outbreeding") and overall genetic quality ("good genes") of a male might influence female mating decisions in the European beewolf. Polymorphic microsatellite markers are presented for the European beewolf that facilitate female choice experiments by genetic paternity analysis.

CHAPTER 13

ZUSAMMENFASSUNG

13.1 BAKTERIELLE ENDOSYMBIONTEN DER BIENENWÖLFE

Symbiontische Interaktionen zwischen verschiedenen Arten stellen allgegenwärtige und essentielle Bestandteile natürlicher Systeme dar und haben wahrscheinlich die Evolution jedes rezenten Lebewesens beeinflusst. Insekten als die diverseste Metazoen-Klasse der Erde profitieren von dem außerordentlichen metabolischen Potenzial vieler Mikroorganismen in einer großen Anzahl mutualistischer Assoziationen. Die große Mehrheit der bisher untersuchten Symbiosen zwischen Insekten und Mikroorganismen stellen Interaktionen dar, in denen die Wirte durch die Symbionten mit essentiellen Nährstoffen versorgt werden. Es sind jedoch auch einige Fälle bekannt, in denen symbiontische Bakterien eine wichtige Rolle für die intraspezifische olfaktorische Kommunikation spielen oder zur Verteidigung gegen Pathogene oder Parasitoide dienen.

Die vorliegende Arbeit untersucht eine hoch spezialisierte Assoziation zwischen einer Grabwespen-Art, dem Europäischen Bienenwolf (*Philanthus triangulum*, Hymenoptera, Crabronidae), und Bakterien aus der Familie der Actinomyceten. Die bakteriellen Symbionten sind an einem einzigartigen Ort zu finden: Sie werden in den Reservoiren spezialisierter Antennendrüsen weiblicher Bienenwölfe kultiviert. Das Weibchen sezerniert vor der Eiablage große Mengen dieser Bakterien in die unterirdischen Brutkammern. Wenn die Bienewolf-Larve einige Tage später ihre Nahrungsaufnahme an den von der Mutter als Nahrungsvorrat bereitgestellten Honigbienen beendet hat, nimmt sie die Bakterien auf und spinnt sie in ihren Kokon mit ein. Dort erfüllen die Symbionten eine wichtige Funktion, indem sie den Schimmelbefall herabsetzen und dadurch die Überlebenschancen der Larve im Kokon während der langen und gefährlichen Winterruhe signifikant erhöhen. Experimente, in denen Bienenwolf-Weibchen ohne die Bakterien aufgezogen wurden, und Beobachtungen an Bienenwolf-Larven deuten darauf hin, dass die Symbionten vertikal von der Mutter an die Töchter weitergegeben werden. Vermutlich werden die Bakterien während des Schlupfes oder kurz davor vom Kokon in die Antennendrüsen-Reservoire aufgenommen. Phylogenetische Untersuchungen von Wirten und Symbionten sowie Transfer-Experimente mit den Bakterien wären notwendig, um herauszufinden, ob ein horizontaler Austausch der Symbionten zwischen verschiedenen Bienenwolf-Arten möglich ist.

Genetische Analysen zeigen, dass die Symbionten einer unbeschriebenen Art der Gattung *Streptomyces* innerhalb der Actinomyceten angehören. 16s rDNA Primer und eine fluoreszenzmarkierte Oligonukleotid-Sonde wurden entwickelt, um die Bienenwolf-Symbionten mittels PCR und Fluoreszenz-in-situ-Hybridisierung (FISH) spezifisch nachweisen zu können. Mit Hilfe von PCR und Sequenzierungen der 16s rDNA konnten nah verwandte Endosymbionten in den Antennen von 28 Arten und Unterarten der Gattung *Philanthus* festgestellt werden, nicht aber in anderen Gattungen der Unterfamilie Philanthinae (*Aphilanthops, Clypeadon, Cerceris*), so dass die Symbiose auf die Gattung *Philanthus* beschränkt zu sein scheint. Phylogenetische Untersuchungen auf der Grundlage nahezu kompletter 16s rDNA-Sequenzen belegen, dass die Symbionten aller analysierten Bienenwolf-Arten eine monophyletische Gruppe innerhalb der Gattung *Streptomyces* bilden, was darauf hindeutet, dass die Symbiose hoch spezifisch ist und wahrscheinlich das Ergebnis einer langen Koevolution und Kospeziation darstellt. Anhand von Sequenzunterschieden zwischen den Symbionten lässt sich das Alter der Assoziation zwischen *Philanthus* und *Streptomyces* auf etwa 26-67 Millionen Jahre schätzen, was der Entstehung der Gattung *Philanthus* entsprechen könnte. Auf der Basis von 16s rDNA Sequenzen und Ultrastruktur-Daten wurden die Antennensymbionten der Bienenwölfe als neues Taxon ‚*Candidatus* Streptomyces philanthi' beschrieben, wobei die Symbionten verschiedener Wirtsarten als Ökotypen behandelt und nach der Wirtsart benannt wurden (z.B. ‚*Candidatus* Streptomyces philanthi triangulum').

Wie die Bakterien von der Assoziation mit Bienenwölfen profitieren, ist noch unklar. Auf jeden Fall wird ihnen vom Wirt eine unbesetzte und wahrscheinlich konkurrenzfreie ökologische Nische in den Antennen sowie eine zuverlässige Weitergabe an die nächste Generation garantiert. Außerdem sprechen einige Hinweise für eine Versorgung der Bakterien mit Nährstoffen durch den Bienenwolf: (1) Weibchen legen manchmal mehrere Brutkammern pro Tag an und sezernieren jedes Mal große Mengen an Bakterien; die Bakterien müssen sich also in den Drüsen-Reservoiren schnell vermehren, um den Vorrat an Symbionten wieder aufzufüllen. (2) Die Reservoire sind von Typ 3-Drüsenzellen umgeben, die die Bakterien mit Nährstoffen versorgen könnten. (3) Eine der Reservoir-Wände weist eine netzartige Struktur auf, die möglicherweise den Eintritt von Hämolymphe und damit von Nährstoffen in das Reservoir zulässt. Dies wird durch chemische Analysen der Kohlenwasserstoffe in der Hämolymphe und in dem Antennendrüsen-Sekret untermauert, die sehr ähnliche Zusammensetzungen aufweisen.

Die Assoziation zwischen Bienenwölfen und Streptomyceten stellt den ersten bekannten Fall einer Symbiose dar, bei der Bakterien in den Antennen von Insekten kultiviert werden, und sie repräsentiert eines von wenigen Beispielen für Actinomyceten als Symbionten von Insekten. Weitere Untersuchungen evolutionärer und ökologischer Aspekte dieser Symbiose werden wertvolle Erkenntnisse über die Bedeutung von Actinomyceten für die Pathogen-Abwehr bei

Insekten liefern und könnten sogar zur Entdeckung neuer Sekundärmetabolite mit antibiotischen Eigenschaften für die Verwendung in der Humanmedizin führen.

13.2 CHEMISCHE KOMMUNIKATION UND PARTNERWAHL BEIM EUROPÄISCHEN BIENENWOLF

Chemische Signale stellen sowohl die älteste als auch die am weitesten verbreitete Form von Kommunikation zwischen Organismen dar. Bei Insekten spielen Pheromone eine essentielle Rolle für die intraspezifische Kommunikation, und eine Vielzahl aktueller Untersuchungen belegt die Bedeutung olfaktorischer Signale für die Balz und Paarung. Die meisten dieser Studien konzentrieren sich jedoch auf Weibchen-Pheromone, während von Männchen produzierte Pheromone trotz ihrer ökologischen und evolutionären Bedeutung für die Partneranlockung und Partnerwahl bisher wenig Beachtung gefunden haben.

Männchen des Europäischen Bienenwolfes etablieren und verteidigen Territorien, die sie mit einem Kopfdrüsen-Sekret markieren. Dieses Sekret wirkt höchstwahrscheinlich als ein Sex-Pheromon und lockt paarungsbereite Weibchen an. Da Männchen-Territorien meist aggregiert in der Nähe von Weibchennestern auftreten, haben die Weibchen die Möglichkeit, zwischen verschiedenen potenziellen Paarungspartnern zu wählen. Die chemischen Analysen der vorliegenden Arbeit zeigen, dass die Zusammensetzung und Menge des männlichen Markierpheromons vom Verwandtschaftsgrad, der Herkunft, dem Alter und der Größe der Männchen abhängen. Das Pheromon beinhaltet demnach Informationen über eine Vielzahl von Eigenschaften der Männchen, die für die Weibchenwahl von Bedeutung sein könnten. Sowohl die genetische Distanz („optimal outbreeding“) als auch die allgemeine genetische Qualität („good genes“) eines Männchens könnte die Partnerwahl der Bienenwolf-Weibchen beeinflussen. In dieser Arbeit für den Europäischen Bienenwolf entwickelte polymorphe Mikrosatelliten legen den Grundstein für Vaterschaftsanalysen und ermöglichen so die Durchführung und Auswertung von Experimenten zur Weibchenwahl bei dieser Art.

DANKSAGUNG

Zahlreiche Menschen haben mich während der Doktorarbeit in vielfältiger Weise unterstützt und zum Gelingen dieser Arbeit beigetragen.

Zunächst einmal möchte ich mich ganz herzlich bei Prof. Erhard Strohm bedanken, der mir während des gesamten Projektes immer mit Rat und Tat zur Seite gestanden hat. Er hat mich in den spannenden Bereich der Bienenwolf-Forschung eingeführt[1], mit der hohen Kunst des Hypothesen-Spinnens vertraut gemacht[2] und mich vor allem immer darin unterstützt, eigene neue Ideen zu entwickeln und zu verfolgen. Dafür vielen Dank!

Prof. Roy Gross bin ich dankbar für wertvolle Ratschläge im Endosymbionten-Projekt und für die Erstellung des Zweitgutachtens. Prof. Karl-Eduard Linsenmair danke ich für die Gelegenheit, meine Dissertation am Lehrstuhl für Zoologie III anfertigen zu können, und für die Erstellung mehrerer Gutachten bei der Bewerbung um Promotionsstipendien. Ganz herzlich möchte ich mich bei Prof. Jürgen Gadau, bei Dr. Heike Feldhaar und bei Karin Möller für die bereitwillige und freundliche Aufnahme in ihr Populationsgenetik-Labor und für die Hilfe bei (phylo-) genetischen Problemen bedanken.

Mein ganz besonderer Dank gilt der Arbeitsgruppe Strohm (Erhard, Guzzi, Thomas, Norbert, Johannes, Kerstin, Wolfgang, Carolin, Joachim, und vielen mehr) für die supernette und witzige Arbeitsatmosphäre, für die „great moments in science“ und für die seelische und moralische Unterstützung bei den „worst moments“[1-12]. Vielen lieben Dank vor allem an meine langjährigen Büro-Kameraden Norbert, Kerstin und Johannes! Mit euren Wortwitzen, Spontan-Exkursionen, Tea Times, Eisesssessions und den Oden an das Leben, das Universum und den ganzen Rest habt ihr die Doktorarbeit zu einer sehr, sehr schönen Zeit in meinem Leben gemacht[7-9,11,12]!

Der ZooIII-Truppe möchte ich ganz herzlich für die alltäglichen Mensa-Gänge, die Grillfeiern und die zahlreichen netten Gang-Gespräche danken (vor allem Johannes, Tilman, Mo und Mini)[13]. Besonders danke ich Andrea Hilpert für ihre liebe und hilfsbereite Art und für die Unterstützung bei zahlreichen Experimenten[14]. Vielen Dank auch an Norbert „Q“ Schneider und die Werkstatt-Truppe, die es immer wieder geschafft haben, möglichst umsonst und bis gestern völlig unmachbare Gerätschaften zu konstruieren[15]! Danken möchte ich auch Thomas Igerst für seine stete Hilfe bei computertechnischen Spontan-Katastrophen. Der ZooIII-Fußball-Mannschaft und der ZooII-/ZooIII-Badminton-Truppe[16] verdanke ich den sportlichen Ausgleich zur Arbeit.

Der Arbeitsgruppe Gadau/Feldhaar danke ich für die freundliche Aufnahme und die nette Arbeitsatmosphäre. Dankbar bin ich vor allem Karin für die Hilfe bei genetischen Methoden und für die ständige Bereitstellung der notwendigen Materialien und Chemikalien! Danke auch an Jürgen und Barbara für die Indonesischen Wüstenameisen[17]!

Bei Prof. Jon Seger (University of Utah, Salt Lake City, USA) und bei Drs. Fred und Sarah Gess (Albany Museum, Grahamstown, Südafrika) möchte ich mich sehr für ihre spontane und herzliche Gastfreundschaft und für die Hilfe beim Bienenwölfe sammeln bedanken.

PD Dr. Ute Hentschel und ihrer Arbeitsgruppe danke ich für zahlreiche symbiontische Ratschläge und die Hilfe bei der Durchführung von Antibiotika-Biotests. Bei den Mikrobiologen (insbesondere bei Michael Pflock) möchte ich mich für die Bereitstellung von Gerätschaften und Chemikalien und für zahlreiche Tipps bedanken.

Ganz besonders möchte ich allen denen danken, die mir immer wieder gezeigt haben, dass es einfach viel Wichtigeres gibt als die Arbeit. Allen voran danke ich Jutta für ihre Geduld, ihre Unterstützung und ihre Liebe. Meinen Eltern und meiner Schwester möchte ich ganz herzlich dafür danken, dass sie immer für mich da sind. Dankbar bin ich auch der Würzburger Pfadfinder-Leiterrunde, den Wös, Jupfis und Pfadis für die vielen schönen gemeinsamen Erlebnisse, das Quatsch machen und den Spaß an der Pfadfinderei. Allen meinen Freunden möchte ich für ihre Freundschaft, ihr Vertrauen, das eine oder andere Feierabend-Bierchen, Hütten-Wochenenden im Schnee, Südafrika-Pfadfinder-Fahrten, Hardcore-Uno-Abende und vieles mehr danken (insbesondere Jutta, Charly, Flo, Andi, Mel, Beni, Käthi, Doro & Michael, Mini, Johanna und Matthias).

Für finanzielle Unterstützung danke ich der Studienstiftung des Deutschen Volkes (Promotions- und Reisestipendium), dem UNIBUND Würzburg (Sachmittel), der Arthur-von-Gwinner Stiftung (Reisestipendium) und der DFG (Sach- und Reisemittel). Prof. Wolfgang Kinzel möchte ich ganz herzlich für die Betreuung in der Studienstiftung, für die netten Gespräche und für mehrere Gutachten bei Stipendienanträgen danken.

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ERKLÄRUNG

gemäß § 4 Abs. 3 Ziff. 3, 5 und 8
der Promotionsordnung der Fakultät für Biologie der
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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 in dieser noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt.

Ich erkläre weiterhin, dass ich außer meinem Diplom in Biologie an der Universität Würzburg keine akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, den 28. Juni 2006