

# Functional morphology in aculeate Hymenoptera: Unique glands and buffered brains



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*Evolution nennen wir das ewige Stolpern der Natur über Irrtümer hinweg zu letztlich phantastischen Ergebnissen.*

Prof. Querulix, (\*1946),  
deutscher Aphoristiker und Satiriker

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## LIST OF PUBLICATIONS

This thesis is based on the following manuscripts:

- Goettler, W., Kaltenpoth, M., Herzner, G., Strohm, E.** (2007). Morphology and ultrastructure of a bacteria cultivation organ: The antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36: 1-9. **(chapter 2)**
- Kaltenpoth, M., Goettler, W., Herzner, G., Strohm, E.** (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479. **(chapter 3)**
- 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: 1403-1411. **(chapter 4)**
- Kaltenpoth, M., Goettler, W., Koehler, S., Strohm, E.** (in preparation). Population dynamics of a protective insect symbiont reveal severe bottlenecks during vertical transmission. **(chapter 5)**
- Goettler, W., Kaltenpoth, M., McDonald, S., Strohm, E.** (in preparation). Antennal glands in female digger wasps of the genus *Philanthus* (Hymenoptera, Crabronidae). **(chapter 6)**
- Strohm, E., Herzner, G., Goettler, W.** (2007). A "social" gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36 (2): 113-122. **(chapter 7)**
- Herzner, G., Goettler, W., Kroiss, J., Porea, A., Webb, A.G., Jakob, P.M., Rössler, W., Strohm, E.** (2007). Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 36 (2): 123-133. **(chapter 8)**
- Goettler, W., Strohm, E.** (2008). Mandibular glands of male European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae): *Arthropod Structure & Development* 37 (5): 363-371. **(chapter 9)**
- Goettler, W., Radmacher, S., Strohm, E.** (submitted). Does rearing temperature affect synaptic organization in the brain of the solitary Red Mason bee, *Osmia bicornis* (Hymenoptera, Megachilidae)? *Brain, Behavior and Evolution*. **(chapter 10)**



## CHAPTER 1

### GENERAL INTRODUCTION

When we look at morphological and physiological traits of extant organisms, we see the result of evolutionary processes caused by selective pressures on their ancestors. Charles Darwin recognized that natural and sexual selection are the major forces behind the evolution of new species with their vast variety of different traits (Darwin, 1859, 1871). Whereas Darwin drew his conclusions from morphological characteristics, today the most frequently used tools to reveal species' phylogenies are genetic and molecular methods. However, the anatomy of an organism and how it interacts with the environment can not be deduced from genome sequences or amino acids. Therefore investigations of morphology, physiology and behaviour are still inevitable to unravel the branches in the tree of life. Moreover, comparative analyses or the investigation of structural changes in response to environmental factors provides important insights into the function of organs and their interaction with the environment.

This thesis deals on the one hand with the unique morphology and functions of specialized exocrine glands in solitary digger wasps called beewolves (*Philanthus spp.*, Hymenoptera, Crabronidae). On the other hand it presents results on how temperature affects the postembryonic brain development of the Red Mason bee (*Osmia bicornis*, Hymenoptera, Megachilidae). Both, glands and brains evolved with regard to morphology, physiology and function as a result of the environmental conditions their owners were confronted with. This first chapter provides a brief introduction to the genus *Philanthus* with emphasis on the European beewolf, *P. triangulum*. It furthermore presents a short overview about the exocrine glands under study and insect-bacteria symbiosis. Finally we introduce the Red Mason bee, *O. bicornis* and the problem of attaining developmental stability at different temperatures.

### 1.1 Exocrine glands and their products

A good part of this thesis deals with insects' exocrine glands and their secretions, thus a short overview of relevant terms appears to be useful. Exocrine glands by definition secrete onto the body surface or into a duct. They are of ectodermal origin and primarily in contact with the cuticle. A universally accepted classification of insects' exocrine glands into three types is based on the type of the cells' connection to the adjacent cuticle (Noirot and Quennedey, 1974, 1991; Quennedey, 1998). Class 1 gland cells are in direct contact with the cuticle as it is found for common epidermal cells. The secretion of these cells therefore has to pass through the cuticle through small canals. In class 1 gland cells we often find an enlarged surface of the cell membrane, e.g. apical microvilli and basal invaginations. Class 2 gland cells are surrounded by epidermal cells which are covered by cuticle. The secretion is first transferred to the epidermal cells, which are themselves class 1 gland cells. Finally the secretion is delivered through the cuticle. The most derived gland cells are those of class 3 where a gland cell is penetrated by a canal cell with a porous cuticle that is surrounded by microvilli. The combination of canal and microvilli forms the so-called end apparatus and can be seen in the gland cells as an elongated slightly fuzzy area. The canal is in contact with the cuticle of the respective secretion organ or body surface. Frequently the class 3 gland cells are clustered in so-called 'acini' with the canals of the gland cells forming a bundle between an acinus and the cuticle [Fig. 1.1]. In this thesis we mostly deal with class 3 cells and also class 1 cells bordering gland reservoirs.

According to their numerous exocrine glands and their highly diverse gland products insects are referred to as "chemists par excellence" (Blum, 1985, p.536) or "walking glandular batteries" (Billen, 1991, p.67). Some examples for the functions of exocrine gland secretions are intra- and interspecific

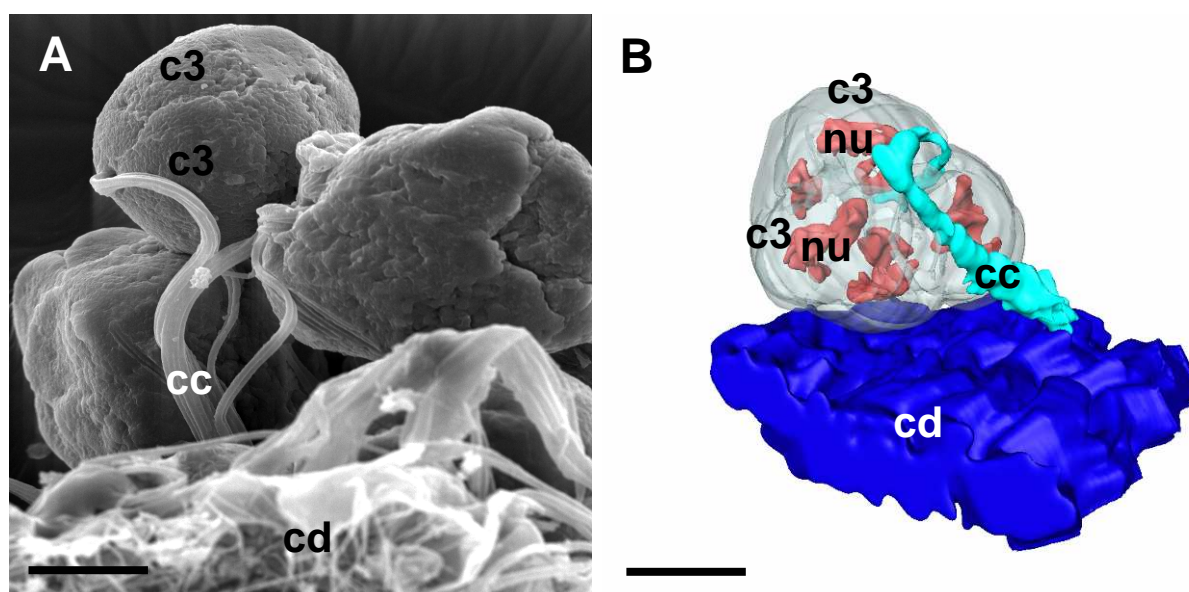


Fig. 1.1. Class 3 gland cell units (acini) of mandibular gland of male *P. triangulum*. (A) SEM micrograph. (B) 3D reconstruction based on semithin sections (by Nathalie Czech). c3 – class 3 gland cells; cc – conducting canals; cd – collecting duct; nu – nucleus. scale bars = 20  $\mu$ m

communication (e.g. Shorey, 1973; Billen and Morgan, 1998; Ayasse et al., 2001), attack and defense (e.g. Beard, 1963; Prestwich, 1984; Gross, 1993; Gunawardena and Bandumathie, 1993), digestion (e.g. Terra, 1990; Swart and Felgenhauer, 2003) as well as host detection (Isidoro et al., 1996; Bin et al., 1999), wax secretion (e.g. Gullan and Kosztarab, 1997; Muller and Hepburn, 1992), silk production (Sehnal and Akai, 1990; Fisher and Robertson, 1999) or the feeding of bacterial symbionts (Currie et al., 2006). In particular social hymenoptera (ants, bees, wasps) show a vast variety of exocrine glands and individuals can bear 20 or more different types located in all body parts from antennae to tarsal segments (e.g. Jackson and Morgan, 1993; Jeanne, 1996; Billen and Morgan, 1998).

Exocrine glands are frequently denominated according to the location of the gland itself (e.g. antennal, postpharyngeal gland (PPG)) or its openings (mandibular gland (MG)). The next paragraphs provide a short overview of the occurrence of the hymenopteran glands that are dealt with in this thesis and their already known functions. These are in particular two male cephalic glands (mandibular gland and postpharyngeal gland), female postpharyngeal glands and antennal glands. The results presented in this thesis refer to the respective glands and functions in our model species, the European beewolf and its congeners.

### *1.1.1 Postpharyngeal glands and nestmate recognition*

It is crucial for social insects to distinguish between nest-mates and foreigners which may threaten brood and food storages inside the nests. As within the dark nests visual cues are of limited use – most termites are blind anyway – social insects rely on olfaction for nestmate-recognition (e.g. Gamboa et al., 1986; Breed, 1998; Vander Meer and Morel, 1998). The nest-specific odour which identifies all members of a colony is composed of complex blends of more or less volatile hydrocarbons (HC) on the insects' cuticle (e.g. Hölldobler and Carlin, 1987; Smith and Breed, 1995; Singer, 1998; Dani et al., 2001).

In ants (Formicidae) the formation and dispersion of the nest-specific 'Gestalt' odour is at least partly accomplished by the postpharyngeal gland (PPG) (e.g. Crozier and Dix, 1979; Hefetz et al., 1992; Oldham et al., 1999; Boulay et al., 2004). Each ant takes up HCs from the cuticle of nestmates during allogrooming, mixes them in the PPG with HCs sequestered from its own hemolymph and again delivers the PPG content to other colony members (Hefetz et al., 1992, Soroker et al., 1994, 1995a,b, 1998; Vienne et al., 1995; Lenoir et al., 2001; Soroker and Hefetz, 2000). Hitherto the PPG had been described only in ants and authors referred to it as an idiosyncratic organ of Formicidae that evolved in response to the requirements of eusociality (Crozier and Dix, 1979; Billen, 1990; Hölldobler and Wilson, 1990; Lenoir et al., 1999; Eelen et al., 2006).



Recently, female European beewolves (Hymenoptera, Crabronidae) have been reported to use the secretions of a large cephalic gland to coat their paralyzed honey bee prey (Strohm and Linsenmair, 1995; Herzner and Strohm, 2007; Herzner et al., 2007). This female beewolf gland was referred to as PPG the first time in 2001 (Strohm and Linsenmair, 2001). However only a detailed morphological investigation of this putative PPG could confirm this assumption and provide a basis for phylogenetic considerations.

### *1.1.2 Male cephalic glands and pheromones*

The exchange of information between conspecifics via volatile chemicals, so-called pheromones, is found in all taxa of insects (e.g. Blum and Brand, 1972; Tillmann et al., 1999). Basically pheromones might be involved in aggregation, dispersal, alarm and sexual behaviour (Shorey, 1973; Ayasse, 2001). Many pheromones are efficient over long distances, inconspicuous to most predators and could contain valuable information about the sender (e.g. Herzner et al., 2006; Kaltenpoth and Strohm, 2006; Kaltenpoth et al., 2007). The origin of pheromones is even more diverse as their function and the respective exocrine glands could be located throughout the insects' body (e.g. Landolt and Akre, 1979; Attygalle and Morgan, 1984; Downing, 1991; Jackson and Morgan, 1993; Jeanne, 1996; Billen and Morgan, 1998).

In aculeate Hymenoptera males frequently use the secretions of mandibular glands to scent mark their territories (Apidae: Cane et al., 1983; Cane and Michener, 1983; Hefetz, 1983; Vinson et al., 1982; Gracioli et al., 2004; Vespidae: Wenzel, 1987; Crabronidae: Evans and O'Neill, 1988). Male digger wasps of the subfamily Philanthinae (Hymenoptera, Crabronidae) use pheromones produced in their mandibular glands (MG) to attract females and to mark their territories (e.g. Evans and O'Neill, 1988; McDaniel et al., 1987, 1992; Clarke et al., 2001; Schmitt et al., 2003; Kroiss et al., 2006; Kaltenpoth et al. 2007). A common morphological feature of males in the subfamily Philanthinae is a clypeal brush which is used to dispense the mandibular gland secretion onto surfaces inside their territories (Evans and O'Neill, 1988; Alexander, 1992). An exception is the non-territorial species *P. albopilosus* where males have been reported to possess only reduced mandibular glands and lack a clypeal brush (Evans and O'Neill, 1988; unpubl. data). The putative contents of mandibular glands in the subfamily Philanthinae were analyzed in a number of species (*P. triangulum*: Kaltenpoth and Strohm, 2006; Kroiss et al., 2006; Schmitt et al., 2003; Schmidt et al., 1990; Borg-Karlson and Tengö, 1980; *P. basilaris/ bicinctus*: McDaniel et al., 1987; Schmidt et al., 1985; *P. crabroniformis/ barbatus/ pulcher*: McDaniel et al., 1992; *Eucerceris conata/ montana/ rubripes/ tricolor*: Clarke et al., 2001). However, knowledge about the morphology of mandibular glands in Philanthinae is only fragmentary (Ågren, 1977; Gwynne, 1978; Evans and O'Neill, 1988).

In addition Kroiss et al. (2006) recently published the chemical analysis of the content of a hitherto unknown cephalic gland in male beewolves, *P. triangulum*. Based on their results they proposed that (1) the contents of the new gland are used for scent marking and that (2) the gland is a PPG (Kroiss et al., 2006). Detailed morphological investigations should reveal whether assumption (2) is true and how the MG and the putative PPG are involved in the process of pheromone production and storage.

### 1.1.3 Antennal glands in Hymenoptera

There is only a small number of descriptions of antennal glands in the order Hymenoptera, most of them were found in males where they play a role in male courtship and mating behaviour (Bin and Vinson, 1986; Isidoro and Bin, 1995; Isidoro et al., 1996, 1999, 2000; Felicioli et al., 1998; Bin et al., 1999; Guerrieri et al., 2001; Battaglia et al., 2002; Romani et al., 2003, 2005). In species with male antennal glands the mating behaviour frequently involves rapid antennal movement (antennation) and physical contact between antennae of both sexes whereby the antennal gland secretion is most likely spread onto the female antennal receptors (Felicioli et al., 1998; Isidoro et al., 1999; Romani et al., 2003, 2005).

In Hymenoptera female antennal glands have been found in the parasitoid *Trissolcus basalis* (Scelionidae) where the secretions are likely involved in host recognition by dissolving kairomones from the host eggs (Isidoro et al., 1996; Bin et al., 1999). In aculeate Hymenoptera female antennal glands have been described in queens and workers of four ant species (Formicidae) where the function is unclear (Isidoro et al., 2000; Romani et al., 2006). All the antennal glands of Hymenoptera described so far consist of only small aggregations of either class 1 or class 3 gland cells (according to Noirot and Quennedey, 1974, 1991) secreting directly onto the outer antennomere cuticle without conspicuous reservoirs or other modifications of the antennal morphology.

Already in the 1960s Rathmayer discovered that female beewolves exhibit antennal glands with unusual morphology, but his results had been unregarded until 1995 the secretion of these antennal glands was proofed to provide directional information for the cocoon alignment of beewolf larvae (Strohm, 1995; Strohm and Linsenmair, 1995). As a part of this thesis it turned out in 2005 that the beewolf glands in fact contain filamentous structures which resemble bacteria.

## 1.2 Insect-bacteria symbiosis

A vast variety of mutualistic associations evolved between insects and bacteria (e.g. Buchner, 1965; Werren and O'Neill, 1997; Bourtzis and Miller, 2003). Symbiosis in general could be classified following several criteria: According to the interdependence between the mutualistic partners a symbiosis could be either facultative (no – weak interdependence) or obligate (strong interdependence

– vitally important) (Ishikawa, 2003). Furthermore bacteria could live as ectosymbionts outside the insect host (Currie et al., 1999, 2006) or as endosymbionts inside the host's body (Buchner, 1965). In the latter case, insects frequently evolved specialized organs which harbour their prokaryotic partners, e.g. specializations of the digestive tract (Billen and Buschinger, 2000; Moran and Baumann, 2000). Some symbiotic bacteria even live intracellular in specialized cells (bacteriocytes) and organs (bacteriomes) (Houk and Griffiths, 1980; Baumann et al., 1995; Braendle et al., 2003). It has been estimated that more than 10% of all insects rely on intracellular bacteria for their development and survival (Baumann et al., 2006).

In this thesis the term “symbiosis” mostly means mutualism, i.e. an interspecific association with benefits for both partners (Paracer and Ahmadjian, 2000). In general the insects provide their bacterial partners a free ecological niche with constant conditions, furthermore nutrients and an assured transmission to the next generation (Margulis and Fester, 1991; Currie, 2001). In return, bacteria support their hosts e.g. in digestion (e.g. Dettner, 1999; Dillon and Dillon, 2004; Zientz et al., 2004), defence against pathogens (Currie et al., 1999; Hu and Webster, 2000; Takatsuka and Kunimi, 2000; Gebhardt et al., 2002; Piel, 2004; Dillon et al., 2005) or supply them with components used for constitution of pheromones (Dillon et al., 2000, 2002; Matsuura, 2003). Frequently blood-sucking, wood-feeding and phloem-sucking insects are associated with bacterial symbionts that supply their hosts with essential nutrients that are originally lacking in their unbalanced diets (e.g. Buchner, 1965; Harington, 1960; Douglas, 1998, 2006; Aksoy, 2003; Zientz et al., 2004; Moran et al., 2005). One example is the aphid-bacteria symbiosis where endosymbionts of the genus *Buchnera* live in specialized body cavities (bacteriomes) and provide their hosts with essential amino acids (e.g. Douglas, 1998, 2006; Braendle et al., 2003).

Only recently symbioses have been described in which bacteria defend their insect hosts and nutrition against pathogens or parasitoids (Currie et al., 1999, 2003; Piel, 2002). Bacterial symbionts in the guts of insects could protect their hosts from pathogenic microorganisms by producing antibiotics (Dillon and Charnley, 1995; Dillon et al., 2000, 2002, 2005; Takatsuka and Kunimi, 2000) or outcompeting pathogens due to a more efficient processing of nutrients (Godfray et al., 1999; Dillon and Dillon, 2004). Besides the *Buchnera* endosymbionts aphids also harbour vertically transmitted bacteria, so-called secondary symbionts, which reduce the aphids' vulnerability against hymenopteran parasitoids, probably by help of a bacteriophage as a third partner (Oliver et al., 2003; Moran et al., 2005).

Another interesting example for a protective symbiosis is found in fungus-growing ants (Hymenoptera, Formicidae, Attini). They use symbiotic bacteria to protect their fungus-gardens against the pathogenic *Escovopsis* fungus (Currie et al., 1999, 2003a,b). The bacteria of the genus *Pseudonocardia* (Currie et al., 2003a; Cafaro and Currie, 2005) proliferate on distinct areas of the

ants' outer cuticle which exhibits unique cuticular crypts in a number of taxa (Currie et al., 2006). Epidermal glands secreting onto the cuticle and into the bacteria-filled crypts probably provide the *Pseudonocardia* with nutrients (Currie et al., 2006). Bioassays demonstrated that the bacteria selectively inhibit the growth of the parasitic *Escovopsis* in the fungus-gardens probably through the antibiotic properties of their metabolites (Currie et al., 1999, 2003a,b). Founding queens transport the bacteria on their cuticle to the new nest and the bacteria are also transferred between nestmates (Currie et al., 2003a; Poulsen et al., 2002).

The symbiosis between fungus-growing ants and mutualistic bacteria exhibits a prime example of how coevolutionary processes between host, symbionts, and pathogens could change the anatomy, physiology, and behaviour of insects. The facts that female beewolves' antennal glands exhibit an unusual morphology with large invaginations and the finding of putative bacteria were the first hints, that beewolves possibly engage a symbiotic association with bacteria, too.

### **1.3 The European beewolf, *Philanthus triangulum***

#### *1.3.1 Systematic and distribution*

The genus *Philanthus* (Hymenoptera, Crabronidae) is distributed with about 136 species in the Holarctic and Ethiopian regions (Bohart and Menke, 1976; Evans and O'Neill, 1988). Some species also occur in the Neotropic (Cuba and Central America) and Oriental regions whereas no *Philanthus* species are found in South America, Australia and Antarctica (Bohart and Menke, 1976). The genus *Trachypus* which inhabits Central and South America (Bohart and Menke, 1976) is recognized as the sister group to the genus *Philanthus* (Alexander, 1992; Roeser-Mueller, pers. comm.). Together with the genus *Philanthinus*, the genera *Philanthus* and *Trachypus* constitute the tribe Philanthini which, together with the Cercerini and Aphilanthopsini, constitute the subfamily Philanthinae (Alexander, 1992).

The distribution of the European beewolf, *Philanthus triangulum* Fabricius, ranges from Northern Europe to South Africa with the eastern border in the middle east (Bohart and Menke, 1976; Blösch, 2000; Ebrahimi, 2005). Under optimal conditions (warm and sandy habitat, abundant honey bees) nest aggregations can contain several hundred nests (Tinbergen and Kuyt, 1938; Simon Thomas and Simon Thomas, 1980).

#### *1.3.2 Female European beewolves*

European beewolves exhibit a strong sexual dimorphism according to body size - females are considerably larger as males (Strohm, 1995). The females build subterranean nests in sandy soil and provision their offspring exclusively with paralyzed honey bees, *Apis mellifera* (e.g. Tinbergen, 1932;

Strohm, 1995). Nests consist of a main burrow from which horizontal side burrows with terminal brood cells branch off (Simon Thomas and Veenendaal, 1978; Strohm, 1995). The female provisions each brood cell with one to five honey bees, lays a single egg on one of the bees and carefully closes the side burrow with sand. Prior to oviposition the female beewolf turns its head to the distal end of the brood cell and applies a white secretion from her antennal glands onto the ceiling. The antennal glands consist of large reservoirs surrounded by numerous gland cell units (Strohm and Linsenmair, 1995).

The whitish secretion is known to provide an orientational cue for the larvae when spinning its cocoon (Strohm, 1995; Strohm and Linsenmair, 1995). After feeding on the paralyzed bees the beewolf larvae attach their cocoon in vicinity of the antennal gland secretion to the wall of the brood cell (Strohm, 1995; Strohm and Linsenmair, 1995). Therefore the pupae lay with their head pointing towards the main burrow. After eclosion the young beewolf reaches the main burrow by digging straight forward through the sand-filled side burrow and leaves the nest easily through the main burrow. Biotests showed that if the antennal gland secretion is relocated within the brood cells, larvae hence attach their cocoon to the wrong side of the brood cell (Strohm, 1995; Strohm and Linsenmair, 1995). When the imagos eclose from the misaligned cocoons they nevertheless dig straight forward, miss the main burrow and under natural conditions would die before they reach the surface.



Fig. 1.2. Female European beewolf with paralyzed honey bee

In the warm and humid brood cells the beewolf offspring is permanently threatened by pathogenic fungi which could infect the eggs, larvae or pupae as well as the provisioned honey bees. Since all of the possible scenarios are lethal or at least harmful, mechanisms which protect the wasps' offspring against fungal infestations would be a great selective advantage (Strohm, 2000; Strohm and Linsenmair, 2001). In fact beewolf females apply large amounts of a secretion from a cephalic gland – the putative postpharyngeal gland – onto the surface of the paralyzed honey bees (Strohm, 2001;



Strohm and Linsenmair, 2001; Herzner and Strohm, 2007; Herzner et al., 2007). This secretion is composed of various hydrocarbons and reduces water condensation on the bees' surface and furthermore lowers the water loss of the bees (Herzner and Strohm, 2007; Herzner et al., 2007; Herzner and Strohm, in press; Strohm et al., in press). As fungi grow best on moist substrate this kind of food-wrapping is an effective method to reduce the risk of fungal infestation. It seems obvious that beewolves should have evolved further strategies to protect also their eggs, larvae and pupae from the fungal menace.

### 1.3.3 Male European beewolves

Male beewolves establish territories in which they scent mark plants and other structures with secretions of cephalic glands (e.g. Evans and O'Neill, 1988; Schmitt et al., 2003; Herzner et al., 2006; Kroiss et al., 2006). The small territories (about 0.25 m<sup>2</sup>) contain no resources essential to females and are defended against intruding conspecific males in prolonged flight interactions (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988; Strohm and Linsenmair, 1995; Strohm and Lechner, 2000). To deliver the secretions onto the surface, males run on plants or other structures with lowered heads and opened mandibles and drag their clypeal brush over the surface (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988; Strohm, 1995). Since the abdomen is lowered, the body forms an inverted V. This behaviour was initially called abdomen dragging, but there is no evidence that the abdomen has any function in the scent marking (Strohm, unpubl. data). Receptive females enter the territories and copulations take place within or in the vicinity of these territories (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988). Since females approach the territories from the downwind side in a zig-zagging flight they probably find the territories by olfactory sensing of the male pheromone (e.g. Evans and O'Neill, 1988; Schmitt et al., 2003; Herzner et al., 2006; Kroiss et al., 2006).



Fig. 1.3. Male European beewolf

#### 1.4 Temperature-dependent brain plasticity

As poikilothermic organisms insects face the problem to adapt their physiological processes to the temperature regime of their environment, no matter whether the temperature is constant or fluctuating. Especially during the postembryonic development of holometabolic insects temperature is a crucial abiotic factor that influences duration and success of larval and pupal stages (e.g. Hagstrum and Milliken, 1988; Weeks and Levine, 1990; Gilbert and Raworth, 1996; Urbaneja et al., 2001).

Most taxa of social insects like termites, ants, bees and wasps evolved mechanisms to regulate the nest temperature (reviewed in Jones and Oldroyd, 2007). Social insects either use passive mechanisms like nest site selection (Seeley, 1982; Jeanne and Morgan, 1992; Chen et al., 2002), nest orientation (Hubbard and Cunningham, 1977; Jacklyn, 1992) and nest architecture (Seeley and Morse, 1978; Navarro and Jaffe, 1985; Korb and Linsenmair, 1998, 1999) or active mechanisms like clustering (Kronenberg and Heller, 1982; Fahrenholz et al., 1989), generating of metabolic heat (Esch et al., 1991; Bujok et al., 2002) and wing fanning (Seeley and Heinrich, 1981; Vogt, 1986; Weidenmüller, 2004). The ability to control their temperature environment is recognized as one reason for the evolutionary success of insect societies (Wilson, 1971; Hölldobler and Wilson, 1990). Honey bees, *Apis mellifera*, are known to control the temperature of their brood combs in a narrow range of around 35 °C (e.g. Himmer, 1927; Heinrich and Esch, 1994; Kleinhenz et al., 2003). The energy used for this task is estimated to be about 40% of the total energy consumption per year (Tautz et al., 2003). This emphasizes that elaborate temperature regulation in insects is based on division of labour as individuals which are concerned with active climate control probably fail for other tasks like foraging or nest building.

Solitary insects can not afford to spend so many resources for active temperature control since it would cost them too much time and energy. Therefore they have to rely on passive mechanisms to obtain optimal temperatures for their brood, like selecting naturally insulated nest sites deep in the substrate, e.g. soil or wood. However in most solitary species no obvious temperature management during postembryonic development is present and the offspring has to cope with the varying temperature conditions that they experience in their environment.

Brains with their complexity and importance for the survival of the individuals are favourable organs to test the influence of such different temperatures during postembryonic development. The complex processes during neurometamorphosis include apoptosis, synaptogenesis, as well as the growth and proliferation of cells (Truman, 1990; Rössler and Bickmeyer, 1993; Fahrbach and Weeks, 2002). Temperature could affect these processes either directly or indirectly via hormonal or enzymatic pathways (Fahrbach and Weeks, 2002).

Recent studies on honey bees, *A. mellifera*, showed that different temperatures during the pupal phase affect the survival rate, the synaptic organization of the brain and the duration of postembryonic development (Groh et al., 2004, 2006) as well as the morphology, behaviour and learning abilities of the adults (Jones et al., 2005; Tautz et al., 2003). Following the results of these studies honey bees adapted their pupal development to the in-hive temperature of about 35 °C. In contrast, solitary bee species that develop in nests without insulation should be adapted to variable ambient temperatures.

### 1.5 The Red Mason bee, *Osmia bicornis*

In our studies we used the solitary Red Mason bee, *Osmia bicornis* ssp. *cornigera* (Hymenoptera, Megachilidae) which is one of the most abundant Megachilid bees in Central Europe (Raw, 1972; Peters, 1977; Westrich, 1989; Kronic and Stanisavljevic, 2006). The species *Osmia bicornis* (syn.: *O. rufa*;) is divided in three subspecies. The ssp. *cornigera* is distributed from the Pyrenees in the West over the whole Eurasian continent, ssp. *rufa* is found on the Iberian Peninsula, England and Scandinavia and ssp. *fracticornis* in Northern Algeria and Morocco (Raw, 1972; Peters, 1977; Westrich, 1989; Kronic and Stanisavljevic, 2006).

In *Osmia bicornis*, both genders can be easily distinguished due to their sexual dimorphism. Females are larger than males and characterized by two cuticular ‘horns’ on their clypeus whereas males’ front heads are covered with white hairs (Fig. 1.4). The females of this univoltin species build nests in small cavities like holes in walls or hollow stems. Each nest consists of several brood cells separated by loam walls (Raw, 1972; Westrich, 1989; Strohm et al., 2002). The female provisions each brood cell primarily with pollen (Maddocks and Paulus, 1987; Strohm et al., 2002), lays a single egg and closes the cell. Larval and pupal development takes place during summer and the completely developed adults hibernate in the cocoon and leave the nest in spring of the following year. We used this polylectic species for investigations as it is quite abundant and easily accepts artificial nests (Strohm et al., 2002).



Fig. 1.4. Red Mason bees during mating

## 1.6 Outline of the thesis

### 1.6.1 Antennal glands and bacterial symbionts

Chapters 2-6 of this thesis deal with the unique symbiosis between beewolves, *Philanthus spp.*, and their bacterial symbionts. We describe the morphology of specialized antennal glands in female European beewolves, *P. triangulum* and discuss their function as cultivation organs for symbiotic *Streptomyces* bacteria (chapter 2). The bacteria proliferate inside the antennal glands and are secreted by the female into the subterranean brood cells where they are actively taken up by the beewolf larvae and applied to the cocoon silk. Biotests revealed that the bacteria protect the cocoon from fungal infestation and therefore are crucial for the survival of the beewolf offspring (chapter 3). Symbiotic bacteria of the genus *Streptomyces* were found with genetic methods in all of the 27 *Philanthus* species investigated, but not in closely related genera of the subfamily Philanthinae. Based on morphological, ecological and genetic data a new monophyletic taxon ‘*Candidatus Streptomyces philanthi*’ is proposed (chapter 4). Most probably the bacterial endosymbionts are transmitted vertically from beewolf mothers to their daughters via the brood cell and the cocoon. The bacteria start to proliferate inside the antennal glands only after eclosure of the beewolf imago from the cocoon. The implications of the genetic bottleneck during the transmission and the growth rate of the bacteria are discussed in chapter 5. Streptomyces were detected with genetic methods in all *Philanthus* species investigated so far (chapter 4). Therefore the morphology of the respective antennal glands was characterized in 15 species to reveal information about the glands’ phylogeny (chapter 6). Some of the 3D-models in chapter 6 are based on high-resolution microtomographic data sets which turned out to be a suitable method for anatomic investigations on insects.

### 1.6.2 Cephalic glands of the European beewolf

Hitherto the postpharyngeal gland (PPG) was thought to be idiosyncratic to ants (Hymenoptera, Formicidae) where it plays a role in allogrooming and nestmate recognition. In chapters 7 and 8 we provide the first descriptions of such glands outside the Formicidae for both sexes of the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae).

The PPG of female European beewolves resembles the PPG in ants in most morphological aspects although the function seems to be quite different on first sight (chapter 7). Female beewolves extensively lick their honey bee prey and thereby apply large amounts of PPG secretion onto the bees’ surface (Herzner et al., 2007). This treatment reduces the condensation of water on the bees’ cuticle and as a consequence the risk of infestation with pathogenic fungi (Herzner and Strohm, 2007). The licking of the honey bee prey can be seen as homologous to the allogrooming behaviour of ants and amongst other things suggests a common evolutionary origin of the PPG and the according behaviour in ants and beewolves.

In chapter 8 the morphology and content of a PPG in male European bees is characterized. The results of the GC-MS analysis (Kroiss et al., 2006) as well as the absence of gland cells suggest that this gland functions as a reservoir for the male sex pheromone, which is most likely produced in the neighbouring mandibular glands. The morphology and ultrastructure of the latter are characterized in chapter 9. The fact that the mandibular glands of male bees are connected to the PPG through a lateral duct supports the hypothesis of distinct pheromone-production and -storage sites within the two glands.

### *1.6.3 Temperature-effects on the brain of the solitary Red Mason bee*

Honey bees maintain a constant temperature within their hive and adapted their postembryonic development to these homeothermic conditions. Previous studies showed, that even small temperature deviations during the pupal development adversely affect the synaptic organization of the adult honey bee workers and queens (Groh et al., 2004, 2006).

In contrast, solitary species like the Red Mason bee should be more tolerant against variable temperatures as they face a wide range of temperatures during postembryonic development. Therefore this species should have evolved mechanisms to buffer the effect of different temperatures on the development of vital organs like the brain. In chapter 10 we tested this hypothesis by exposing Red Mason bees to different temperatures during postembryonic development and investigated, whether the synaptic organization in adult brains was influenced.



## 1.7 References

- Agren, L. (1977). Mandibular gland morphology of *Cerceris rybyensis* (L.), (Hymenoptera: Philanthidae). *Zoon* 5: 91-95.
- Aksoy, S. (2003). Symbiosis in tsetse. in Bourtzis, K., Miller, T.A., eds. *Insect symbiosis*. CRC Press, Boca Raton, Florida.
- Alexander, B.A. (1992). A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 17: 91-108.
- Attygalle, A.B., Morgan, E.D. (1984). Chemicals from the glands of ants. *Chemical Society Reviews* 13: 245-278.
- Ayasse, M., Paxton, R.J., Tengö, J. (2001). Mating behaviour and chemical communication in the order Hymenoptera. *Annual Review of Entomology* 46: 31-78.
- Battaglia, D., Isidoro, N., Romani, R., Bin, F., Pennacchio, F. (2002). Mating behaviour of *Aphidius ervi* (Hymenoptera: Braconidae): The role of antennae. *European Journal of Entomology* 99 (4): 451-456.
- Baumann, P., Baumann, L., Lai, C.Y., Rouhbakhsh, D. (1995). Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: Intracellular symbionts of Aphids. *Annual Review of Entomology* 49: 55-94.
- Baumann, P., Moran, N.A., Baumann, L. (2006). Bacteriocyte-associated endosymbionts of insects. in Dworkin, M., ed *The Prokaryotes*. Springer, New York.
- Beard, R.L. (1963). Insect toxins and venoms. *Annual Review of Entomology* 8: 1-18.
- Billen, J. (1990). Phylogenetic aspects of exocrine gland development in the Formicidae. Pages 317-318 in Veeresh, G.K., Mallik, B., Viraktamath, C.A., eds. *Social insects and environment*. Oxford and IBH Publishing, New Delhi, Bombay, Calcutta.
- Billen, J. (1991). Ultrastructural organization of the exocrine glands in ants. *Ethology Ecology & Evolution* (1): 67-73.
- Billen, J., Morgan, E.D. (1998). Pheromone communication in social insects: sources and secretions. in Vander Meer, R.K., Breed, M.D., Espelie, K.E., Winston, M.L., eds. *Pheromone Communication in Social Insects. Ants, Wasps, Bees and Termites*. Westview Press, Boulder, CO.
- Billen, J., Buschinger, A. (2000). Morphology and ultrastructure of a specialized bacterial pouch in the digestive tract of *Tetraponera* ants (Formicidae, Pseudomyrmecinae). *Arthropod Structure & Development* 29: 259-266.
- Bin, F., Vinson, S.B. (1986). Morphology of the antennal sex-gland in male *Trissolcus basalis* (Woll.) (Hymenoptera: Scelionidae), an egg parasitoid of the green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae). *International Journal of Insect Morphology & Embryology* 15: 129-138.
- Bin, F., Isidoro, N., Romani, R. (1999a). Antennal structures of Hymenoptera: Sensilla or glands? *Atti dell'Accademia Nazionale Italiana di Entomologia Rendiconti, Anno XLVII - 1999*: 250-263.
- Bin, F., Wackers, F., Romani, R., Isidoro, N. (1999b). Tyloids in *Pimpla turionellae* (L.) are release structures of male antennal glands involved in courtship behaviour (Hymenoptera: Ichneumonidae). *International Journal of Insect Morphology & Embryology* 28 (1-2): 61-68.
- Blösch, M. (2000). Die Grabwespen Deutschlands. Die Tierwelt Deutschlands 71. Goecke & Evers, Keltern.
- Blum, M.S., Brand, J.M. (1972). Social insect pheromones: their chemistry and function. *American Zoologist* 12: 553-576.
- Blum, M.S. (1985). Exocrine systems. Pages 535-579 in Blum, M.S., ed *Fundamentals of insect physiology*. Wiley, New York.
- Bohart, R.M., Menke, A.S. (1976). Sphecid wasps of the world. University of California Press, Berkeley and Los Angeles.
- Borg-Karlson, A.K., Tengö, J. (1980). Pyrazines as marking volatiles in philanthine and nyssonine wasps (Hymenoptera: Sphecidae). *Journal of Chemical Ecology* 6 (4): 827-835.
- Boulay, R., Katzav-Gozansky, T., Hefetz, A., Lenoir, A. (2004). Odour convergence and tolerance between nestmates through trophallaxis and grooming in the ant *Camponotus fellah* (Dalla Torre). *Insectes Sociaux* 51 (1): 55-61.
- Bourtzis, K., Miller, T.A. (2003). *Insect Symbiosis*. CRC Press, Boca Raton.
- Braendle, C., T., M., Bickel, R., Shingleton, A.W., Kambhampati, S., Stern, D.L. (2003). Developmental Origin and Evolution of Bacteriocytes in the Aphid-*Buchnera* Symbiosis. *PLoS Biology* 1 (1): 70-76.
- Breed, M.D. (1998). Recognition pheromones of the honey bee. *BioScience* 48 (6): 463-470.
- Buchner, P. (1965). Endosymbiosis of animals with plant microorganisms. Interscience Publishers, New York.
- Bujok, B., Kleinhenz, M., Fuchs, S., Tautz, J. (2002). Hot spots in the bee hive. *Naturwissenschaften* 89: 299-301.
- Cafaro, M.J., Currie, C.R. (2005). Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Canadian Journal of Microbiology* 51 (6): 441-446.

- Cane, J.H., Gerdin, S., Wife, G. (1983). Mandibular gland secretions of solitary bees (Hymenoptera, Apoidea) - Potential for nest cell disinfection. *Journal of the Kansas Entomological Society* 56 (2): 199-204.
- Cane, J.H., Michener, C.D. (1983). Chemistry and function of mandibular gland products of bees of the genus *Exoneura* (Hymenoptera, Anthophoridae). *Journal of Chemical Ecology* 9 (12): 1525-1531.
- Chen, Y., Hansen, L.D., Brown, J.J. (2002). Nesting sites of the carpenter ant, *Camponotus vicinus* (Mayr)(Hymenoptera: Formicidae) in northern Idaho. *Environmental Entomology* 31 (6): 1037-1042.
- Clarke, S.R., Dani, F.R., Jones, G.R., Morgan, E.D., Schmidt, J.O. (2001). (Z)-3-Hexenyl(R)-3-hydroxybutanoate: A male specific compound in three North American decorator wasps *Eucerceris rubripes*, *E. conata* and *E. tricolor*. *Journal of Chemical Ecology* 27: 1437-1447.
- Crozier, R.H., Dix, M.W. (1979). Analysis of two genetic models for the innate components of colony odour in social Hymenoptera. *Behavioral Ecology and Sociobiology* 4: 217-224.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398 (6729): 701-704.
- Currie, C.R. (2001). A community of ants, fungi, and bacteria: A multilateral approach to studying symbiosis. *Annual Review of Entomology* 55: 357-380.
- Currie, C.R., Bot, A.N.M., Boomsma, J.J. (2003a). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101 (1): 91-102.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (2003b). corrigendum: Fungus-growing ants use antibiotic-producing bacteria to control garden parasites (vol 398, pg 701, 1999). *Nature* 423 (6938): 461-461.
- Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J., Billen, J. (2006). Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311 (28): 81-83.
- Dahms, E.C. (1984). An interpretation of the structure and function of the antennal sense organs of *Melittobia australica* (Hymenoptera: Eulophidae) with the discovery of a large dermal gland in the male scape. *Mem. Queensland Museum* 21: 361-377.
- Dani, F.R., Jones, G.R., Destri, S., Spencer, S.H., Turrillazzi, S. (2001). Deciphering the recognition signature within the cuticular chemical profile of paper wasps. *Animal Behaviour* 62: 165-171.
- Darwin, C. (1859). On the origin of species by means of natural selection. Murray, London.
- Darwin, C. (1871). The descent of man, and selection in relation to sex. Murray, London.
- Dettner, K. (1999). Insekten und Mikroorganismen. in Dettner, K., Peters, W., eds. *Lehrbuch der Entomologie*. Gustav Fischer Verlag, Stuttgart.
- Dillon, R.J., Charnley, A.K. (1995). Chemical barriers to gut infection in the desert locust: *In vivo* production of antimicrobial phenols associated with the bacterium *Pantoea agglomerans*. *Journal of Invertebrate Pathology* 66 (1): 72-75.
- Dillon, R.J., Vennard, C.T., Charnley, A.K. (2000). Exploitation of gut bacteria in the locust. *Nature* 403 (6772): 851-851.
- Dillon, R.J., Vennard, C.T., Charnley, A.K. (2002). A note: Gut bacteria produce components of a locust cohesion pheromone. *Journal of Applied Microbiology* 92 (4): 759-763.
- Dillon, R.J., Dillon, V.M. (2004). The gut bacteria of insects: nonpathogenic interactions. *Annual Review of Entomology* 49: 71-92.
- Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K. (2005). Diversity of locust gut bacteria protects against pathogen invasion. *Ecology Letters* 8 (12): 1291-1298.
- Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* 43: 17-37.
- Douglas, A.E. (2006). Phloem-sap feeding by animals: problems and solutions. *Journal of Experimental Botany* 57 (4): 747-754.
- Downing, H.A. (1991). The function and evolution of exocrine glands. Pages 540-569 in Ross, K.G., Matthews, R.W., eds. *The social biology of wasps*. Cornstock, Ithaca.
- Ebrahimi, E. (2005). An identification guide to the Sphecidae of Iran (Insecta, Hymenoptera). *Journal of the Entomological Society of Iran* 24 (2): 109-135.
- Eelen, D., Borgesen, L., Billen, J. (2006). Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 87: 101-111.
- Esch, H., Goller, F., Heinrich, B. (1991). How do bees shiver? *Naturwissenschaften* 78: 325-328.
- Evans, H.E., O'Neill, K.M. (1988). The Natural History and Behavior of North American Beewolves. Cornell University Press, Ithaca, New York.
- Fahrbach, S.E., Weeks, J.C. (2002). Hormonal regulation of neuronal and behavioral plasticity in insects. Pages 331-358 in Pfaff, D.W., Arnold, A.P., Etgen, A.M., Fahrbach, S.E., Rubin, R.T., eds. *Hormones, Brain and Behavior*. Elsevier, Oxford.

- Fahrenholz, L., Lamprecht, I., Schrickler, B. (1989). Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes. *Journal of Comparative Physiology B* 159: 551-560.
- Felicioli, A., Isidoro, N., Romani, R., Bin, F. (1998). Ethological and morphological analysis of mating behavior in *Osmia cornuta* Latr. (Hymenoptera: Megachilidae). *Insect Social Life* 2: 137-144.
- Fisher, B.L., Robertson, H.G. (1999). Silk production by adult workers of the ant *Melissotarsus emeryi* (Hymenoptera, Formicidae) in South African fynbos. *Insectes Sociaux* 46: 78-83.
- Gamboa, G.J. (1986). The evolution and ontogeny of nestmate recognition in social wasps. *Annual Review of Entomology* 31: 431-454.
- Gebhardt, K., Schimana, J., Muller, J., Fiedler, H.P., Kallenborn, H.G., Holzenkämpfer, M., Krastel, P., Zeeck, A., Vater, J., Hotzel, A., Schmid, D.G., Rheinheimer, J., Dettner, K. (2002). Screening for biologically active metabolites with endosymbiotic bacilli isolated from arthropods. *FEMS Microbiology Letters* 217 (2): 199-205.
- Gilbert, N., Raworth, D.A. (1996). Insects and temperature - A general theory. *Canadian Entomologist* 128 (1): 1-13.
- Godfray, H.C.J., Briggs, C.J., Barlow, N.D., O'Callaghan, M., Glare, T.R., Jackson, T.A. (1999). A model of insect-pathogen dynamics in which a pathogenic bacterium can also reproduce saprophytically. *Proceedings of the Royal Society of London Series B* 266 (1416): 233-240.
- Gracioli, L.F., Silva de Moraes, R.L.M., Cruz-Landim, C. (2004). Ultrastructural aspects of the mandibular gland of *Melipona bicolor* Lepeletier, 1836 (Hymenoptera: Apidae, Meliponini) in the castes. *Micron* 35 (5): 331-336.
- Groh, C., Tautz, J., Rössler, W. (2004). Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. *PNAS* 101 (12): 4268-4273.
- Groh, C., Ahrens, D., Rössler, W. (2006). Environment- and age-dependent plasticity of synaptic complexes in the mushroom bodies of honeybee queens. *Brain Behavior & Evolution* 68: 1-14.
- Gross, P. (1993). Insect behavioral and morphological defenses against parasitoids. *Annual Review of Entomology* 38: 251-273.
- Guerrieri, E., Pedata, P.A., Romani, R., N., I., Bin, F. (2001). Functional anatomy of male antennal glands in three species of Encyrtidae (Hymenoptera: Chalcidoidea). *Journal of Natural History* 35 (1): 41-54.
- Gullan, P.J., Kosztarab, M. (1997). Adaptions in scale insects. *Annual Review of Entomology* 42: 23-50.
- Gunawardena, N.E., Bandumathie, M.K. (1993). Defensive secretion of Rice Bug, *Leptocoris oratorius* Fabricius, (Hemiptera: Coreidae): a unique chemical combination and its toxic, repellent, and alarm properties. *Journal of Chemical Ecology* 19 (4): 1993.
- Gwynne, D. (1978). Male territoriality in the bumblebee wolf, *Philanthus bicinctus* (Mickel)(Hymenoptera, Sphecidae): observations on the behaviour of individual males. *Zeitschrift für Tierpsychologie* 47: 89-103.
- Hagstrum, D.W., Milliken, G.A. (1988). Quantitative analysis of temperature, moisture, and diet factors affecting insect development. *Annals of the Entomological Society of America* 81 (4): 539-546.
- Harrington, J.S. (1960). Synthesis of thiamine and folic acid by *Nocardia rhodnii*, the micro-symbiont of *Rhodnius prolixus*. *Nature* 188 (4755): 1027-1028.
- Hefetz, A. (1983). Function of secretion of mandibular gland of male in territorial behavior of *Xylocopa sulcatipes* (Hymenoptera:Anthophoridae). *Journal of Chemical Ecology* 9 (7): 923-931.
- Hefetz, A., Errard, C., Cojocar, M. (1992). The occurrence of heterospecific substances in the postpharyngeal gland secretion of ants reared in mixed species colonies (Hymenoptera: Formicidae). *Naturwissenschaften* 79: 417-420.
- Heinrich, B., Esch, H. (1994). Thermoregulation in bees. *American Scientist* 82 (2): 164-170.
- Herzner, G., Schmitt, T., Heckel, F., Schreier, P., Strohm, E. (2006). Brothers smell similar: variation in the sex pheromone of male European beewolves *Philanthus triangulum* F. (Hymenoptera: Crabronidae) and its implication for inbreeding avoidance. *Biological Journal of the Linnean Society* 89: 433-442.
- Herzner, G., Schmitt, T., Peschke, K., Hilpert, A., Strohm, E. (2007). Food wrapping with the post pharyngeal gland secretion by females of the european beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 33 (4): 849-859.
- Herzner, G., Strohm, E. (2007). Fighting fungi with physics: food wrapping by a solitary wasp prevents water condensation. *Current Biology* 17: R46-R47.
- Himmer, A. (1927). Ein Beitrag zur Kenntnis des Wärmehaushalts im Nestbau sozialer Hautflügler. *Zeitschrift für vergleichende Physiologie* 5: 375-389.
- Hölldobler, B., Carlin, N.F. (1987). Anonymity and specificity in the chemical communication signals of social insects. *Journal of Comparative Physiology A* 161: 567-581.
- Hölldobler, B., Wilson, E.O. (1990). The ants. Harvard University Press, Cambridge.

- Houk, E.J., Griffiths, G.W. (1980). Intracellular symbiotes of the homoptera. *Annual Review of Entomology* 25: 161-187.
- Hu, K., Webster, J.M. (2000). Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus heterorhabditis* infected *Galleria melonella* larvae. *FEMS Microbiology Letters* 189 (2): 219-223.
- Hubbard, M.D., Cunningham, W.G. (1977). Orientation of mounds of the ant *Solenopsis invicta* (Hymenoptera, Formicidae, Myrmicinae). *Insectes Sociaux* 24 (1): 3-7.
- Ishikawa, H. (2003). Insect symbiosis: an introduction. in Bourtzis, K., Miller, T.A., eds. *Insect symbiosis*. CRC Press, Boca Raton, Florida.
- Isidoro, N., Bin, F. (1995). Male antennal gland of *amitus spiniferus* (Brethes) (Hymenoptera: Platygasteridae), likely involved in courtship behavior. *International Journal of Insect Morphology & Embryology* 24 (4): 365-373.
- Isidoro, N., Bin, F., Colazza, S., Vinson, B.S. (1996). Morphology of antennal gustatory sensilla and glands in some parasitoid hymenoptera with hypothesis on their role in sex and host recognition. *Journal of Hymenoptera Research* 5 (1996): 206-239.
- Isidoro, N., Bin, F., Romani, R., Pujade-Villar, J., Ros-Farre, P. (1999). Diversity and function of male antennal glands in Cynipoidea (Hymenoptera). *Zoologica Scripta* 28 (1-2): 165-174.
- Isidoro, N., Romani, R., Velasquez, D., Renthal, R., Bin, F., Vinson, S.B. (2000). Antennal glands in queen and worker of the fire ant, *Solenopsis invicta* Buren: first report in female social Aculeata (Hymenoptera, Formicidae). *Insectes Sociaux* 47 (3): 236-240.
- Jacklyn, P.M. (1992). "Magnetic" termite mound surfaces are orientated to suit wind and shade conditions. *Oecologia* 91: 385-395.
- Jackson, B.D., Morgan, E.D. (1993). Insect chemical communication: pheromones and exocrine glands of ants. *Chemoecology* 4: 125-144.
- Jeanne, R.L., Morgan, R.C. (1992). The influence on nest site choice and reproductive strategies *Polistes* wasps. *Ecological Entomology* 17 (2): 135-141.
- Jeanne, R.L. (1996). The evolution of exocrine gland function in wasps. in Turillazzi, S., West-Eberhard, M.J., eds. *Natural history and evolution of paper wasps*. Oxford University Press, Oxford.
- Jones, J.C., Helliwell, P., Beekmann, M., Maleszka, R., Oldroyd, B.P. (2005). The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*. *Journal of Comparative Physiology A* 191: 1121-1129.
- Jones, J.C., Oldroyd, B.P. (2007). Nest thermoregulation in social insects. *Advances in Insect Physiology* 33: 153-191.
- Kaltenpoth, M., Strohm, E. (2006). The scent of senescence: Age-dependent changes in the composition of the marking pheromone of the male European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae). *Journal of Insect Science* 6: 20.
- Kaltenpoth, M., Kroiss, J., Strohm, E. (2007). The odor of origin: kinship and geographical distance are reflected in the marking pheromone of male beewolves (*Philanthus triangulum* F., Hymenoptera, Crabronidae). *BMC Ecology* 7:11.
- Kleinhenz, M., Bujok, B., Fuchs, S., Tautz, J. (2003). Hot bees in empty broodnest cells: heating from within. *The Journal of Experimental Biology* 206: 4217-4231.
- Korb, J., Linsenmair, K.E. (1998). The effects of temperature on the architecture and distribution of *Macrotermes bellicosus* (Isoptera, Macrotermitinae) mounds in different habitats of a West African Guinea savanna. *Insectes Sociaux* 45: 51-65.
- Korb, J., Linsenmair, K.E. (1999). The architecture of termite mounds: a result of a trade-off between thermoregulation and gas exchange? *Behavioral Ecology* 10 (3): 312-316.
- Kroiss, J., Schmitt, T., Schreier, P., Strohm, E., Herzner, G. (2006). A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 32: 2763-2776.
- Kronenberg, F., Heller, H.C. (1982). Colonial Thermoregulation in Honey Bees (*Apis mellifera*). *Journal of Comparative Physiology* 148: 65-76.
- Kronic, M.D., Stanisavljevic, L.Z. (2006). The biology of European Orchard bee, *Osmia cornuta*. Faculty of Biology University of Belgrade, Belgrade.
- Landolt, P.J., Akre, R.D. (1979). Occurrence and location of exocrine glands in some social vespidae (Hymenoptera). *Annals of the Entomological Society of America* 72 (1): 141-148.
- Lenoir, A., Fresneau, D., Errard, C., Hefetz, A. (1999). Individuality and colonial identity in ants: the emergence of the social representation concept. in Detrain, C., Deneubourg, J.L., Pasteels, J.M., eds. *Information processing in social insects*. Birkhäuser, Basel.

- Lenoir, A., Hefetz, A., Simon, T., Soroker, V. (2001). Comparative dynamics of gestalt odour formation in two ant species *Camponotus fellah* and *Aphaenogaster senilis* (Hymenoptera: Formicidae). *Physiological Entomology* 26: 275-283.
- Margulis, L., Fester, R. (1991). Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. MIT Press, Cambridge.
- Matsuura, K. (2003). Symbionts affecting termite behavior. in Bourtzis, K., Miller, T.A., eds. *Insect symbiosis*. CRC Press, Boca Raton, Florida.
- McDaniel, C.A., Howard, R.W., O'Neill, K.M., Schmidt, J.O. (1987). Chemistry of male mandibular gland secretions of *Philanthus basilaris* (Cresson) and *Philanthus bicinctus* (Mickel)(Hymenoptera: Sphecidae). *Journal of Chemical Ecology* 13: 227-235.
- McDaniel, C.A., Schmidt, J.O., Howard, R.W. (1992). Mandibular gland secretions of the male beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera: Sphecidae). *Journal of Chemical Ecology* 18 (1): 27-37.
- Moran, N.A., Baumann, P. (2000). Bacterial endosymbionts in animals. *Current Opinion in Microbiology* 3: 270-275.
- Moran, N.A., Degnan, P.H., Santos, S.R., Dunbar, H.E., Ochman, H. (2005). The players in a mutualistic symbiosis: Insects, bacteria, viruses, and virulence genes. *PNAS* 102 (47): 16919-16926.
- Muller, W.J., Hepburn, H.R. (1992). Temporal and spatial patterns of wax secretion and related behavior in the division of labour of the honeybee (*Apis mellifera capensis*). *Journal of Comparative Physiology A* 171: 111-1158.
- Navarro, J.G., Jaffe, K. (1985). On the adaptive value of nest features in the grass-cutting ant *Acromyrmex landolti*. *Biotropica* 17 (4): 347-348.
- Noirot, C., Quennedey, A. (1974). Fine-structure of insect epidermal glands. *Annual Review of Entomology* 19: 61-80.
- Noirot, C., Quennedey, A. (1991). Glands, gland-cells, glandular units - some comments on terminology and classification. *Annales De La Societe Entomologique De France* 27 (2): 123-128.
- Oldham, N.J., Morgan, E.D., Agosti, D., Wehner, R. (1999). Species recognition from postpharyngeal gland contents of ants of the *Cataglyphis bicolor* group. *Journal of Chemical Ecology* 25 (6): 1383-1393.
- Oliver, K.M., Russell, J.A., Moran, N.A., Hunter, M.S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *PNAS* 100 (4): 1803-1807.
- Paracer, S., Ahmadjian, V. (2000). Symbiosis: an introduction to biological associations. Oxford University Press, Oxford, New York.
- Peters, D.S. (1977). Systematik und Zoogeographie der west-paläarktischen Arten von *Osmia* s str, *Monosmia*, und *Orientosmia*. *Senckenbergiana Biologica* 58: 287-346.
- Piel, J. (2002). A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. *PNAS* 99 (22): 14002-14007.
- Piel, J. (2004). Metabolites from symbiotic bacteria. *Natural Product Reports* 21: 519-538.
- Poulsen, M., Bot, A.N.M., Currie, C.R., Boomsma, J.J. (2002). Mutualistic bacteria and a possible trade-off between alternative defence mechanisms in *Acromyrmex* leaf-cutting ants. *Insectes Sociaux* 49: 15-19.
- Prestwich, G.D. (1984). Defense Mechanisms of Termites. *Annual Review of Entomology* 1984 (29).
- Quennedey, A. (1998). Insect epidermal gland cells: ultrastructure and morphogenesis.
- Rathmayer, W. (1962). Das Paralysisproblem beim Bienenwolf *Philanthus triangulum* F. (Hym. Sphec.). *Zeitschrift für vergleichende Physiologie* 6: 105-122.
- Raw, A. (1972). The biology of the solitary bee *Osmia rufa* (L.) (Megachilidae). *Transcriptions of the Royal Entomological Society of London* 124 (3): 213-229.
- Romani, R., Isidoro, N., Riolo, P., Bin, F. (2003). Antennal glands in male bees: structures for sexual communication by pheromones? *Apidologie* 34 (6): 603-610.
- Romani, R., Isidoro, N., Riolo, P., Bin, F., Fortunato, A., Turillazzi, S., Beani, L. (2005). A new role for antennation in paper wasps (Hymenoptera, Vespidae): antennal courtship and sex dimorphic glands in antennomeres. *Insectes Sociaux* 52: 96-102.
- Romani, R., Grasso, D.A., Mori, A., Isidoro, N., Le Moli, F. (2006). Antennal glands of the slave-making ant *Polyergus rufescens* and its slave species *Formica cunicularia* (Hymenoptera, Formicidae). *Canadian Journal of Zoology* 84: 1-5.
- Rössler, W., Bickmeyer, U. (1993). Locust medial neurosecretory cells *in vitro*: morphology, electrophysiological and effects of temperature. *Journal of Experimental Biology* 183: 323-339.
- Schmidt, J.O., O'Neill, K.M., Fales, H.M., McDaniel, C.A., Howard, R.W. (1985). Volatiles from mandibular glands of male beewolves (Hymenoptera: Sphecidae, *Philanthus*) and their possible roles. *Journal of Chemical Ecology* 11 (7): 895-901.
- Schmidt, J.O., McDaniel, C.A., Simon Thomas, R.T. (1990). Chemistry of male mandibular gland secretions of *Philanthus triangulum*. *Journal of Chemical Ecology* 16 (7): 2135-2143.

- Schmitt, T., Strohm, E., Herzner, G., Bicchi, C., Krammer, G., Heckel, F., Schreier, P. (2003). (S)-2,3-dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum*. *Journal of Chemical Ecology* 29 (11): 2469-2479.
- Seeley, T., Heinrich, B. (1981). *Insect thermoregulation*. Wiley, New York.
- Seeley, T.D., Morse, R.A. (1978). Nest site selection by the honey bee, *Apis mellifera*. *Insectes Sociaux* 25: 323-337.
- Seeley, T.D. (1982). How honeybees find a home. *Sci. Am.* 247 (4): 158-169.
- Sehnal, F., Akai, H. (1990). Insect silk glands: their types, development and function, and effects of environmental factors and morphogenetic hormones on them. *International Journal of Insect Morphology & Embryology* 19 (2): 79-132.
- Shorey, H.H. (1973). Behavioral responses to insect pheromones. *Annual Review of Entomology* 18: 349-380.
- Simon Thomas, R.T., Poorter, E.P.R. (1972). Notes on the behaviour of males of *Philanthus triangulum* (F.) (Hymenoptera, Sphecidae). *Tijdschrift voor Entomologie* 115: 141-151.
- Simon Thomas, R.T., Veenendaal, R.L. (1978). Observations on the underground behavior of *Philanthus triangulum* (Hymenoptera, Sphecidae). *Entomologische Berichten Amsterdam* 38 (1): 3-8.
- Simon Thomas, R.T., Simon Thomas, A.M.J. (1980). *Philanthus triangulum* and its recent eruption as a predator of honeybees in an Egyptian oasis. *Bee World* 61 (3): 97-107.
- Singer, T.L. (1998). Roles of Hydrocarbons in the recognition systems of insects. *American Zoologist* 38: 394-405.
- Smith, B.H., Breed, M.D. (1995). The chemical basis for nestmate recognition and mate discrimination in social insects. in Cardé, R.T., Bell, W.J., eds. *Chemical Ecology of Insects vol. 2*. Chapman and Hall, New York.
- Soroker, V., Vienne, C., Hefetz, A. (1994). The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 81 (11): 510-513.
- Soroker, V., Hefetz, A., Cojocar, M., Billen, J., Franke, S., Francke, W. (1995a). Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 20 (4): 323-329.
- Soroker, V., Vienne, C., Hefetz, A. (1995b). Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera: Formicidae). *Journal of Chemical Ecology* 21: 365-378.
- Soroker, V., Fresneau, D., Hefetz, A. (1998). Formation of colony odor in Ponerine ant *Pachycondyla apicalis*. *Journal of Chemical Ecology* 24: 1077-1090.
- Soroker, V., Hefetz, A. (2000). Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. *Journal of Insect Physiology* 46: 1097-1102.
- Strohm, E. (1995). Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). *Wissenschaftliche Schriftenreihe Biologie* 6.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Lechner, K. (2000). Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 59: 183-191.
- Strohm, E., Linsenmair, K.E. (2000). Allocation of parental investment among individual offspring in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Biological Journal of the Linnean Society* 69 (2): 173-192.
- Strohm, E., Linsenmair, K.E. (2001). Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 26 (2): 198-203.
- Strohm, E., Marliani, A. (2002). The cost of parental care: prey hunting in a digger wasp. *Behavioral Ecology* 13 (1): 52-58.
- Strohm, E., Herzner, G., Goettler, W. (2007). A "social" gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36 (2): 113-122.
- Swart, C.C., Felgenhauer, B.E. (2003). Structure and function of the mouthparts and salivary gland complex of the Giant Waterbug, *Belostoma lutarium* (Stål) (Hemiptera: Belostomatidae). *Annals of the Entomological Society of America* 96 (6): 870-882.
- Takatsuka, J., Kunimi, Y. (2000). Intestinal bacteria affect growth of *Bacillus thuringiensis* in larvae of the oriental tea tortrix, *Homona magnanima* Diakonoff (Lepidoptera: Tortricidae). *Journal of Invertebrate Pathology* 76 (3): 222-226.
- Tautz, J., Maier, S., Groh, C., Rössler, W., Brockmann, A. (2003). Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. *PNAS* 100 (12): 7343-7347.
- Terra, W.R. (1990). Evolution of digestive systems of insects. *Annual Review of Entomology* 35: 181-200.

- Tillmann, J.A., Seybold, S.J., Jurenka, R.A., Blomquist, G.J. (1999). Insect pheromones - an overview of biosynthesis and endocrine regulation. *Insect Biochemistry and Molecular Biology* 29: 481-514.
- Tinbergen, N., Kruyt, W. (1938). Über die Orientierung des Bienenwolfes (*Philanthus triangulum* Fabr.). *Journal of Comparative Physiology A* 25 (3): 292-334.
- Truman, J.W. (1990). Metamorphosis of the central nervous system of *Drosophila*. *Journal of Neurobiology* 21 (7): 1072-1084.
- Urbaneja, A., Llácer, E., Garrido, A., Jacas, J.A. (2001). Effect of temperature on the life history of *Cirrospilus* sp. near *lyncus* (Hymenoptera: Eulophidae), a parasitoid of *Pylocnistis citrella* (Lepidoptera: Gracillariidae). *Biological Control* 21 (3): 293-299.
- Vander Meer, R.K., Morel, L. (1998). Nestmate recognition in ants. Pages 79-103 in Vander Meer, R.K., Breed, M.D., Espie, K.E., Winston, M.L., eds. *Pheromone communication in social insects*. Westview Press, Boulder, CO.
- Vienne, C., Soroker, V., Hefetz, A. (1995). Congruency of hydrocarbon patterns in heterospecific groups of ants - transfer and/or biosynthesis. *Insectes Sociaux* 42 (3): 267-277.
- Vinson, S.B., Williams, H.J., Frankie, G.W., Wheeler, J.W., Blum, M.S., Coville, R.E. (1982). Mandibular glands of male *Centris adani*, (Hymenoptera; Anthophoridae) their morphology, chemical constituents, and function in scent marking and territorial behavior. *Journal of Chemical Ecology* 8 (2): 319-327.
- Vogt, F.D. (1986). Thermoregulation in bumblebee colonies, I: thermoregulatory versus brood-maintenance behaviors during acute changes in ambient temperatures. *Physiological Zoology* 59: 55-59.
- Weeks, J.C., Levine, R.B. (1990). Postembryonic neuronal plasticity and its hormonal control during insect metamorphosis. *Annual Review of Entomology* 13: 183-194.
- Weidenmüller, A. (2004). The control of nest climate in bumblebee (*Bombus terrestris*) colonies: interindividual variability and self reinforcement in fanning response. *Behavioral Ecology* 15 (1): 120-128.
- Wenzel, J.W. (1987). Male reproductive behavior and mandibular glands in *Polistes major* (Hymenoptera: Vespidae). *Insectes Sociaux* 34 (1): 44-57.
- Werren, J.H., O'Neill, S.L. (1997). Influential passengers: inherited microorganisms and arthropod reproduction. Pages 1-41 in O'Neill, S.L., Hoffman, A.A., Werren, J.H., eds. *The evolution of heritable symbionts*. Oxford University Press, Oxford.
- Wilson, E.O. (1971). *The Insect Societies*. Harvard University Press, Cambridge, MA.
- Zientz, E., Dandekar, T., Gross, R. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiology and Molecular Biology Reviews* 68 (4): 745-770.

## CHAPTER 2

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

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### 2.1 Summary

Females of a solitary digger wasp, the European beewolf (*Philanthus triangulum* F.), cultivate symbiotic bacteria of the genus *Streptomyces* in specialized antennal glands. The streptomycetes are secreted in the subterranean brood cells and protect the offspring against mould fungi. We reconstructed the complex morphology of the antennal glands using 3D-visualization software, investigated the ultrastructure of the glands, and examine the role of the antennal glands as organs for the cultivation of the symbiotic bacteria. The bacteria are cultivated 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 when completely filled with bacteria it comprises about half of the antennomere volume. The opening of the reservoir is covered by gelatinous appendage of the cuticle. The cells of the monolayered epithelium bordering each reservoir show basal invaginations, apical microvilli and numerous vesicles. Each reservoir is surrounded by approximately 400 class 3 gland units that 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. In the reservoir lumen, large numbers of symbiotic *Streptomyces* bacteria are embedded in secretion droplets. Thus, the bacteria are apparently provided with large amounts of nutrients via the gland epithelium and the class 3 gland cell units.



## 2.2 Introduction

Symbioses are important components of the natural world and insects have evolved a variety of symbiotic relationships with microorganisms (Bourtzis and Miller, 2003; Moran and Baumann, 2000). Symbiotic bacteria occur either intra- or extracellularly and enhance the fitness of their hosts, e.g. by supplying essential nutrients (e.g. Buchner, 1965; Douglas, 1998; Paracer and Ahmadjian, 2000) or by providing defence against pathogens (Charnley et al., 1985; Currie et al., 1999; Takatsuka and Kunimi, 2000; Dillon et al., 2005). Frequently, symbioses with bacteria are 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 and Buschinger, 2000). Recently, a symbiosis between a digger wasp, the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae) and bacteria of the genus *Streptomyces* has been described (Kaltenpoth et al., 2005). This association is unusual with regard to the localization of the cultivation organ: specialized glands in the antennae of the 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 and 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 (Kaltenpoth et al., 2005; Strohm and Linsenmair, 1995). Then the female beewolf lays an egg and closes the brood cell.

The white substance serves at least two functions: First it provides a necessary cue for the orientation of the cocoon spinning of the beewolf larvae. This orientation eventually facilitates the emergence of the adult beewolf from the brood cell (Strohm and Linsenmair, 1995). The second function of the whitish antennal 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; Kaltenpoth et al., 2006). Thus, the bacteria are cultivated in the antennal glands of beewolf females.

Antennal glands have been rarely described in the Hymenoptera. After the first report of glands in the antennae of males of the parasitoid wasp *Melittobia australica* (Eulophidae; Dahms, 1984) only a few other studies found such glands. In male Hymenoptera, antennal glands are known to play a role in mating and courtship behaviour by secreting volatile or paste-like substances acting as sex-

pheromones. In various taxa like Cynipoidea (Isidoro et al., 1999), Chalcidoidea (Guerrieri et al., 2001), Platygasteridae (Isidoro and Bin, 1995), Scelionidae (Bin and Vinson, 1986), Vespidae (Bin et al., 1999a; Isidoro et al., 1996; 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, 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 directly 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.

## 2.3 Materials and Methods

### 2.3.1 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 brood cell provisioning. Female beewolves were anaesthetised with CO<sub>2</sub> and killed with diethyl ether.

### 2.3.2 Semithin sections and 3D-reconstruction

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.

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 using the 3D-visualization software Amira<sup>®</sup> (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 the reconstruction of the three-dimensional arrangement of the antennal structures. The volume of certain structures of the 3D-model can then be calculated.

### 2.3.3 Electron microscopy

For scanning electron microscopy (SEM), the antennae of freshly killed females 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.

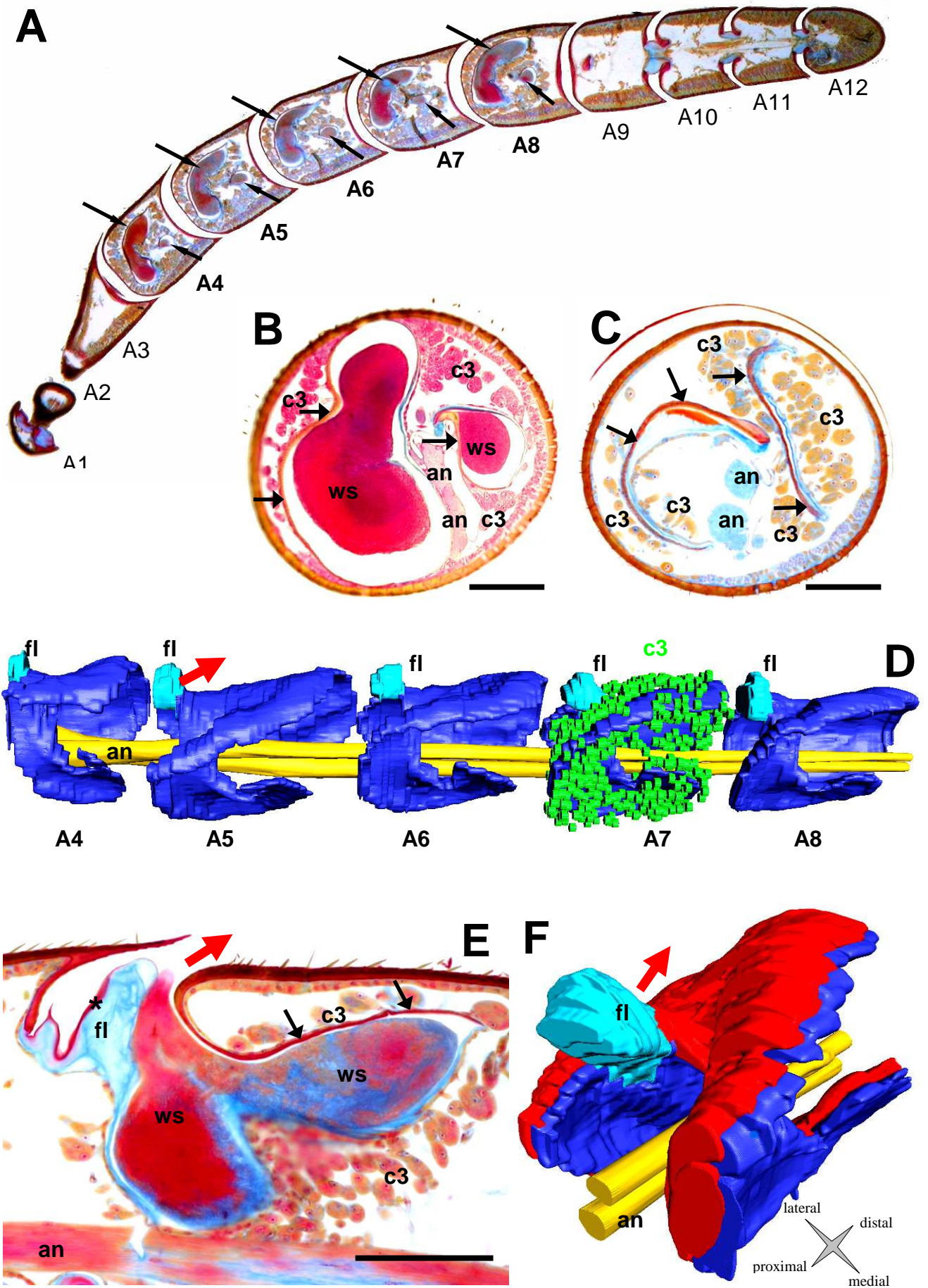
For transmission electron microscopy (TEM), the antennae were fixed overnight at 4 °C in a solution of 4% glutardialdehyd in 0,1 M Sörensen phosphate buffer at pH 7.4 (Sörensen, 1909), followed by postfixation with 2% osmium tetroxide. After dehydration in a graded ethanol series and propylene oxide 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.

## 2.4 Results

### 2.4.1 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. 2.1A]. 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 and Quenedey, 1974). The 3D-reconstruction reveals that such a gland reservoir consists of two lobes, that more or less describe a bent figure "S" [Fig. 2.1D,F] surrounding the antennal nerv. 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. 2.1D,F].

The reservoir's opening is located dorsally in the proximal intersegmental gap of the antennomere [Figs. 2.1E, 2.2] and is visible as a small hole when the adjacent proximate antennal segment is removed [Fig. 2.2]. Longitudinal semithin sections revealed that the opening is covered by a flap-like



projecting part of the intersegmental cuticle with gelatinous appendages [Fig. 2.1E]. 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. Completely filled with white substance a reservoir makes up more than 50% of the antennomere's volume and apparently even squeezes the antennal nerve between its two parts [Fig. 2.1B]. An empty reservoir, by contrast, appears completely collapsed with the opposing sides of the reservoir cuticle close to each other [Fig. 2.1C]. The 3D-reconstruction of filled reservoirs showed a maximum volume of 0.07  $\mu\text{l}$  (data not shown). Thus, the ten reservoirs of both antennae have a remarkable maximum volume of approx. 0.7  $\mu\text{l}$ . 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. 2.1B,C], whereas on the lateral side the cuticle is reinforced and has a net-like structure [Fig. 2.1B,C,F, see also Fig. 2.4]. The transition between the reinforced, net-like cuticle and thin cuticle is abrupt at the dorsal and ventral side of the reservoir tubes [Fig. 2.1B,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 bearing the glands (A4 to A8). 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).

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. 2.1B-E]. The acini are spherical or drop shaped with diameters up to 30  $\mu\text{m}$ . They are almost evenly distributed over the surface of the reservoir, but

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(previous page) Fig. 2.1. **A** Longitudinal semithin section through the antenna of a female European beewolf with 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 nerv (an) in figure (B) is squeezed between the two parts of the reservoir filled with white substance (ws). Arrows indicate the reinforced net like cuticle bordering the reservoir, scale bars 100  $\mu\text{m}$ . **D** 3D-reconstruction of almost empty reservoirs (dark blue) in antennomere A4 to A8 surrounding the ventral antennal nerv (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 light blue. The route of the reservoir content when pressed out is indicated by a red arrow. **E** Longitudinal semithin section through an antennomere showing the flap (fl) with gelatinous part (light blue) and projecting cuticle (\*) controlling the reservoir outlet. Black arrows indicate the reinforced cuticle, scale bar 100  $\mu\text{m}$ . (red arrow) route of reservoir content when pressed out. **F** Proximal view on 3D-reconstruction of a single reservoir showing the position of the reinforced cuticle (red) bordering the lumen. (A1-A12) antennomeres, (an) antennal nerv, (c3) class 3 gland cell units, (fl) flaps at reservoir openings, (ws) white substance, (red arrow) route of reservoir content when pressed out.

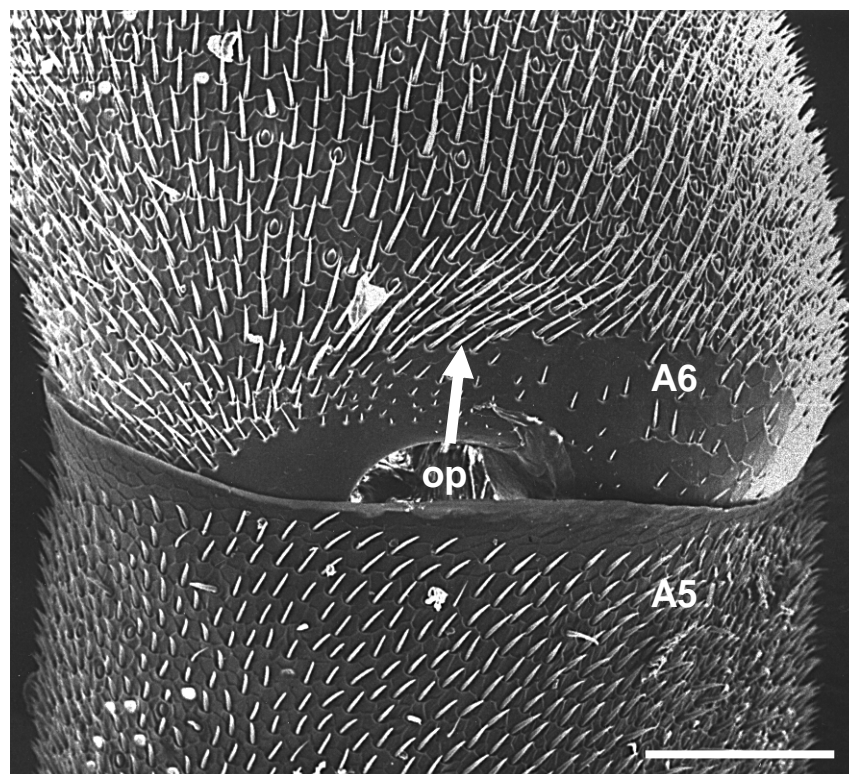


Fig. 2.2. SEM micrograph of antennomeres A5 and A6 (left antennae). Antennomere A6 is bent downwards, so the dorsal opening (op) of the gland reservoir in A6 is visible, scale bar 100  $\mu\text{m}$ . (white arrow) route of reservoir content when pressed out.

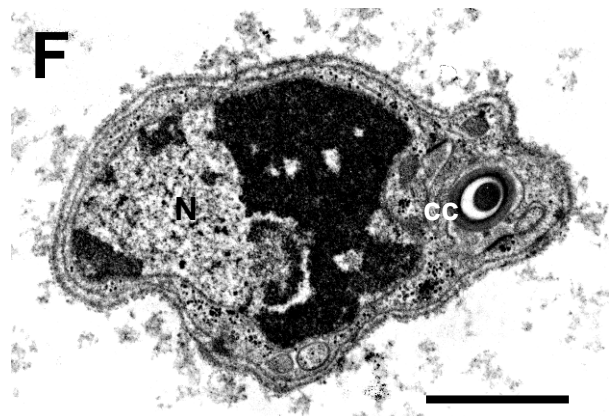
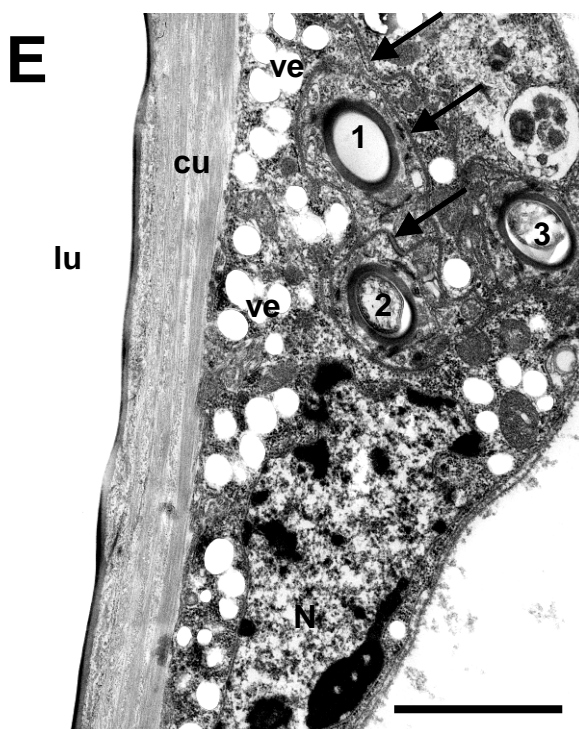
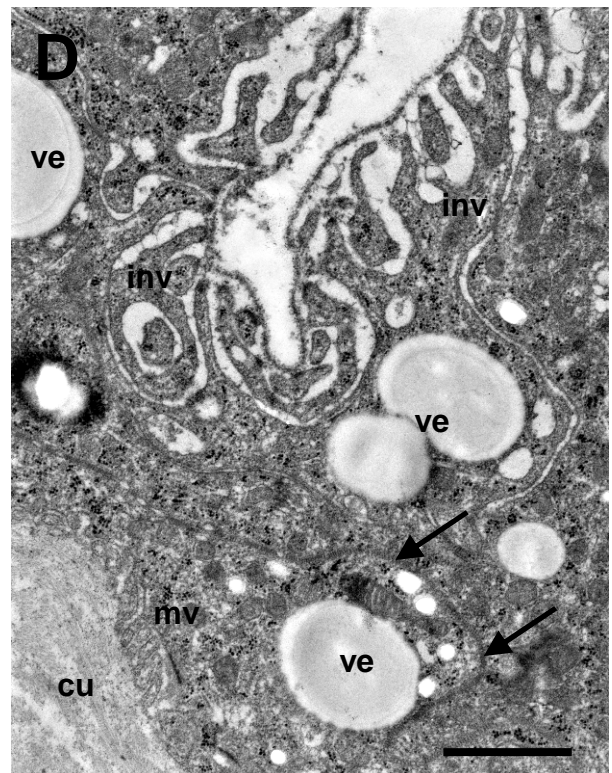
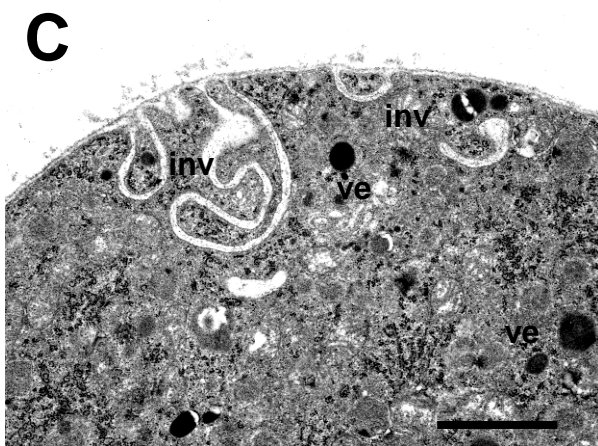
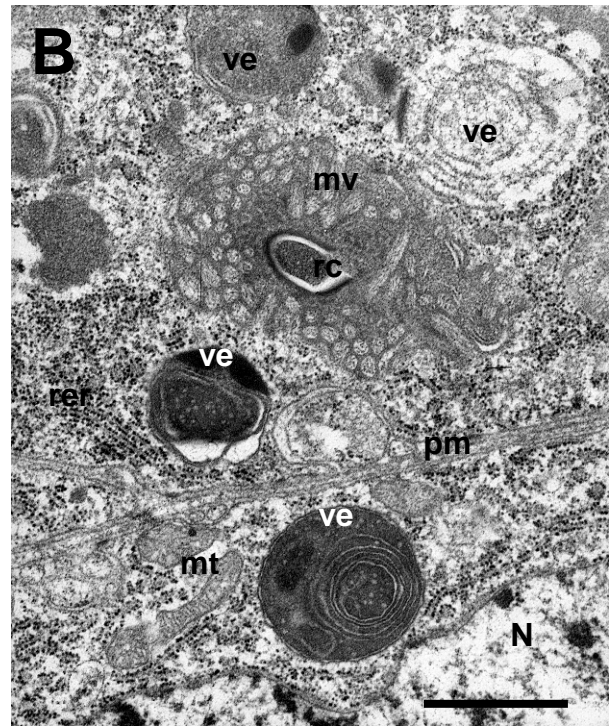
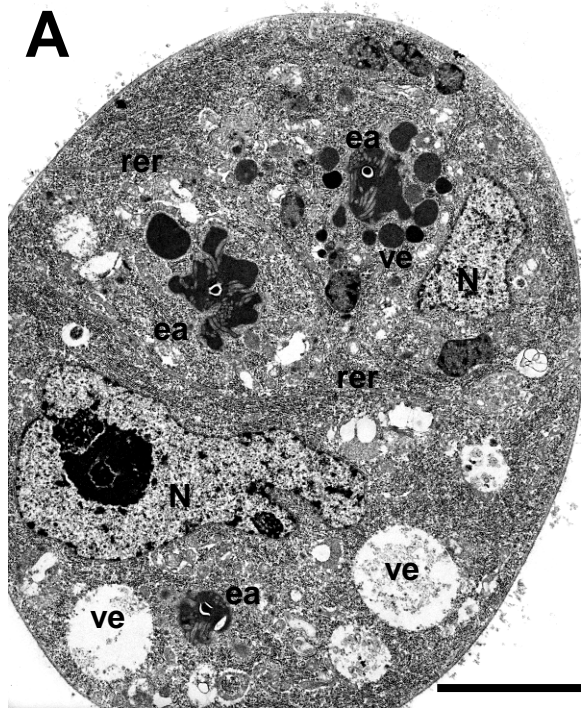
slightly more abundant at the membranous cuticle of the medial side of the reservoir. Conducting canals connect the class 3 cells to the reservoir and the canals of each acinus frequently form a bundle and open into the reservoir in groups [Fig. 2.4].

#### 2.4.2 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. 2.4C]. Where the bordering epithelium was removed during preparation the net like structure of the reinforced cuticle is clearly visible [Fig. 2.4A]. This net like structure of the endo- and exocuticle even shines through the epicuticle when scanning the inner side of the reservoir lumen [Fig. 2.4C]. 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  $\mu\text{m}$  thick [Fig. 2.4B].

TEM micrographs confirm the existence of a cuticle lining the reservoir lumen. The membranous part of the reservoir cuticle is about 1  $\mu\text{m}$ , the reinforced net-like cuticle up to 3  $\mu\text{m}$  thick [Figs. 2.3E, 2.4B]. The monolayered epithelium bordering the reservoir is 2 to 5  $\mu\text{m}$  thick and also fills the interspaces of the net-like reinforced parts of the cuticle [Fig. 2.4B].





The cells of the epithelium are flat with comparatively large nuclei and are connected by septate desmosomes [Fig. 2.3D,F]. Especially the epithelium of the membranous cuticle contains numerous electron lucent vesicles with diameters up to 1  $\mu\text{m}$  [Fig. 2.3D,E]. In the epithelium bordering the gelatinous projection at the opening of the reservoir there are invaginations of the basal cell membrane and microvilli at the apical side [Fig. 2.3D].

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. 2.3A,B]. The majority of these vesicles are about 1  $\mu\text{m}$  in diameter, whereas some show diameters of more than 4  $\mu\text{m}$  [Fig. 2.3A,B]. Frequently vesicles bear membranous structures [Fig. 2.3B]. Sometimes the class 3 cells show invaginations of the parts of the plasma membrane that are in contact with the hemolymph [Fig. 2.3C]. 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. 2.3A,B]. Canal cells encircle the conducting canals leading from the acini to the reservoir lumen [Fig. 2.3F]. The content of the receiving and conducting canals seems to be a mixture of electron dense and lucent secretion [Figs. 2.3A,B,E,F, 2.4B]. No nerves or axons linked to class 3 gland cells or the bordering epithelium were observed. Tracheoles were found in the bordering epithelium, but they never penetrated the lumen of the reservoirs.

#### 2.4.3 Reservoir contents

The content of a reservoir consists of the primary secretion of the class 3 gland cells and the bordering epithelium, seen as vesicles, and the symbiotic bacteria of the genus *Streptomyces* (Kaltenpoth et al., 2005; Kaltenpoth et al., 2006). The bacteria are about 0.5  $\mu\text{m}$  in diameter and form long and sometimes branched filaments. The branching is characteristic for actinomycetes [Fig. 2.5A,B]. The bacterial filaments are mostly aligned parallel and are embedded in secretion droplets of irregular shape and diameters of up to 5  $\mu\text{m}$ , whereas smaller droplets are more electron dense than large droplets [Fig. 2.5A,B].

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(previous page) Fig. 2.3. TEM micrographs of secretory cells/organelles. **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  $\mu\text{m}$ . (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  $\mu\text{m}$ . (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  $\mu\text{m}$ . (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 1  $\mu\text{m}$ . **E** Mono-layered 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  $\mu\text{m}$ . **F** Conducting canal (cc) with electron dense content surrounded by canal cell, scale bar 1  $\mu\text{m}$ . (N) nucleus



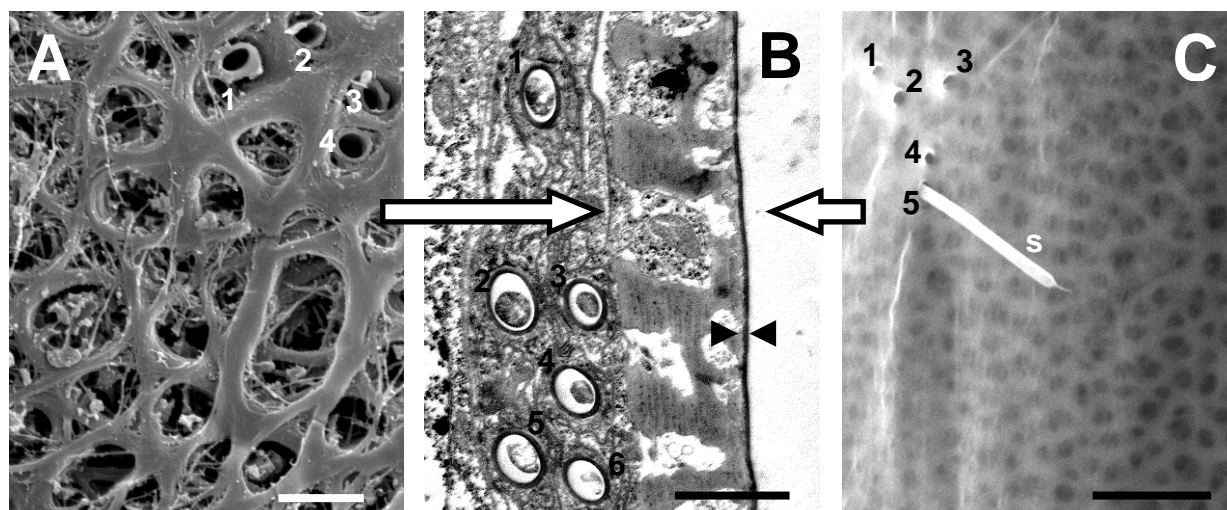


Fig. 2.4. Electron micrographs of net-like cuticle bordering the reservoir. **A** SEM: Exterior view of the cuticle with net-like structure (cu). The monolayered epithelium was removed during preparation, scale bar 2  $\mu\text{m}$ . (1-4) conducting canals. **B** TEM: Monolayered epithelium covered 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  $\mu\text{m}$ . 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 endo- and exocuticle shining through the thin epicuticle, scale bar 5  $\mu\text{m}$ .

## 2.5 Discussion

The antennal gland reservoirs of female European bees represent unique bacteria cultivation organs with a highly elaborated ultrastructure. The lumen of the reservoir has two parts that make up a considerable fraction of the antennomere. The reservoir is enclosed by a monolayered epithelium with a cuticle and many class 3 gland cells secrete into the 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 the hemolymph pressure in the antennae. The transport of white substance out of the inter-antennomere space might be facilitated by the observed bending of the antennae. The projecting cuticle with gelatinous appendages that covers the reservoir's opening probably acts as a closing device that possibly prevents invasion of the glands by undesirable bacteria.

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, beewolf females

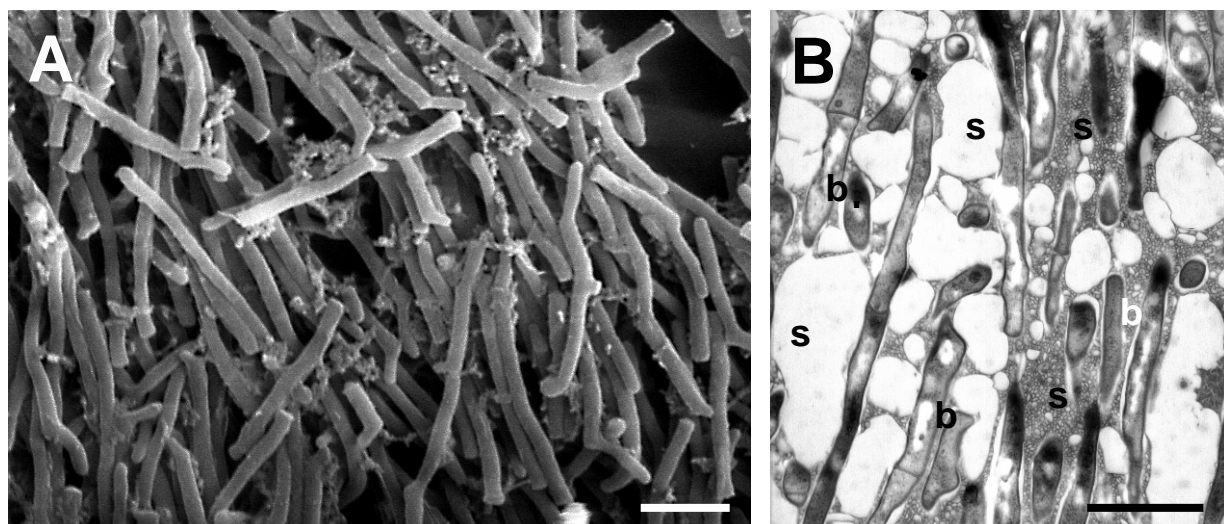


Fig. 2.5. Contents of the antennal gland reservoir. **A** SEM micrograph of the filamentous *Streptomyces* bacteria. Due to preparation the secretion between the bacteria is reduced to some flakes, scale bar 2 $\mu$ m. **B** TEM micrograph of the white substance inside the gland reservoir. The *Streptomyces* bacteria (b) are embedded in secretion droplets (s) of various sizes. Daughter cells remain connected after cell division and form long filaments, scale bar 2 $\mu$ m.

have to provide the symbiotic bacteria with essential nutrients to allow rapid growth and, thus, replenishment of the gland reservoir. 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. In the reservoir, the Streptomyces are embedded in a matrix of electron dense and electron lucent vesicles that may contain these nutrients. The content of the vesicles is most probably 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. The appearance of the vesicles in these class 3 cells as well as the content of the conducting canals suggest that the class 3 cells produce most of the material found in the gland reservoir. Additionally, substances may be sequestered from the hemolymph, as suggested by the invaginations of the cell membranes, stored in vesicles of the class 3 gland cells and transported into the reservoir.

Sequestration of substances from the hemolymph may also occur via the monolayered epithelium bordering the reservoir. 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 possessed only simple glands without a reservoir that produced only the primary secretion of either the epithelium or the class 3 gland units and delivered it as a directional cue for the cocoon alignment of the larvae (Strohm and 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 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 some benefits for the larvae by protecting them against fungal infestation (Kaltenpoth et al., 2005). Subsequently, natural selection could have changed the morphology of the antennal glands by forming large reservoirs that now function as cultivation organs for the bacterial partners.

A comparable relationship is known from fungus-growing ants which cultivate mutualistic *Pseudonocardia* bacteria on their body surface (Currie et al., 2006). Both, *Pseudonocardia* on ants' bodies and *Streptomyces* in beewolf antennae are members of the antibiotic-producing group Actinomycetes. The *Pseudonocardia* bacteria protect the ants' fungus gardens from a parasitic fungus, as *Streptomyces* protect the beewolf offspring from mould fungi. The *Pseudonocardia* are reared in cavities of the ants' cuticle and class 3 gland cells beneath the cuticle are connected via canals to the cavities (Currie et al., 2006). Therefore it seems that fungus-growing ants and beewolves use analogue alliances with bacteria to combat the fungal menace.

Using genetic analysis, endosymbiotic streptomycetes have recently been found in the antennae of 27 species of *Philanthus*, but not in closely related taxa 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.

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## 2.6 References

- Billen, J., Buschinger, A. (2000). Morphology and ultrastructure of a specialized bacterial pouch in the digestive tract of *Tetraponera* ants (Formicidae, Pseudomyrmecinae). *Arthropod Structure & Development* 29: 259-266.
- Bin, F., Vinson, S.B. (1986). Morphology of the antennal sex-gland in male *Trissolcus basalis* (Woll.) (Hymenoptera:Scelionidae), an egg parasitoid of the green stink bug, *Nezara viridula* (Hemiptera: Pentatomidae). *International Journal of Insect Morphology & Embryology* 15: 129-138.
- Bin, F., Isidoro, N., Romani, R. (1999a). Antennal structures of Hymenoptera: Sensilla or glands? *Atti dell'Accademia Nazionale Italiana di Entomologia Rendiconti, Anno XLVII - 1999*: 250-263.
- Bin, F., Wackers, F., Romani, R., Isidoro, N. (1999b). Tyloids in *Pimpla turionellae* (L.) are release structures of male antennal glands involved in courtship behaviour (Hymenoptera: Ichneumonidae). *International Journal of Insect Morphology & Embryology* 28 (1-2): 61-68.
- Böck, P. (1989). *Romeis Mikroskopische Technik*. Urban & Schwarzenberg, München, Wien, Baltimore.
- Bourtzis, K., Miller, T.A. (2003). *Insect Symbiosis*. CRC Press, Boca Raton.
- Brothers, D.J. (1999). Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). *Zoologica Scripta* 28 (1-2): 233-249.
- Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. Interscience Publishers, New York.
- Charnley, A.K., Hunt, J., Dillon, R.J. (1985). The germ-free culture of desert locusts, *Schistocerca gregaria*. *Journal of Insect Physiology* 31: 477-485.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398 (6729): 701-704.
- Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J., Billen, J. (2006). Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311 (28): 81-83.
- Dahms, E.C. (1984). An interpretation of the structure and function of the antennal sense organs of *Melittobia australica* (Hymenoptera: Eulophidae) with the discovery of a large dermal gland in the male scape. *Mem. Queensland Museum* 21: 361-377.
- Dillon, R.J., Vennard, C.T., Buckling, A., Charnley, A.K. (2005). Diversity of locust gut bacteria protects against pathogen invasion. *Ecology Letters* 8 (12): 1291-1298.
- Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* 43: 17-37.
- Guerrieri, E., Pedata, P.A., Romani, R., N., I., Bin, F. (2001). Functional anatomy of male antennal glands in three species of Encyrtidae (Hymenoptera: Chalcidoidea). *Journal of Natural History* 35 (1): 41-54.
- Herzner, G., Schmitt, T., Linsenmair, K.E., Strohm, E. (2003). Flagellar sensilla in male and female European beewolves, *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Entomologica Fennica* 14: 237-247.
- Isidoro, N., Bin, F. (1995). Male antennal gland of *amitus spiniferus* (Brethes) (Hymenoptera: Platygasteridae), likely involved in courtship behavior. *International Journal of Insect Morphology & Embryology* 24 (4): 365-373.
- Isidoro, N., Bin, F., Colazza, S., Vinson, B.S. (1996). Morphology of antennal gustatory sensilla and glands in some parasitoid hymenoptera with hypothesis on their role in sex and host recognition. *Journal of Hymenoptera Research* 5 (1996): 206-239.
- Isidoro, N., Bin, F., Romani, R., Pujade-Villar, J., Ros-Farre, P. (1999). Diversity and function of male antennal glands in Cynipoidea (Hymenoptera). *Zoologica Scripta* 28 (1-2): 165-174.
- Isidoro, N., Romani, R., Velasquez, D., Renthall, R., Bin, F., Vinson, S.B. (2000). Antennal glands in queen and worker of the fire ant, *Solenopsis invicta* Buren: first report in female social Aculeata (Hymenoptera, Formicidae). *Insectes Sociaux* 47 (3): 236-240.
- Kaltenpoth, M., Goettler, W., Herzner, G., Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479.
- 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: 1403-1411.
- Moran, N.A., Baumann, P. (2000). Bacterial endosymbionts in animals. *Current Opinion in Microbiology* 3: 270-275.
- Noirot, C., Quennedey, A. (1974). Fine-structure of insect epidermal glands. *Annual Review of Entomology* 19: 61-80.
- Paracer, S., Ahmadian, V. (2000). *Symbiosis: an introduction to biological associations*. Oxford University Press, Oxford, New York.
- Rathmayer, W. (1962). Paralysis caused by the digger wasp *Philanthus*. *Nature* 196: 1149-1151.

- Romani, R., Isidoro, N., Riolo, P., Bin, F. (2003). Antennal glands in male bees: structures for sexual communication by pheromones? *Apidologie* 34 (6): 603-610.
- Romani, R., Isidoro, N., Riolo, P., Bin, F., Fortunato, A., Turillazzi, S., Beani, L. (2005). A new role for antennation in paper wasps (Hymenoptera, Vespidae): antennal courtship and sex dimorphic glands in antennomeres. *Insectes Sociaux* 52: 96-102.
- Romani, R., Grasso, D.A., Mori, A., Isidoro, N., Le Moli, F. (2006). Antennal glands of the slave-making ant *Polyergus rufescens* and its slave species *Formica cunicularia* (Hymenoptera, Formicidae). *Canadian Journal of Zoology* 84: 1-5.
- Snodgrass, R.E. (1935). Principles of insect morphology. McGraw-Hill, New York, London.
- Sørensen, S. (1909). The measurement of the hydrogen ion concentration and its importance for enzymatic process. *Biochemische Zeitschrift* 21: 131-304.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (1997). Female size affects provisioning and sex allocation in a digger wasp. *Animal Behaviour* 54: 23-34.
- Takatsuka, J., Kunimi, Y. (2000). Intestinal bacteria affect growth of *Bacillus thuringiensis* in larvae of the oriental tea tortrix, *Homona magnanima* Diakonoff (Lepidoptera: Tortricidae). *Journal of Invertebrate Pathology* 76 (3): 222-226.



## CHAPTER 3

**SYMBIOTIC BACTERIA PROTECT WASP LARVAE  
FROM FUNGAL INFESTATION**

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**3.1 Summary**

Symbiotic associations between different organisms are of great importance for evolutionary and ecological processes (Buchner, 1921; Maynard-Smith, 1989; Margulis and 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 and 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. Behavioural 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*.



### 3.2 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 and 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 and 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.

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 and Linsenmair, 1995). The white substance is thus pressed out of specialized antennal glands and appears as white particles on the antennae [Fig. 3.1, see supplemental video 1 of the online publication]. 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 and 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  $\mu\text{m}$  [Fig. 3.1B]. 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. 3.2].

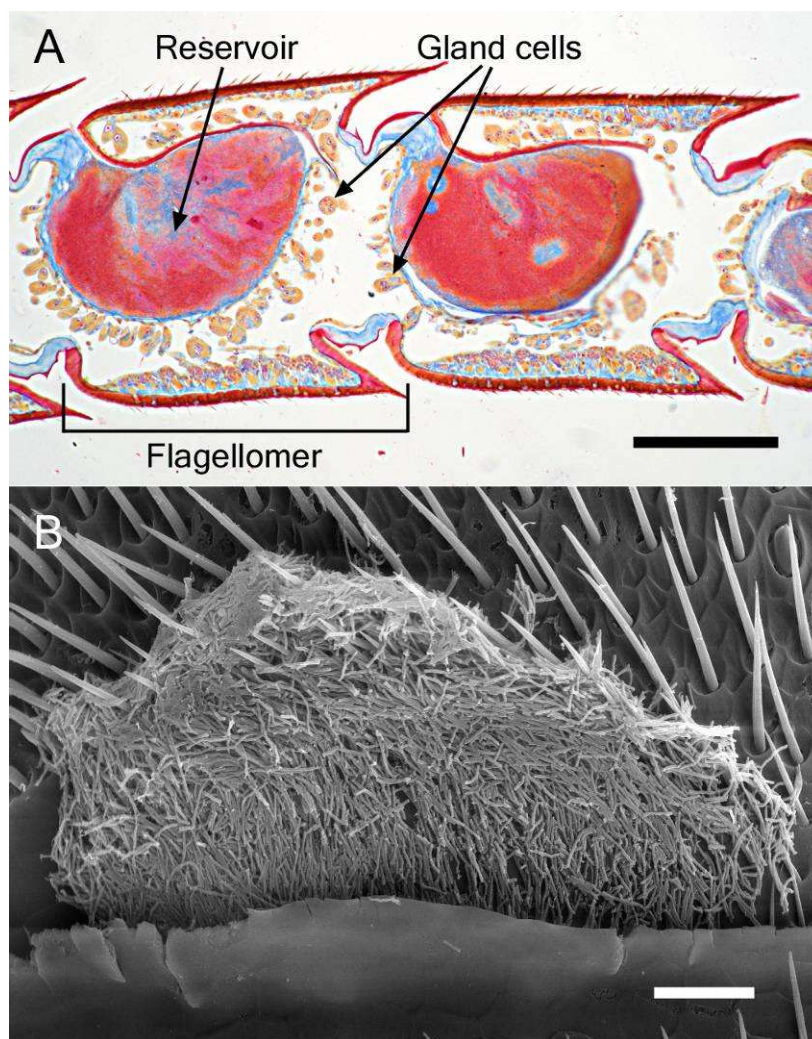


Fig. 3.1. Antennae of female beewolves, *Philanthus triangulum*, with endosymbiotic streptomycetes. **A** Light microscopic picture of a semi-thin section of a female antenna, with endosymbiotic bacteria (red) in the reservoir of antennal glands. Scale bar = 0.3 mm. **B** Scanning electron micrograph of a female antenna. White substance is secreted from the opening of the gland at the joint between two flagellomers. Scale bar = 20  $\mu$ m.

The new type is most closely related to the *S. armeniacus* group (*S. griseocarneau*, *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. 3.3] as well as

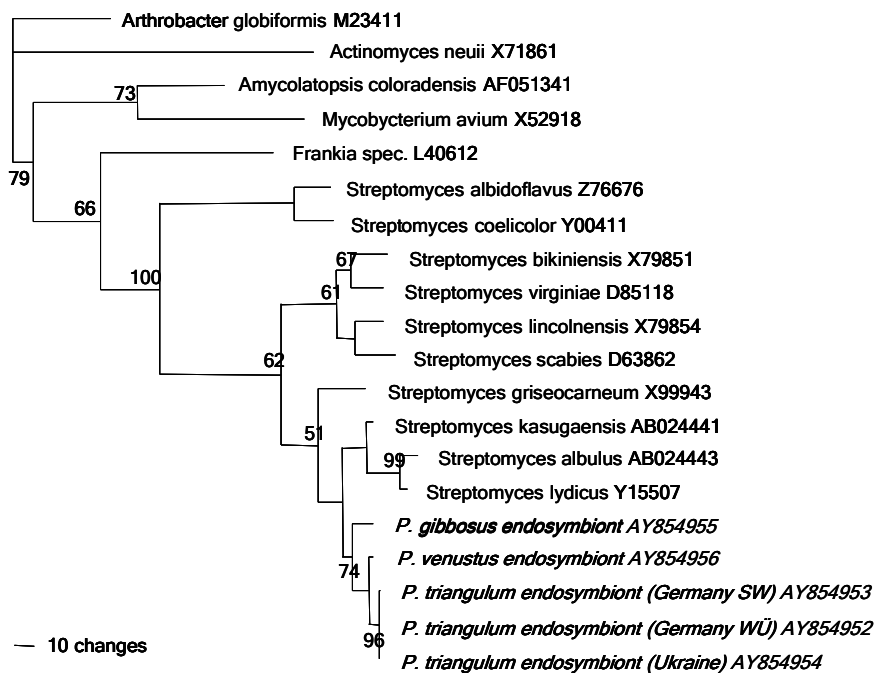


Fig. 3.2. Phylogenetic position of beewolf endosymbionts within the actinomycetes: First of three equally parsimonious trees from a full heuristic search with tree bisection and reconnection (TBR) branch swapping and random addition sequence (100 replicates). Analysis is based on 1324 bp of 16S rDNA, 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.

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.

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 and 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

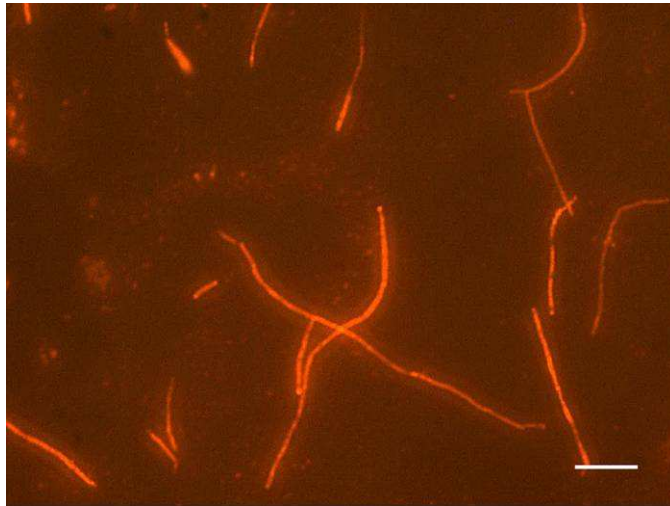


Fig. 3.3. Fluorescence in-situ hybridization (FISH) of endosymbiotic *Streptomyces* in the white substance after secretion by a beewolf female. Scale bar = 5  $\mu$ m.

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).

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. 3.4]. 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).

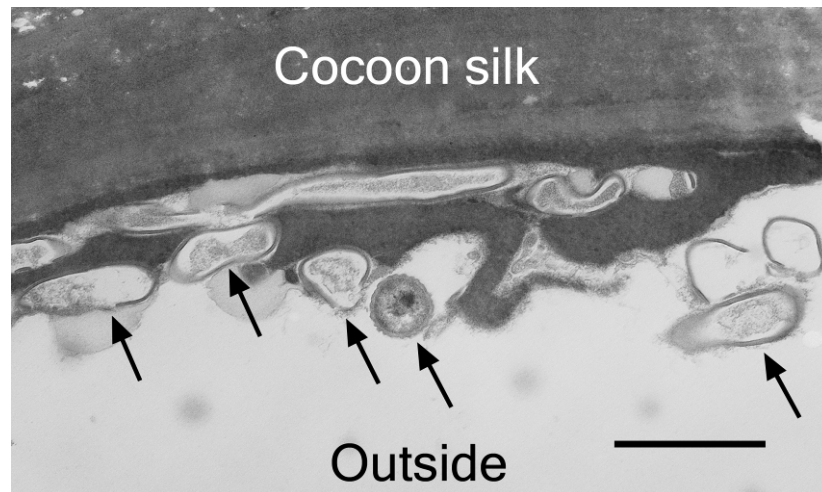


Fig. 3.4. Transmission electron micrograph of a cocoon cross-section with bacteria on the outside (arrows). Scale bar = 1  $\mu\text{m}$ .

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. 3.5, 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 and 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 (see supplemental video 2 of the online publication). 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.

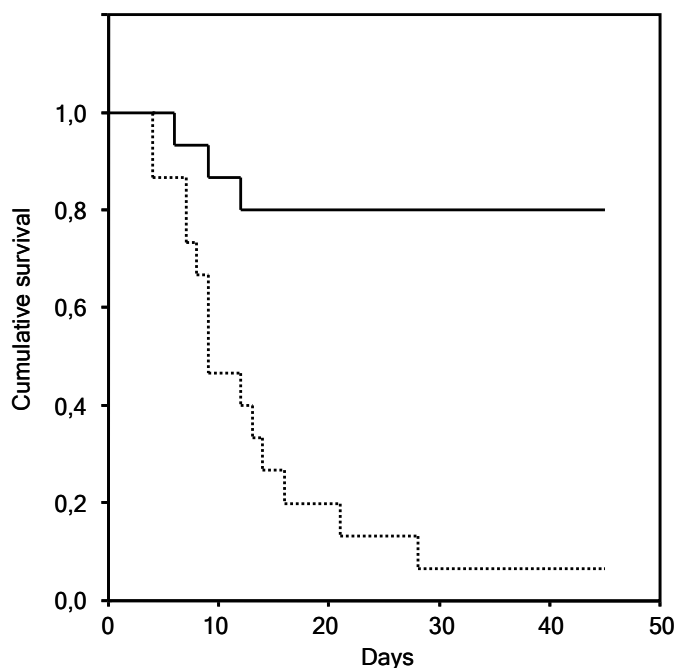


Fig: 3.5. Cumulative survival of larvae with (solid line) and without white substance (dotted line) in the brood cell. The experiment was terminated after 45 days.

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. 3.2]. 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.

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 behaviour. 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.

### 3.3 Methods

#### 3.3.1 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® Mastercylers 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 MgCl<sub>2</sub>, 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.

#### 3.3.2 Phylogenetic analysis

Partial 16S rDNA sequences of the endosymbionts and representative actinomycete genera from the GenBank database (accession numbers are given in Fig. 3.2) 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.

#### 3.3.3 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.

#### *3.3.4 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).

#### *3.3.5 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.

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*Accession numbers*

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.

### 3.4 References

- Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A. (1990). Combination of 16S ribosomal RNA targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* 56 (6): 1919-1925.
- Behal, V. (2000). Bioactive products from *Streptomyces*. Pages 113-156 in Neidleman, S., Laskin, A., Bennett, J., Gadd, G., eds. *Advances in applied microbiology. Volume 47*. Academic Press, New York.
- Buchner, P. (1921). Tier und Pflanze in intrazellulärer Symbiose. Verlag Gebrüder Borntraeger, Berlin.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398 (6729): 701-704.
- Currie, C.R. (2001). A community of ants, fungi, and bacteria: A multilateral approach to studying symbiosis. *Annual Review of Entomology* 55: 357-380.
- Currie, C.R., Bot, A.N.M., Boomsma, J.J. (2003). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101 (1): 91-102.
- Goodfellow, M., Cross, T. (1984). The biology of actinomycetes. Academic Press, London.
- Grimm, D., Merkert, H., Ludwig, W., Schleifer, K.H., Hacker, J., Brand, B.C. (1998). Specific detection of *Legionella pneumophila*: Construction of a new 16S rRNA-targeted oligonucleotide probe. *Applied and Environmental Microbiology* 64: 2686-2690.
- Kutzner, H.J. (1981). The family Streptomycetaceae. Pages 2028-2090 in Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G., eds. *The Prokaryotes*. Springer, Berlin.
- Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., Tiedje, J.M. (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Research* 29 (1): 173-174.
- Margulis, L., Fester, R. (1991). Symbiosis as a source of evolutionary innovation: speciation and morphogenesis. MIT Press, Cambridge.
- Moran, N.A., Baumann, P. (2000). Bacterial endosymbionts in animals. *Current Opinion in Microbiology* 3: 270-275.
- Pernodet, J.L., Boccard, F., Alegre, M.T., Gagnat, J., Guerineau, M. (1989). Organization and nucleotide sequence analysis of a ribosomal RNA gene cluster from *Streptomyces ambofaciens*. *Gene* 79 (1): 33-46.
- Poulsen, M., Bot, A.N.M., Currie, C.R., Nielsen, M.G., Boomsma, J.J. (2003). Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. *Functional Ecology* 17: 260-269.
- Rintala, H., Nevalainen, A., Ronka, E., Suutari, M. (2001). PCR primers targeting the 16S rRNA gene for the specific detection of streptomycetes. *Molecular & Cellular Probes*.
- Sapp, J. (1994). Evolution by association: a history of symbiosis. Oxford University Press, New York.
- Sauer, C., Dudaczek, D., Hölldobler, B., Gross, R. (2002). Tissue localization of the endosymbiotic bacterium '*Candidatus Blochmannia floridanus*' in adults and larvae of the carpenter ant *Camponotus floridanus*. *Applied and Environmental Microbiology* 68 (9): 4187-4193.
- Stach, J.E.M., Maldonado, L.A., Ward, A.C., Goodfellow, M., Bull, A.T. (2003). New primers for the class Actinobacteria: application to marine and terrestrial environments. *Environmental Microbiology* 5 (10): 828-841.
- Strohm, E. (1995). Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). *Wissenschaftliche Schriftenreihe Biologie* 6.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (1999). Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behavioral Ecology and Sociobiology* 47: 76-88.
- Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991). 16s ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173 (2): 697-703.



## CHAPTER 4

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

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#### 4.1 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 streptomyces 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.

## 4.2 Introduction

Many insects have evolved associations with endosymbiotic bacteria that are essential for reproduction or survival of the host (Moran and Baumann, 1994). Most of these bacteria are intracellular symbionts in specialist feeders, e.g. phloem-feeding, blood-sucking, or wood-feeding insects (Baumann and Moran, 1997; Priest and 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 and 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 and Linsenmair, 1995). After feeding on the provisioned prey, larvae spin a cocoon in which they usually overwinter and emerge the following summer (Strohm and 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 and 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 and 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 and 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 and 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 and 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.

### 4.3 Methods

#### 4.3.1 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 4.1]. 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 and Grissell (1975) and Ferguson (1983a,b). Because males lack the relevant glands (Strohm and 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.

#### 4.3.2 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<sub>4</sub> 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.

#### 4.3.3 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 Mastercylers in a total reaction volume of 25  $\mu$ l [containing 2  $\mu$ l of template, 1x PCR buffer (10 mM Tris-HCl, pH 8.8; 50 mM KCl; 0.08% NP-40), 2.5 mM MgCl<sub>2</sub>, 240  $\mu$ M 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<sup>2+</sup> 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<sub>2</sub> was used for Strep\_phil\_fwd4/Act-A19. The annealing temperature was set to 65°C for Strep\_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 4.1] were used as templates. Extracted DNA from cultures of *Streptomyces rimosus* DSM 40260<sup>T</sup>, *S. aureofaciens* DSM 40631, and *S. venezuelae* DSM 40230<sup>T</sup> was included to assess the specificity of the primers for *Philanthus* endosymbiont DNA.

#### 4.3.4 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<sup>T</sup>, *S. rimosus* DSM 40260<sup>T</sup> 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.

#### 4.3.5 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<sup>T</sup> was defined as the outgroup. Using the same settings, bootstrap values were obtained from a search with 1000 replicates.

## 4.4 Results

### 4.4.1 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. 4.1]. 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.

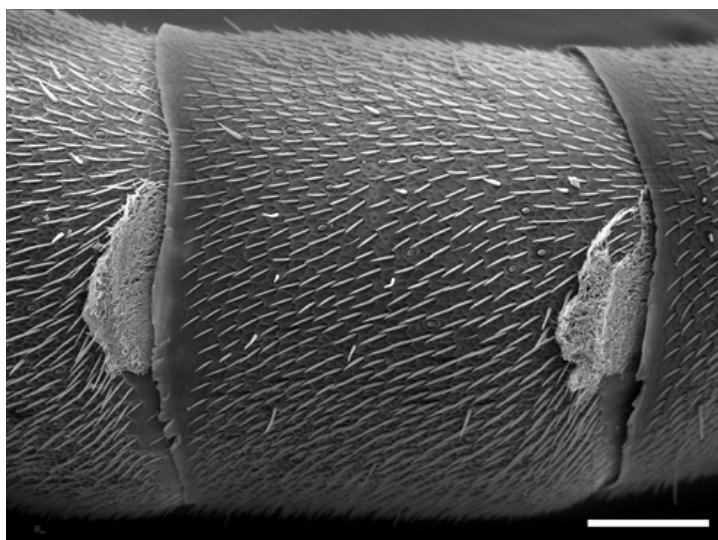


Fig. 4.1. SEM image of an antenna of a female European beewolf (*P. triangulum*) with symbiotic *Streptomyces* bacteria being secreted from the antennal glands. Scale bar = 100  $\mu\text{m}$ .



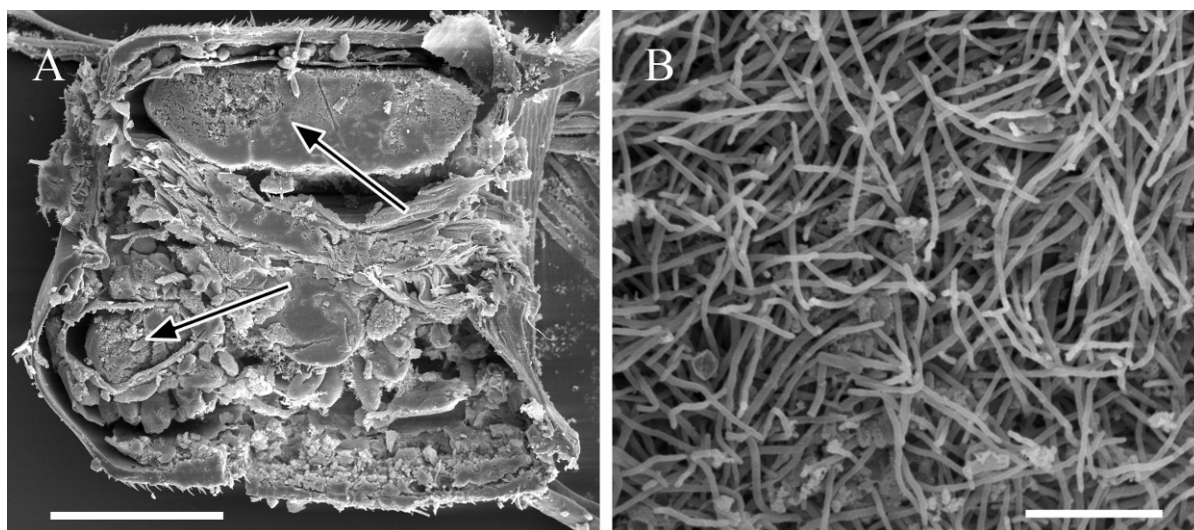


Fig. 4.2. SEM image of the interior of an antennal segment of a female *P. loefflingi*. (A) Longitudinal section of a flagellomer. The reservoir of the antennal gland is indicated by arrows. (B) Symbiotic *Streptomyces* bacteria forming a dense cluster within the antennal gland. Scale bars = 200  $\mu\text{m}$  (A) and 10  $\mu\text{m}$  (B).

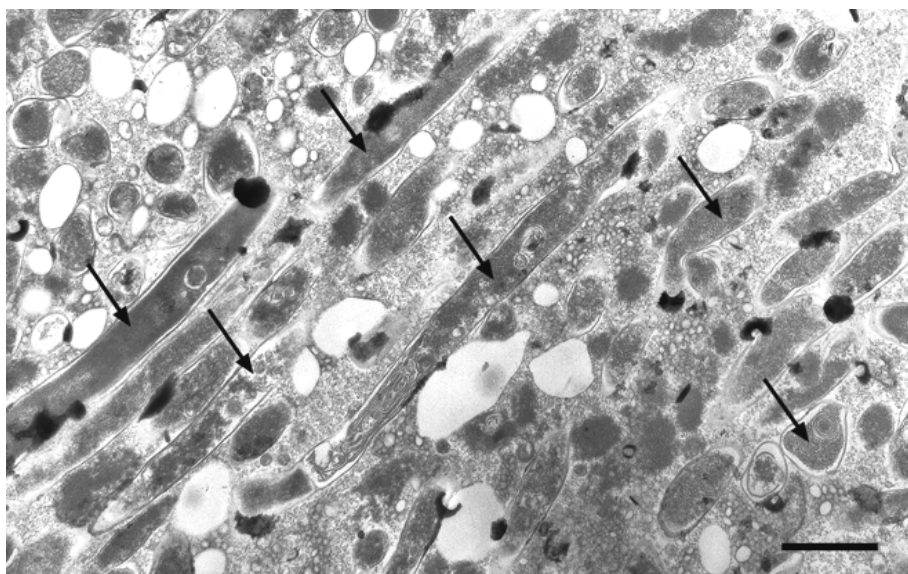


Fig. 4.3 TEM image of a cross-section through the antennal gland of a female *P. triangulum*. Some endosymbiotic *Streptomyces* are indicated by arrows. Scale bar = 1  $\mu\text{m}$ .

When a flagellomer was cut open, filamentous bacteria were clearly visible in large numbers within the gland reservoir [Fig. 4.2A], where they formed a dense cluster of cells [Fig. 4.2B]. 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. 4.3]. 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  $\mu\text{m}$  wide and highly variable in length (5 – 20  $\mu\text{m}$ ). 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. 4.4].

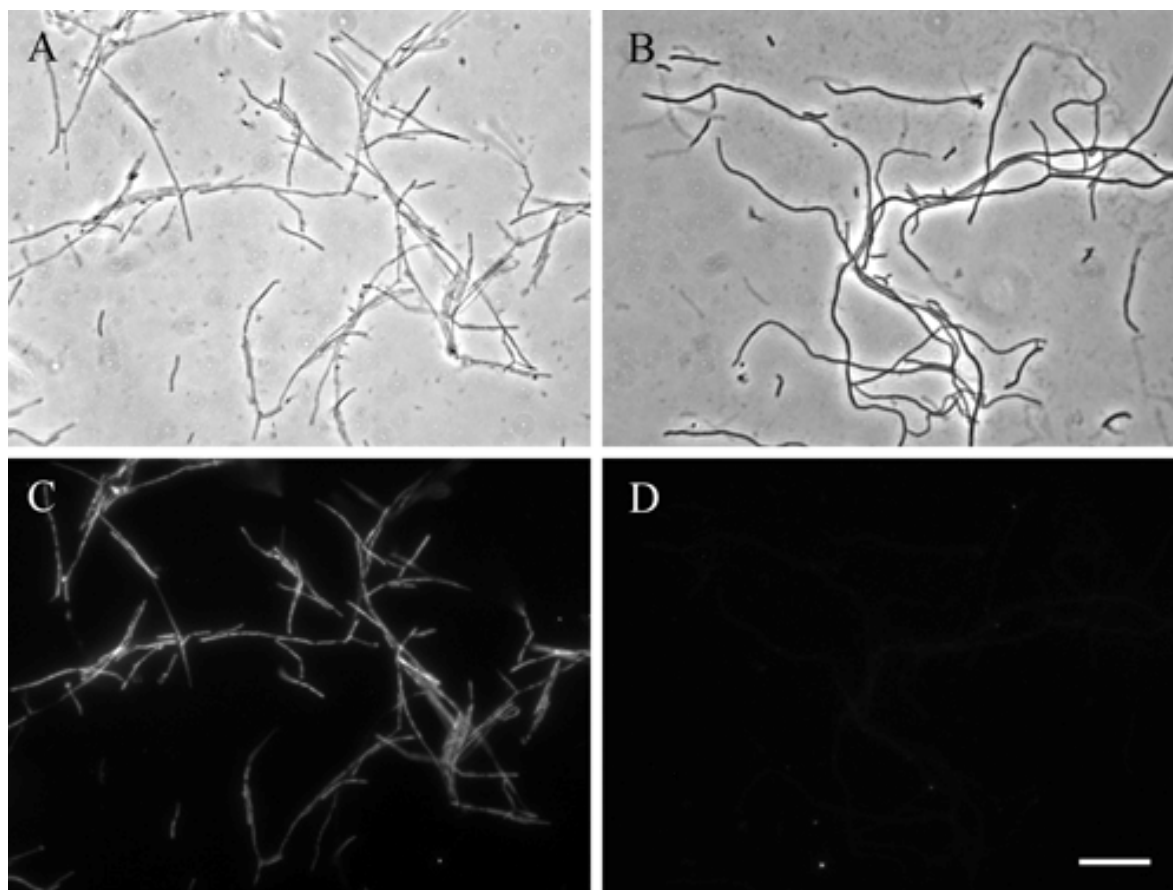


Fig. 4.4. FISH of antennal *Streptomyces* endosymbionts. Phase-contrast micrograph of symbiotic bacteria in the antennal gland secretion of a female beewolf (A) and of a negative control strain of *Streptomyces rimosus* DSM 40260<sup>T</sup> (B). (C, D) Epifluorescence micrographs of the same areas after staining with the specific Cy3-labeled probe SPT177. Scale bar = 10  $\mu$ m.

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. 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. 4.4]. 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.

#### 4.4.2 Distribution of symbionts among philanthine wasps

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 4.1]. 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.

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

Table 4.1. 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.

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.

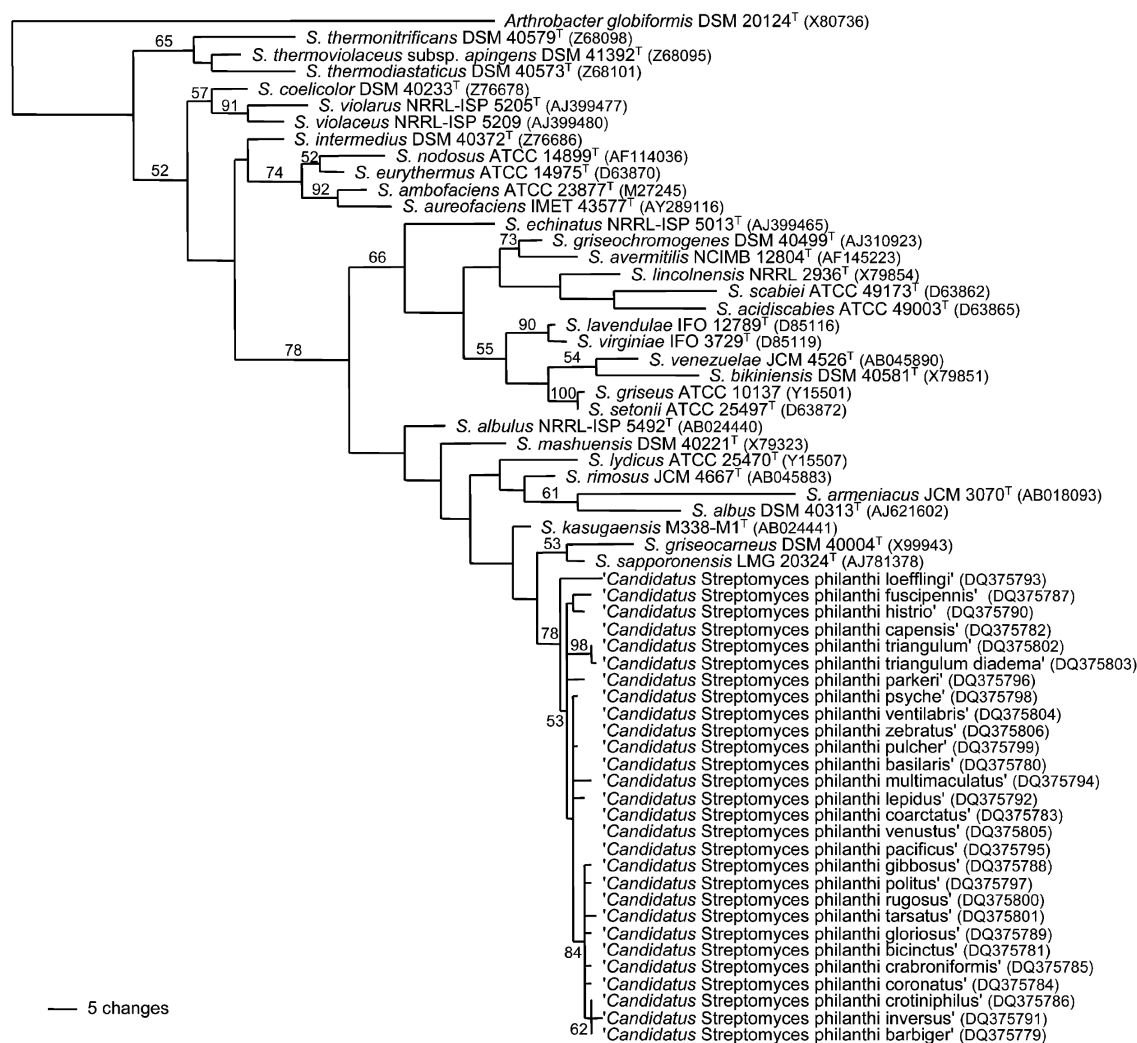


Fig. 4.5. Phylogenetic position of *Philanthus* endosymbionts within the genus *Streptomyces* based on 1324 bp of 16S rRNA gene sequence (104 parsimony-informative characters). First of 600 equally parsimonious trees from a full heuristic search with random addition sequence (10 replicates) and TBR branch swapping. *Arthrobacter globiformis* was defined as the outgroup. Bootstrap values at nodes are percentages of 1000 replicates. GenBank accession numbers are given in parentheses. Scale bar = 5 changes.

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<sup>T</sup>, a close relative of the *Philanthus* symbionts [Fig. 4.5] 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.

#### 4.4.3 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. 4.5]. 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. 4.5 and Table 4.1.

## 4.5 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 and Moran, 1997; Moran and 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 online supplementary data, chapter 4.7).

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 and 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 and 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 and 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 and Ruby, 1991; Nyholm et al., 2000; Nishiguchi, 2002; Nyholm and 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 and 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 and 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.

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#### **4.6 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 as supplementary material in IJSEM Online. 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.

#### 4.7 Online supplementary data

##### *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 and 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*.

*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 <sub>2</sub> HPO <sub>4</sub>	0.7 g
KH <sub>2</sub> PO <sub>4</sub>	0.3 g
MgSO <sub>4</sub> • 5 H <sub>2</sub> O	0.5 g
FeSO <sub>4</sub> • 7 H <sub>2</sub> O	0.01 g
ZnSO <sub>4</sub>	0.001 g
MnCl <sub>2</sub>	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 <sub>3</sub>	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 <sub>2</sub>	0.15 g
MgCl <sub>2</sub>	0.05 g
KCl	0.2 g
NaHPO <sub>4</sub>	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.

## 4.8 References

- Aksoy, S., Chen, X., Hypsa, V. (1997). Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). *Insect Molecular Biology* 6 (2): 183-190.
- Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A. (1990). Combination of 16S ribosomal RNA targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology* 56 (6): 1919-1925.
- Bandi, C., Damiani, G., Magrassi, L., Grigolo, A., Fani, R., Sacchi, L. (1994). Flavobacteria as intracellular symbionts in cockroaches. *Proceedings of the Royal Society of London Series B* 257 (1348): 43-48.
- Bandi, C., Sironi, M., Damiani, G., Magrassi, L., Nalepa, C.A., Laudani, U., Sacchi, L. (1995). The establishment of intracellular symbiosis in an ancestor of cockroaches and termites. *Proceedings: Biological Sciences* 259: 293-299.
- Baumann, P., Moran, N.A. (1997). Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Antonie van Leeuwenhoek* 72: 39-48.
- Baumann, P., Moran, N.A., Baumann, L. (1997). The evolution and genetics of aphid endosymbionts. *Bioscience* 47 (1): 12-20.
- Bohart, R.M., Grissell, E.E. (1975). California wasps of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Bulletin of the California Insect Survey* 19: 1-57.
- Bourtzis, K., Miller, T.A. (2003). *Insect Symbiosis*. CRC Press, Boca Raton.
- Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. Interscience Publishers, New York.
- Cafaro, M.J., Currie, C.R. (2005). Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Canadian Journal of Microbiology* 51 (6): 441-446.
- Chen, X.O., Li, C.B., Zhao, S.Y., Aksoy, S. (1999). Phylogeny of the symbionts of tsetse and the evolutionary relationships with their hosts. *Prog. Nat. Sci.* 9 (12): 922-928.
- Clark, J.W., Hossain, S., Burnside, C.A., Kambhampati, S. (2001). Coevolution between a cockroach and its bacterial endosymbiont: a biogeographical perspective. *Proceedings of the Royal Society of London Series B* 268 (1465): 393-398.
- Clark, M.A., Moran, N.A., Baumann, P., Wernegreen, J.J. (2000). Cospeciation between bacterial endosymbionts (*Buchnera*) and a recent radiation of aphids (*Uroleucon*) and pitfalls of testing for phylogenetic congruence. *Evolution* 54 (2): 517-525.
- Cohan, F.M. (2002). What are bacterial species? *Annual Review of Microbiology* 56: 457-487.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398 (6729): 701-704.
- Currie, C.R., Bot, A.N.M., Boomsma, J.J. (2003). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101 (1): 91-102.
- Dale, C., Jones, T.M., Pontes, M. (2005). Degenerative evolution and functional diversification of type-III secretion systems in the insect endosymbiont *Sodalis glossinidius*. *Molecular Biology and Evolution* 22 (3): 758-766.
- Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* 43: 17-37.
- Ferguson, G.R. (1983a). Two new species in the genus *Philanthus* and a key to the *politus* group (Hymenoptera, Philanthidae). *Pan-Pacific Entomologist* 59: 55-63.
- Ferguson, G.R. (1983b). Revision of the *Philanthus zebratus* group (Hymenoptera, Philanthidae). *Journal of the New York Entomological Society* 91 (4): 289-303.
- Grimm, D., Merkert, H., Ludwig, W., Schleifer, K.H., Hacker, J., Brand, B.C. (1998). Specific detection of *Legionella pneumophila*: Construction of a new 16S rRNA-targeted oligonucleotide probe. *Applied and Environmental Microbiology* 64: 2686-2690.
- Hsu, S.C., Lockwood, J.L. (1975). Powdered chitin agar as a selective medium for enumeration of actinomycetes in water and soil. *Applied Microbiology* 29 (3): 422-426.
- Hugenholtz, P., Pitulle, C., Hershberger, K.L., Pace, N.R. (1998). Novel division level bacterial diversity in a Yellowstone hot spring. *Journal of Bacteriology* 180 (2): 366-376.
- Ishikawa, H. (2003). Insect symbiosis: an introduction. in Bourtzis, K., Miller, T.A., eds. *Insect symbiosis*. CRC Press, Boca Raton, Florida.
- Kaltenpoth, M., Goettler, W., Herzner, G., Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479.
- Konstantinidis, K.T., Tiedje, J.M. (2005). Genomic insights that advance the species definition for prokaryotes. *PNAS* 102 (7): 2567-2572.

- Liu, Z.H., Shi, Y.L., Zhang, Y.M., Zhou, Z.H., Lui, Z.T., Li, W., Huang, Y., Rodriguez, C., Goodfellow, M. (2005). Classification of *Streptomyces griseus* (Krainsky 1914) Waksman and Henrici 1948 and related species and the transfer of '*Microstreptospora cinerea*' to the genus *Streptomyces* as *Streptomyces yanii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 55: 1605-1610.
- Lo, N., Bandi, C., Watanabe, H., Nalepa, C.A., Beninati, T. (2003). Evidence for cocladogenesis between diverse dictyopteran lineages and their intracellular endosymbionts. *Molecular Biology and Evolution* 20 (6): 907-913.
- Maidak, B.L., Cole, J.R., Lilburn, T.G., Parker, C.T., Saxman, P.R., Farris, R.J., Garrity, G.M., Olsen, G.J., Schmidt, T.M., Tiedje, J.M. (2001). The RDP-II (Ribosomal Database Project). *Nucleic Acids Research* 29 (1): 173-174.
- Manfio, G.P., Atalan, E., Zakrzewska-Czerwinska, J., Mordarski, M., Rodriguez, C., Collins, M.D., Goodfellow, M. (2003). Classification of novel soil streptomycetes as *Streptomyces aureus* sp. nov., *Streptomyces laceyi* sp. nov. and *Streptomyces sanglieri* sp. nov. *Antonie van Leeuwenhoek* 83 (3): 245-255.
- McFall-Ngai, M.J., Ruby, E.G. (1991). Symbiont recognition and subsequent morphogenesis as early events in an animal-bacterial mutualism. *Science* 254 (5037): 1491-1493.
- Moran, N.A., Munson, M.A., Baumann, P., Ishikawa, H. (1993). A molecular clock in endosymbiotic bacteria is calibrated using the insect hosts. *Proceedings: Biological Sciences* 253: 167-171.
- Moran, N.A., Baumann, P. (1994). Phylogenetics of cytoplasmically inherited microorganisms of arthropods. *Trends in Ecology and Evolution* 9 (1): 15-20.
- Moran, N.A., Telang, A. (1998). Bacteriocyte-associated symbionts of insects - A variety of insect groups harbor ancient prokaryotic endosymbionts. *Bioscience* 48 (4): 295-304.
- Moran, N.A., Baumann, P. (2000). Bacterial endosymbionts in animals. *Current Opinion in Microbiology* 3: 270-275.
- Nishiguchi, M.K. (2002). Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. *Microbial Ecology* 44 (1): 10-18.
- Nyholm, S.V., Stabb, E.V., Ruby, E.G., McFall-Ngai, M.J. (2000). Establishment of an animal-bacterial association: Recruiting symbiotic vibrios from the environment. *PNAS* 97 (18): 10231-10235.
- Nyholm, S.V., McFall-Ngai, M.J. (2004). The winnowing: establishing the squid-*Vibrio* symbiosis. *Nature Reviews Microbiology* 2 (8): 632-642.
- Ochman, H., Wilson, A.C. (1987). Evolution in bacteria: Evidence for a universal substitution rate in cellular genomes. *Journal of Molecular Evolution* 26: 74-86.
- Pernodet, J.L., Boccard, F., Alegre, M.T., Gagnat, J., Guerinéau, M. (1989). Organization and nucleotide sequence analysis of a ribosomal RNA gene cluster from *Streptomyces ambofaciens*. *Gene* 79 (1): 33-46.
- Priest, F.G., Dewar, S.J. (2000). Bacteria and insects. Pages 165-202 in Priest, F.G., Goodfellow, M., eds. *Applied microbial systematics*. Kluwer Academic Publications, Dordrecht.
- Rintala, H., Nevalainen, A., Ronka, E., Suutari, M. (2001). PCR primers targeting the 16S rRNA gene for the specific detection of streptomycetes. *Molecular & Cellular Probes*.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, New York.
- Sauer, C., Stackebrandt, E., Gadau, J., Hölldobler, B., Gross, R. (2000). Systematic relationships and cospeciation of bacterial endosymbionts and their carpenter ant host species: proposal of the new taxon *Candidatus Blochmannia* gen. nov. *International Journal of Systematic and Evolutionary Microbiology* 50: 1877-1886.
- Sauer, C., Dudaczek, D., Hölldobler, B., Gross, R. (2002). Tissue localization of the endosymbiotic bacterium '*Candidatus Blochmannia floridanus*' in adults and larvae of the carpenter ant *Camponotus floridanus*. *Applied and Environmental Microbiology* 68 (9): 4187-4193.
- Semiring, L., Ward, A.C., Goodfellow, M. (2000). Selective isolation and characterisation of members of the *Streptomyces violaceusniger* clade associated with the roots of *Paraserianthes falcataria*. *Antonie van Leeuwenhoek* 78: 353-366.
- Stach, J.E.M., Maldonado, L.A., Ward, A.C., Goodfellow, M., Bull, A.T. (2003). New primers for the class Actinobacteria: application to marine and terrestrial environments. *Environmental Microbiology* 5 (10): 828-841.
- Strohm, E. (1995). Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). *Wissenschaftliche Schriftenreihe Biologie* 6.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (2001). Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 26 (2): 198-203.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., Lane, D.J. (1991). 16s ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* 173 (2): 697-703.

## CHAPTER 5

**POPULATION DYNAMICS OF A PROTECTIVE INSECT SYMBIONT REVEAL  
SEVERE BOTTLENECKS DURING VERTICAL TRANSMISSION**

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**5.1 Summary**

Insects engage in mutualistic relationships with a wide variety of microorganisms that they usually transmit vertically from one generation to the next. Many of the obligate symbionts are transmitted transovarially, but post-hatch symbiont transmission via egg-smearing, coprophagy, or symbiont-capsules has also been described, especially in Heteroptera. Vertical symbiont transmission usually involves significant bottlenecks that may entail major genetic and genomic consequences for symbiotic organisms. Here we investigated the severity of transmission bottlenecks in a symbiotic system with an unusual way of post-hatch vertical transmission: European beewolves (*Philanthus triangulum*, Hymenoptera, Crabronidae) secrete symbiotic bacteria ('*Candidatus* *Streptomyces philanthi*') from specialized antennal gland reservoirs into their brood cells. The beewolf larva incorporates the symbionts into the cocoon material, from where they are presumably much later taken up by the adult beewolf. We reconstructed the development of the symbiont-containing antennal gland reservoirs of female European beewolves and the growth of the symbionts before and after emergence from the cocoon. The results provide evidence for an uptake of the bacteria during emergence. The bacterial growth follows a logistic growth curve, with a maximum specific growth rate of about 0.101 to 0.111 h<sup>-1</sup>, which lies within the lower range of free-living relatives. With a reduction in cell numbers of about 2.5x10<sup>-5</sup> to 2.5x10<sup>-6</sup>, the symbiont population experiences one of the most severe transmission bottlenecks known for any symbiotic system to date. This extreme bottleneck may have significantly affected the evolution of the beewolf-*Streptomyces* symbiosis by increased genetic drift, an accumulation of mildly deleterious mutations and genome erosion and streamlining.

## 5.2 Introduction

Many insect taxa engage in intimate symbiotic associations with bacteria that reside in the digestive tract or in specialized cells or organs of the host (Buchner, 1965; Bourtzis and Miller, 2003). While some facultative symbionts (e.g. *Wolbachia*) cause reproductive alterations in the insect host or confer other negative effects, most obligate and several facultative symbioses are of mutualistic nature and significantly enhance the fitness of the host (Douglas, 1998; Bourtzis and Miller, 2003). In the majority of these cases, the symbionts provide their hosts with limiting essential nutrients that the insects can neither synthesize themselves nor obtain in sufficient quantities from the diet (Douglas, 1998; Shigenobu et al., 2000; Gil et al., 2003; Ishikawa, 2003; Zientz et al., 2004; Douglas, 2006). However, several cases of defensive alliances have recently been described in which the symbionts confer protection to their hosts against pathogens or parasitoids (Currie et al., 1999; Oliver et al., 2003; Kaltenpoth et al., 2005).

Although environmental uptake of symbionts in each host generation has recently been reported for a mutualistic insect symbiont (Kikuchi et al., 2007), vertical transmission from mother to offspring appears to be the predominant mode of symbiont transfer in insects (e.g. Buchner, 1965; Baumann and Moran, 1997; Bourtzis and Miller, 2003). However, the routes of vertical transmission differ among symbiotic systems: The intracellular primary symbionts of aphids, carpenter ants, tse-tse flies, weevils and several other insect taxa are generally unable to survive outside of the hosts' cells and have to be transmitted transovarially to the eggs (Buchner, 1965; Schröder et al., 1996; Douglas, 1998; Sauer et al., 2002; Nardon, 2006) or via the milk glands to the developing larvae, as it seems to be the case in the pupiparous tse-tse flies (Buchner, 1965; Aksoy et al., 1997). Many heteropteran species, however, have evolved posthatch transmission mechanisms to pass extracellular gut symbionts on to their offspring. In these cases, the symbionts are transmitted via egg smearing, coprophagy, or the deposition of specialized symbiont-containing capsules (Buchner, 1965; Fukatsu and Hosokawa, 2002; Hosokawa et al., 2005; Prado et al., 2006).

Regardless of the route of vertical transmission, the number of symbiont cells transmitted to the next generation is usually substantially lower than the population size of the symbionts within adult insects (Buchner, 1965; Mira and Moran, 2002; Sauer et al., 2002; Hosokawa et al., 2007). Thus, the symbiont populations experience significant bottlenecks with each transmission event, and the severity of the bottlenecks is expected to have important consequences for the evolutionary genetics of the symbionts (Rispe and Moran, 2000; Mira and Moran, 2002). Theoretical considerations as well as empirical evidence suggest that narrow bottlenecks in combination with the effective lack of recombination in strictly vertically transmitted symbionts lead to increased genetic drift, an accumulation of mildly deleterious mutations, faster sequence evolution, and a shift in base composition due to mutational bias in symbiont lineages (Lynch and Gabriel, 1990; Moran, 1996;

Rispe and Moran, 2000; Degnan et al., 2004; Gil et al., 2004). Earlier studies revealed substantial differences in the severity of bottlenecks experienced by symbiont populations, ranging from  $3 \times 10^{-1}$  to about  $5 \times 10^{-5}$  (Nardon and Grenier, 1988; Mira and Moran, 2002; Anbutsu and Fukatsu, 2003; Hosokawa et al., 2007).

Beewolves of the genus *Philanthus* (Hymenoptera, Crabronidae) are solitary digger wasps that engage in a highly specialized symbiotic association with high-GC gram-positive bacteria of the order Actinomycetales (*Candidatus Streptomyces philanthi*) that protect the wasp offspring against pathogenic microorganisms (Kaltenpoth et al., 2005; Kaltenpoth et al., 2006; Goettler et al., 2007). The symbionts are cultivated in specialized antennal gland reservoirs that constitute invaginations of the outer antennal cuticle and are present in five antennal segments (Goettler et al., 2007). The reservoirs are in contact with numerous gland cell units and sealed with a membranous flap mechanism (for a detailed gland morphology see Goettler et al., 2007). Reservoirs are very flexible and the filling status varies within a broad range depending on the amount of bacteria (Goettler, Kaltenpoth, Strohm, unpubl. data).

The antennal symbionts are transmitted vertically by an unusual mechanism of posthatch transfer (Kaltenpoth et al., 2005). Female beewolves catch and paralyze other hymenoptera and supply them as larval provisions into subterranean brood cells. Each brood cell is supplied with the symbiotic bacteria from the antennal gland reservoirs (Strohm and Linsenmair, 1995; Kaltenpoth et al., 2005; Goettler et al., 2007). Later, the larva transfers the symbionts to its cocoon, where they provide protection against detrimental fungi (Kaltenpoth et al., 2005). Additionally, the antennal gland secretion serves a second purpose by providing a directional cue to the larva during cocoon spinning that is necessary for successful adult emergence (Strohm and Linsenmair, 1995). Although the exact route of vertical transmission is not yet known, preliminary studies suggest that the symbionts are taken up by adult females from the cocoon during emergence (Kaltenpoth, unpubl. data).

Here we studied the ontogeny of the symbiont-containing antennal gland reservoir of female European beewolves (*Philanthus triangulum*) before and after emergence from the cocoon, and we measured the growth of the symbiont population in newly emerged beewolves. The results do not only allow us to assess the growth rate of this symbiotic actinomycete in comparison to free-living relatives, but they also shed light on the severity of the bottleneck that the symbiont population is likely to experience during each transmission event.



### 5.3 Materials and methods

#### 5.3.1 *Beewolf specimens*

Cocoons with female larvae were obtained from a laboratory culture of the European beewolf (*P. triangulum*). After hibernation six to nine months at 10°C, they were transferred to room temperature to induce further development. Pupation occurred after three to four weeks. About two to three weeks later, pupae had undergone complete metamorphosis and emerged from the cocoon. The day of emergence was recorded as day 0. After emergence, females were kept individually in polystyrol vials with moist sand and honey *ad libitum*. Female antennae were removed and processed at two-day intervals to yield two data points for each female, since female beewolves are generally rare and difficult to obtain in large numbers.

The pre-emergence specimens (age<0) were ordered chronologically by the degree of melanization of their cuticle that was visible through the cocoon, and the time before emergence was estimated by comparison with individuals in which the process of melanization was observed until emergence (see online supplementary figure 1). Thus, the age of the pre-emergence specimens (in days before emergence) represents an approximate value. After removing the first antenna, the pre-emergence specimens were placed back into the cocoon until removal of the second antenna two days later.

#### 5.3.2 *Semithin sections and 3D-reconstruction*

Semithin sections of beewolf antennae and 3D-reconstructions of the antennal gland reservoirs and the symbionts were obtained as described earlier (Goettler et al., 2007). Briefly, the antennae were fixed in alcoholic Bouin, dehydrated in a graded ethanol series and embedded in Poly/Bed<sup>®</sup> 812 (Polysciences, Eppelheim, Germany). Serial sections of 4 µm thickness were cut with a diamond knife on a Reichert 2040 Autocut and stained with 1% toluidine blue buffered with 1% Di-sodium-tetraborate in distilled water. For each antenna, digital photos of the sections of the eighth segment were obtained with a Nikon Coolpix 990 camera attached to a Zeiss Axiophot M45 light microscope. Due to technical difficulties with the semithin sectioning of the antennae, complete sets of high-quality slices for the eighth segment were not available for all specimens. Thus, the seventh or sixth antennal segment had to be used in some cases. The image stack of the slices was automatically aligned with the 3D-visualization software Amira<sup>®</sup> (Mercury Computer Systems, Berlin), and the alignment was checked by eye and corrected manually. The antennal gland reservoirs and the bacteria within the reservoirs were manually marked with different colors in every slice to allow 3D-reconstruction.

#### 5.3.3 *Measurement of reservoir and bacterial volumes*

The volume of the gland reservoirs and the volume of the bacteria within the reservoirs (in nl) were calculated with Amira based on the 3D-reconstructions. The width of the antennal segment was measured in the median section, and the volume of the antennal segment was approximated as a

cylinder ( $V_A = \pi r^2 h$ ), using half of the antennal width as the radius and the number of sections multiplied by  $4 \mu\text{m}$  (=thickness of the sections) as the height (in  $\mu\text{m}$ ). The relative reservoir volume was calculated by dividing the reservoir volume by the volume of the whole antennal segment. Likewise, the relative volume of the bacteria was calculated as the bacterial volume divided by the reservoir volume.

#### 5.3.4 Calculation of bacterial cell number

Based on the volume of bacteria within the reservoirs, the number of bacterial cells could be approximated. Cells of '*Ca. S. philanthi*' exhibit a length of  $13.7 \pm 9.3 \mu\text{m}$  (mean  $\pm$  SD) when measured in the antennal gland secretion (M. Kaltenpoth, unpubl. data), and a mean diameter of about  $0.5 \mu\text{m}$  (Kaltenpoth et al., 2005). Assuming a cylindrical shape of the bacterial cells, the volume can be calculated as  $V_C = \pi r^2 h$ . This yields a mean volume of about  $2.69 \times 10^{-6}$  nl for a single cell. Thus, the bacterial cell number in the antennal reservoirs could be approximated as

$$(1) \quad N_B = V_B \cdot \frac{10^6}{2.69}$$

with  $N_B$  representing the cell number, and  $V_B$  the volume of the bacteria within the antennal reservoir. However, these values constitute only a rough approximation of the true cell number, since the calculation does not allow for interspaces between bacterial cells.

#### 5.3.5 Statistical analysis, Calculation of specific growth rate and generation time

Logistic regression curves were fit to the data using SigmaPlot 8.0 following equation (2):

$$(2) \quad f(x) = f(x_0) + \frac{a}{1 + \left(\frac{x}{x_0}\right)^b}$$

To calculate the specific growth rate of the symbionts, the part of the data for the absolute and the relative volume of bacterial cells within the antennal gland reservoirs that represented the exponential growth phase (day 0 to day 2) were ln-transformed and plotted against the age. The slopes of the linear regression lines fitted to these data were used as estimates of the specific growth rate. From the growth rate estimates, the doubling time or generation time  $t_D$  was calculated as follows:

$$(3) \quad t_D = \frac{\ln 2}{\mu_{\max}}$$

## 5.4 Results

### 5.4.1 Development of the antennal gland reservoir

The antennal gland reservoirs of 26 female *Philanthus triangulum* antennae and their symbiotic bacteria were reconstructed [for some representative reconstructions see Fig. 5.1]. Seven antennae were obtained from specimens before emergence from the cocoon, three immediately after emergence (on the same day), and 16 one to nine days later. Up to about one day prior to emergence, the antennal sections clearly showed the pupal skin still surrounding the antennal cuticle. Any invasion of bacteria into the antennal gland reservoirs from outside of the insect body seems impossible before shedding of the pupal skin.

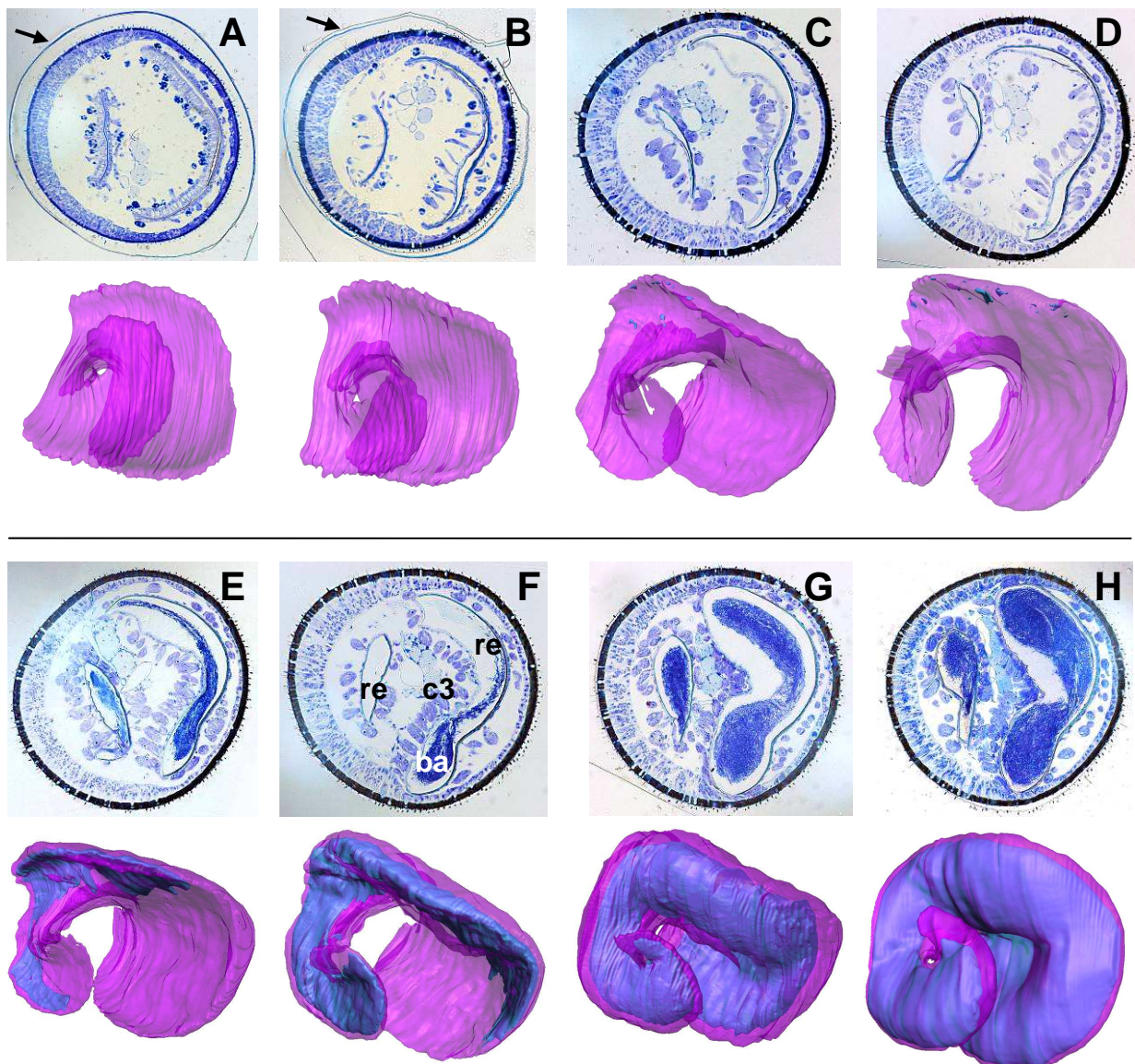


Fig. 5.1. Representative sections and 3D-reconstructions of antennal gland reservoirs (pink, half-transparent) and symbiotic bacteria (blue) from female European beewolves (*P. triangulum*) of different ages. **A** About eight days prior to emergence (day -8), **B** about five days prior to emergence (day -5), **C** day of emergence (day 0), **D** one day after emergence (day 1), **E** two days after emergence (day 2), **F** three days after emergence (day 3), **G** five days after emergence (day 5), **H** nine days after emergence (day 9). Note the pupal skin (arrows) in (A) and (B). (ba) bacteria; (c3), gland cell units; (re) gland reservoir.

The volume of the antennal gland reservoir increased significantly from about 2-8 nl at emergence to about 18 nl at day 9. The increase in volume could be approximated by a logistic growth model (see equation (2);  $R^2=0.644$ ,  $F=13.3$ ,  $p<0.001$ ) (Fig. 5.2). After nine days, the antennal gland reservoir accounted for about 25% of the total antennomere volume (Fig. 5.3). As should be expected, the antennomere volume was independent of the age of the beewolf females (linear regression:  $F=1.34$ ,  $p=0.258$ ; four parameter logistic regression (see equation(2)):  $F=0.400$ ,  $p=0.754$ ).

#### 5.4.2 Uptake of symbiotic bacteria

No bacterial cells could be detected in any of the pre-emergence specimens. The first bacterial cells were visible in the antennal gland reservoirs on the day of emergence, suggesting that the symbionts are taken up by the adult female during or very shortly before emergence. Observations of beewolf females within the cocoon revealed that they repeatedly press and rub their antennae against the inside of the cocoon shortly prior to emergence (see online supplementary video 1). During this procedure, the antennae are strongly bent, which probably exposes the openings of the antennal glands (see Goettler et al., 2007) and thereby facilitates the infection of the reservoirs with the symbiotic bacteria. Counting of individual bacterial cells in the reservoirs of newly emerged beewolves suggested that only 10 to 100 cells are taken up by the females per antennal segment.

#### 5.4.3 Growth of symbiont cells within the reservoir

Starting with very few bacterial cells on the day of emergence, the cell biomass followed a logistic growth curve that began to approach a maximum of about 10-15 nl (or an estimated  $4.5-6.5 \times 10^6$  cells) at day 9 (see equation (2);  $R^2=0.802$ ,  $F=29.8$ ,  $p<0.001$ ) (Fig. 5.4). Finally, the symbiont cells accounted for about 75-80% of the total antennal gland reservoir volume (Fig. 5.5). The remaining 20-25% were probably filled with other components of the antennal gland secretion, mainly hydrocarbons and protein droplets (Kaltenpoth, Goettler, Strohm, unpubl. data).

The growth of the symbiont cells was approximately exponential from day 0 to day 2, so the data in this range were used for estimating the specific growth rate of the symbionts. Linear regressions fitted to the ln-transformed data yielded growth rate estimates of  $\mu=0.111 \text{ h}^{-1}$  and  $\mu=0.101 \text{ h}^{-1}$  for the absolute and the relative volume of bacterial cells in the reservoir, respectively. These growth rates translate into generation times of  $t_D=6.27 \text{ h}$  and  $t_D=6.83 \text{ h}$ , respectively (see equation (3)). The maximum number of symbiont cells in a full reservoir was approaching  $4 \times 10^6$  cells (see equation (1)). Thus, the total number of symbionts in all ten reservoirs of a female beewolf (five per antenna) may reach  $4 \times 10^7$ .

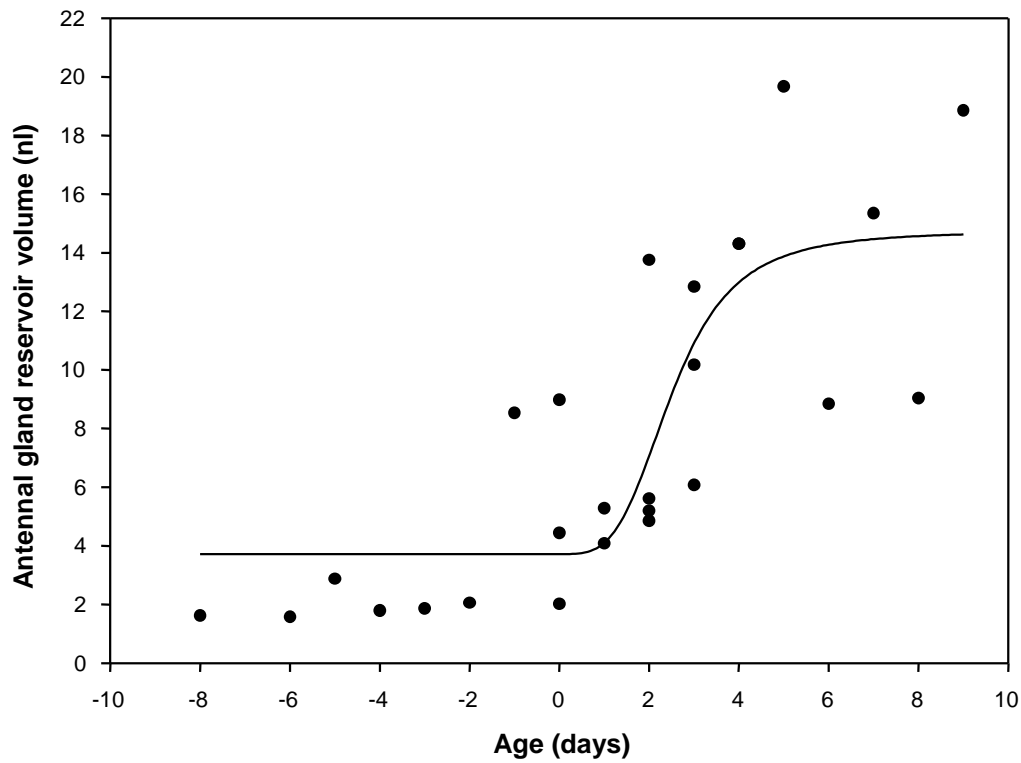


Fig. 5.2. Changes in the volume of the antennal gland reservoir of female European bees before and after emergence from the cocoon (in nl). The logistic growth curve fitted to the data follows equation (2) ( $r^2 = 0.644$ ,  $F = 13.3$ ,  $p < 0.001$ ).

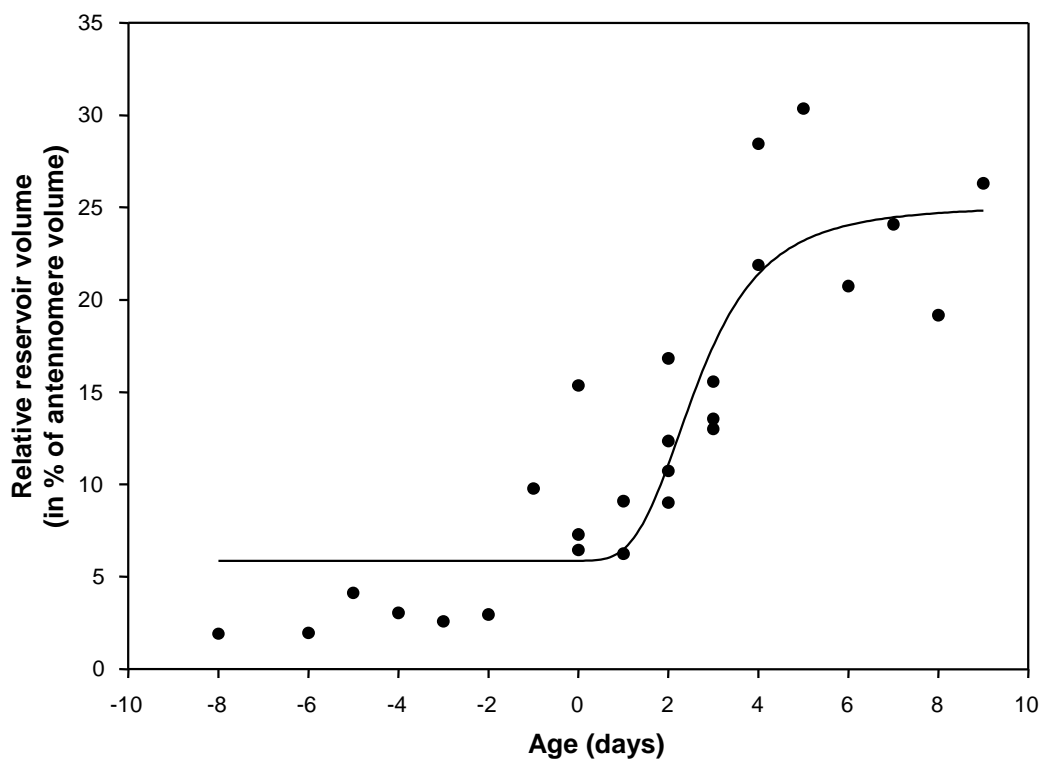


Fig. 5.3. Changes in the relative volume of the antennal gland reservoir of female European bees before and after emergence from the cocoon (in percent of the total antennomere volume). The logistic growth curve fitted to the data follows equation (2) ( $r^2 = 0.783$ ,  $F = 26.5$ ,  $p < 0.001$ ).

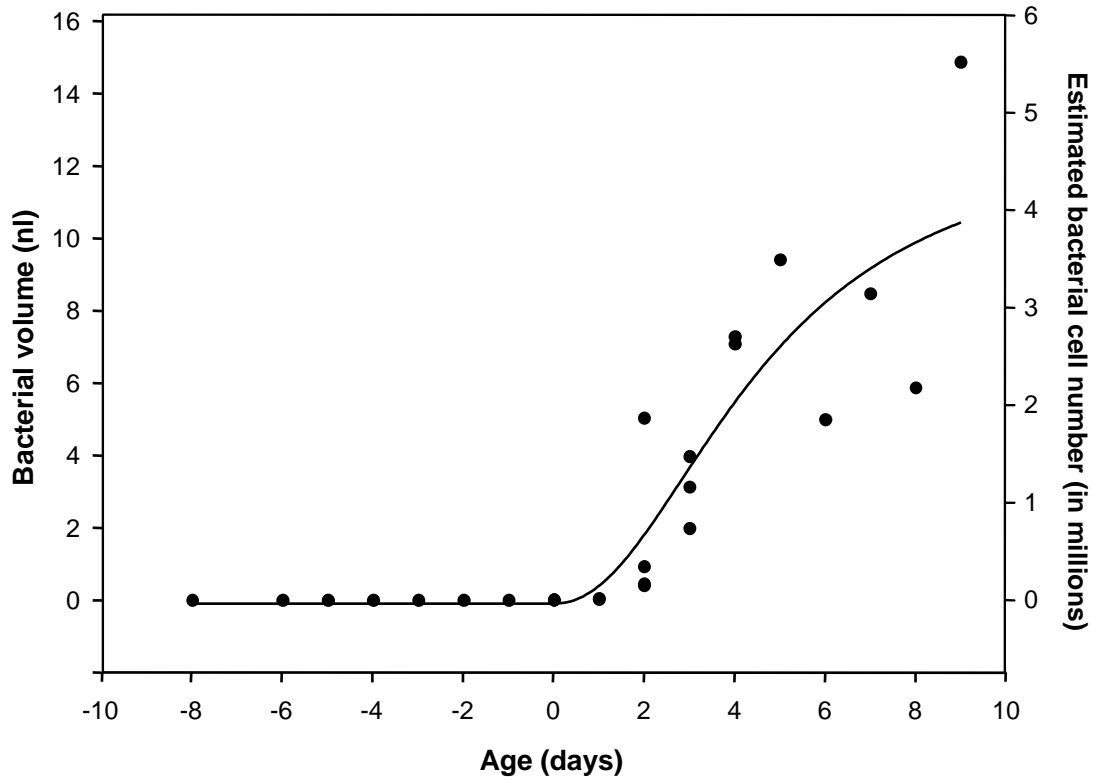


Fig. 5.4. Changes in the volume (left axis) and estimated number (right axis) of symbiont cells within the antennal gland reservoir of female European beeswolves before and after emergence from the cocoon. The logistic growth curve fitted to the data follows equation (2) ( $r^2 = 0.802$ ,  $F = 29.8$ ,  $p < 0.001$ ).

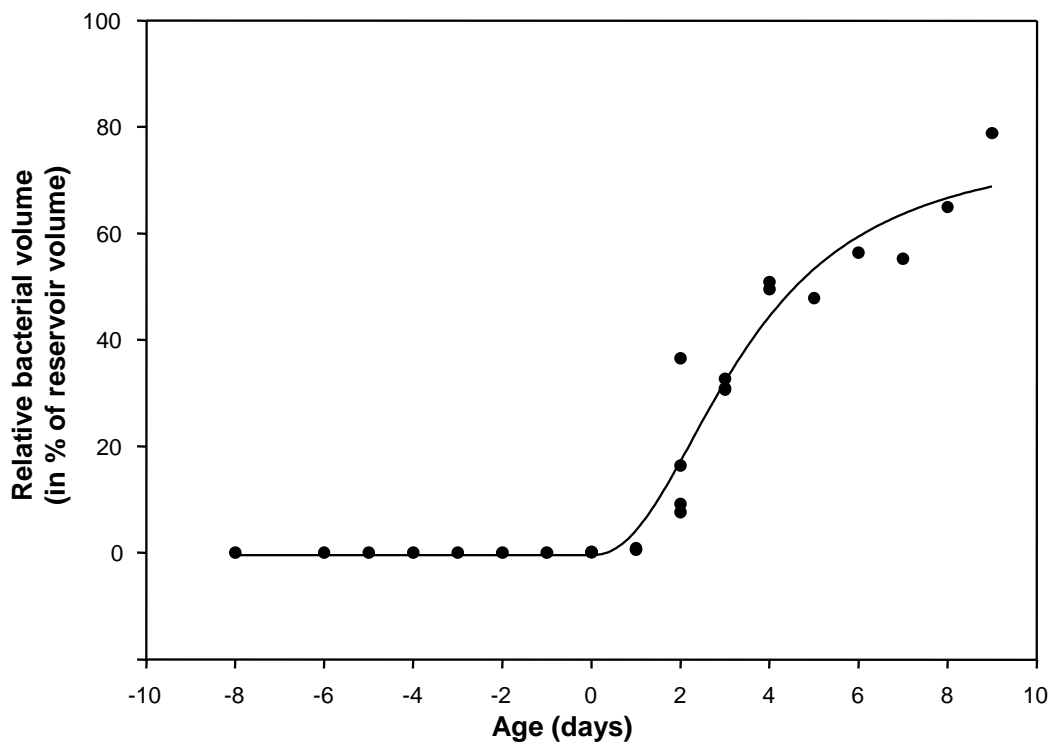


Fig. 5.5. Changes in the relative volume of symbiont cells within the antennal gland reservoir of female European beeswolves before and after emergence from the cocoon (in percent of the total reservoir volume). The logistic growth curve fitted to the data follows equation (2) ( $r^2 = 0.948$ ,  $F = 133.6$ ,  $p < 0.001$ ).

## 5.5 Discussion

In the present study, we analyzed the growth dynamics of the symbiotic bacteria in the antennal gland reservoirs of female European beewolves before and after emergence from the cocoon, and we analyzed the growth dynamics of the symbiotic bacteria cultivated in these reservoirs. The results provide evidence that the bacteria are taken up by female beewolves from the cocoon around the time of emergence, and they subsequently undergo logistic growth within the reservoirs.

The beewolf-*Streptomyces* symbiosis constitutes a specialized association with an unusual vertical transmission route of the symbionts via the brood cell and the cocoon. In insect-bacteria associations with post-hatch vertical transmission, the symbionts must be able to survive outside of the host for the time between oviposition and symbiont uptake by the offspring. In the European beewolf, the symbiont life stage outside of the host is significantly longer: After secretion into the brood cell, the bacteria are taken up and applied to the cocoon by the beewolf larva (about 7-12 days later), where they grant protection against pathogenic fungi (Kaltenpoth et al., 2005). After hibernation in the cocoon for about nine months, the larva pupates and later emerges from the cocoon as an adult beewolf. The results of the present study suggest that the symbionts are incorporated into the antennal gland reservoirs around the time of emergence from the cocoon. Thus, the phase the symbionts spend outside of the beewolf's body usually lasts several months. To our knowledge, this is the longest time for specific symbionts to survive outside of the host's body in any insect-bacteria symbiosis with vertical transmission.

Within the antennal gland reservoirs of beewolf females, the endosymbionts grow exponentially during the first few days. The maximum specific growth rate of 0.101 to 0.111 h<sup>-1</sup> lies within the range of growth rates found for free-living *Streptomyces spp.*: Depending on the species and the growth medium, streptomycetes exhibit specific growth rates between 0.024 and 1.13 h<sup>-1</sup> (Reichl et al., 1992; Shahab et al., 1996; Daae and Ison, 1998; Jonsbu et al., 2002; Anukool et al., 2004; Cox, 2004). The specific growth rate calculated for '*Ca. S. philanthi*' in this study may underestimate the true growth rate, since the measurement of bacterial volumes does not account for changes in the density of bacterial cultures within the antennal gland reservoirs.

The results of the present study indicate that full antennal gland reservoirs of female beewolves can contain about 4x10<sup>6</sup> symbiont cells and, thus, a single female can harbor up to 4x10<sup>7</sup> symbionts in all ten reservoirs. However, the number of bacterial cells at any given time is probably considerably lower under natural conditions, since females usually construct one to three brood cells per day, and our results suggest that the time between brood cell constructions is not long enough for the symbionts to reach the maximum number in the gland reservoirs. Earlier experiments have shown that between 4.7x10<sup>3</sup> and 6.2x10<sup>6</sup> symbiont cells (mean ± SD = 3.4x10<sup>6</sup> ± 1.8x10<sup>6</sup> cells) are secreted by females

into each brood cell (Kaltenpoth, unpubl. data). Thus, a considerable proportion of the bacterial cells within the reservoirs is secreted during the construction of each brood cell, and the number of bacteria may become a limiting factor for females.

During the transmission of the bacteria from beewolf females to their offspring via the brood cell and the cocoon, the symbiont population suffers from a severe bottleneck of about  $2.5 \times 10^{-5}$  to  $2.5 \times 10^{-6}$  from the mother ( $4 \times 10^7$  cells in all ten reservoirs) to the emerging offspring ( $10^2$  to  $10^3$  cells in all ten reservoirs, with  $10^1$  to  $10^2$  cells per reservoir). This bottleneck is significantly more severe than in most other symbiotic systems investigated so far: The symbiont populations of aphids (*Acyrtosiphon pisum*), weevils (*Sitophilus oryzae*), and stinkbugs (*Megacopta punctatissima*) suffer reductions of  $1.6 \times 10^{-2}$  to  $4.6 \times 10^{-3}$  during transmission events (Nardon and Grenier, 1988; Mira and Moran, 2002; Hosokawa et al., 2007). To our knowledge, only symbiotic *Spiroplasma* spp. in fruit flies (*Drosophila melanogaster*) have as yet been reported to experience transmission bottlenecks of similar severity as beewolves ( $3 \times 10^{-4}$  to  $5 \times 10^{-5}$ ) (Anbutsu and Fukatsu, 2003). In *Drosophila*, however, the absolute number of *Spiroplasma* symbionts in both eggs and adult insects is much larger than that of *Streptomyces* in European beewolves (about  $10^2$  to  $10^3$  times larger) (Anbutsu and Fukatsu, 2003), which is expected to reduce the deleterious effects of the transmission bottleneck.

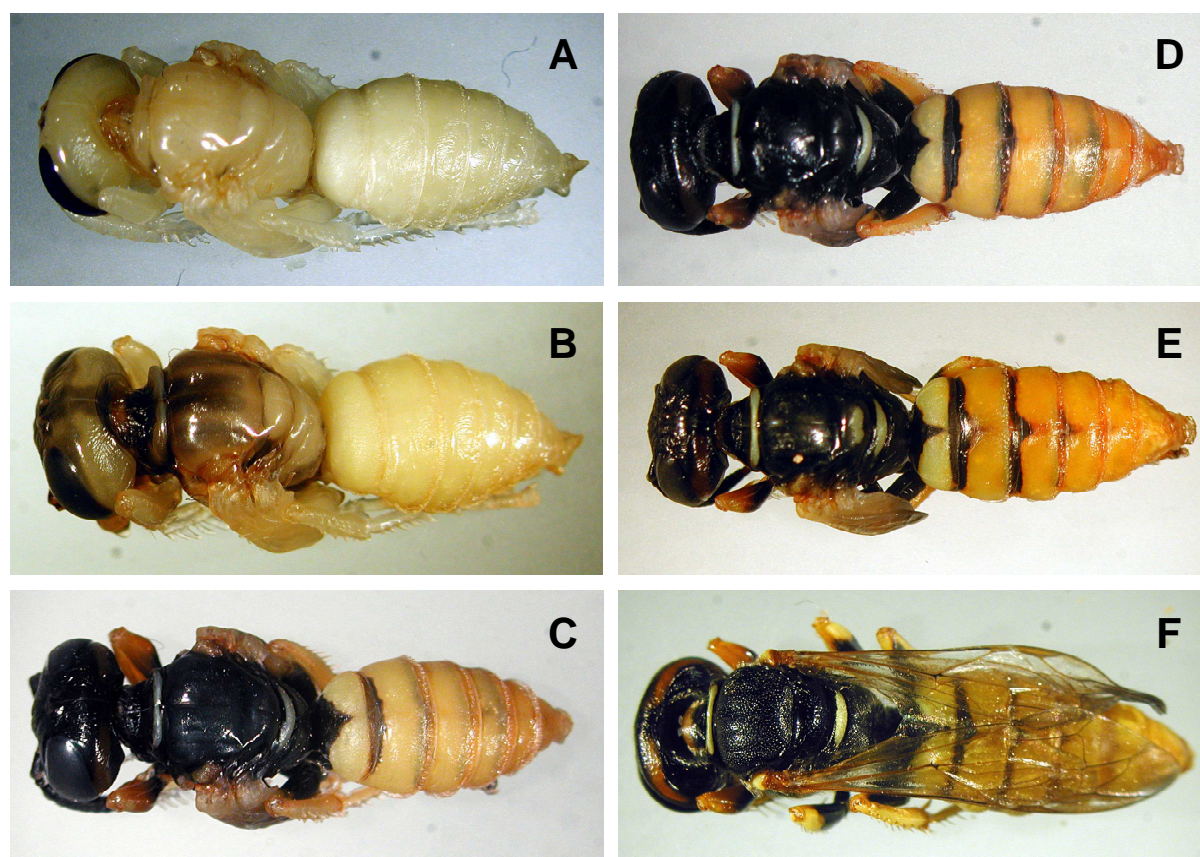
Severe transmission bottlenecks in insect endosymbiont populations probably entail major genetic and genomic consequences for the symbionts. Generally, asexual populations undergoing strong bottlenecks are expected to suffer increased genetic drift and an accumulation of deleterious mutations, a process known as Muller's ratchet, that can ultimately lead to extinction by mutational meltdown (Lynch and Gabriel, 1990; Gabriel et al., 1993; Lynch et al., 1993; Andersson and Kurland, 1998). In the case of obligate endosymbionts, however, selection on the host probably limits the accumulation of deleterious mutations in the symbionts and thereby counteracts this process (Andersson and Kurland, 1998; Pettersson and Berg, 2007). Complete genome sequences of endosymbiotic bacteria provide evidence for significant genome reduction and erosion as well as shifts in base composition towards AT-rich sequences reflecting mutational bias (Shigenobu et al., 2000; Akman et al., 2002; Tamas et al., 2002; Gil et al., 2003; Tamas and Andersson, 2003; van Ham et al., 2003; Moran and Plague, 2004; Degnan et al., 2005; Toh et al., 2006; Wu et al., 2006; McCutcheon and Moran, 2007). However, genome sequencing projects of insect endosymbionts have so far been restricted to  $\gamma$ -Proteobacteria and one species of Bacteroidetes (McCutcheon and Moran, 2007). Our results suggest that genomic analyses of '*Ca. Streptomyces philanthi*', the high-GC gram-positive endosymbiont of European beewolves, will yield interesting insights into the general consequences of an endosymbiotic lifestyle, especially with regard to the effects of severe transmission bottlenecks on genome erosion and streamlining.



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### 5.6 Online supplementary material



Online supplementary Figure 1. Melanization of the cuticle in female European beewolves prior to emergence. Approximate time before emergence: **A** eight days, **B** six days, **C** five days, **D** three days, **E** two days, **F** one day.

Online supplementary Video 1. Putative symbiont uptake behavior by a female European beewolf in the cocoon prior to emergence. The antennae are bent backwards and rubbed against the cocoon silk, so symbiotic bacteria may be inoculated into the antennal gland reservoirs.

## 5.7 References

- Akman, L., Yamashita, A., Watanabe, H., Oshima, K., Shiba, T., Hattori, M., Aksoy, S. (2002). Genome sequence of the endocellular obligate symbiont of tsetse flies, *Wigglesworthia glossinidia*. *Nature Genetics* 32 (3): 402-407.
- Aksoy, S., Chen, X., Hypsa, V. (1997). Phylogeny and potential transmission routes of midgut-associated endosymbionts of tsetse (Diptera: Glossinidae). *Insect Molecular Biology* 6 (2): 183-190.
- Anbutsu, H., Fukatsu, T. (2003). Population dynamics of male-killing and non-male-killing spiroplasmas in *Drosophila melanogaster*. *Applied and Environmental Microbiology* 69 (3): 1428-1434.
- Andersson, S.G., Kurland, C.G. (1998). Reductive evolution of resident genomes. *Trends in Microbiology* 6 (7): 263-268.
- Anukool, U., Gaze, W.H., Wellington, E.M. (2004). In situ monitoring of streptothricin production by *Streptomyces rochei* F20 in soil and rhizosphere. *Applied and Environmental Microbiology* 70 (9): 5222-5228.
- Baumann, P., Moran, N.A. (1997). Non-cultivable microorganisms from symbiotic associations of insects and other hosts. *Antonie van Leeuwenhoek* 72: 39-48.
- Bourtzis, K., Miller, T.A. (2003). *Insect Symbiosis*. CRC Press, Boca Raton.
- Buchner, P. (1965). *Endosymbiosis of animals with plant microorganisms*. Interscience Publishers, New York.
- Cox, R.A. (2004). Quantitative relationships for specific growth rates and macromolecular compositions of *Mycobacterium tuberculosis*, *Streptomyces coelicolor* A3(2) and *Escherichia coli* B/r: an integrative theoretical approach. *Microbiology* 150: 1413-1426.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398 (6729): 701-704.
- Daae, E.B., Ison, A.P. (1998). A simple structured model describing the growth of *Streptomyces lividans*. *Biotechnology and Bioengineering* 58: 263-266.
- Degnan, P.H., Lazarus, A.B., Brock, C.D., Wernegreen, J.J. (2004). Host-symbiont stability and fast evolutionary rates in an ant-bacterium association: Cospeciation of *Camponotus* species and their endosymbionts, *Candidatus Blochmannia*. *Systematic Biology* 53 (1): 95-110.
- Degnan, P.H., Lazarus, A.B., Wernegreen, J.J. (2005). Genome sequence of *Blochmannia pennsylvanicus* indicates parallel evolutionary trends among bacterial mutualists of insects. *Genome Research* 15 (8): 1023-1033.
- Douglas, A.E. (1998). Nutritional interactions in insect-microbial symbioses: Aphids and their symbiotic bacteria *Buchnera*. *Annual Review of Entomology* 43: 17-37.
- Douglas, A.E. (2006). Phloem-sap feeding by animals: problems and solutions. *Journal of Experimental Botany* 57 (4): 747-754.
- Fukatsu, T., Hosokawa, T. (2002). Capsule-transmitted gut symbiotic bacterium of the Japanese common plataspid stinkbug, *Megacopta punctatissima*. *Applied and Environmental Microbiology* 68 (1): 389-396.
- Gabriel, W., Lynch, M., Burger, R. (1993). Muller's ratchet and mutational meltdowns. *Evolution* 47 (6): 1744-1757.
- Gil, R., Silva, F.J., Zientz, E., Delmotte, F., Gonzalez-Candelas, F., Latorre, A., Rausell, C., Kamerbeek, J., Gadau, J., Hölldobler, B., et al. (2003). The genome sequence of *Blochmannia floridanus*: Comparative analysis of reduced genomes. *PNAS* 100 (16): 9388-9393.
- Gil, R., Latorre, A., Moya, A. (2004). Bacterial endosymbionts of insects: insights from comparative genomics. *Environmental Microbiology* 6 (11): 1109-1122.
- Goettler, W., Kaltenpoth, M., Herzner, G., Strohm, E. (2007). Morphology and ultrastructure of a bacteria cultivation organ: The antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36: 1-9.
- Hosokawa, T., Kikuchi, Y., Meng, X.Y., Fukatsu, T. (2005). The making of symbiont capsule in the plataspid stinkbug *Megacopta punctatissima*. *FEMS Microbiol. Ecol.* 54 (3): 471-477.
- Hosokawa, T., Kikuchi, Y., Fukatsu, T. (2007). How many symbionts are provided by mothers, acquired by offspring, and needed for successful vertical transmission in an obligate insect-bacterium mutualism? *Molecular Ecology* 16 (24): 5316-5325.
- Ishikawa, H. (2003). Insect symbiosis: an introduction. in Bourtzis, K., Miller, T.A., eds. *Insect symbiosis*. CRC Press, Boca Raton, Florida.
- Jonsbu, E., McIntyre, M., Nielsen, J. (2002). The influence of carbon sources and morphology on nystatin production by *Streptomyces noursei*. *Journal of Biotechnology* 95 (2): 133-144.
- Kaltenpoth, M., Goettler, W., Herzner, G., Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479.

- 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: 1403-1411.
- Kikuchi, Y., Hosokawa, T., Fukatsu, T. (2007). Insect-microbe mutualism without vertical transmission: a stinkbug acquires a beneficial gut symbiont from the environment every generation. *Applied and Environmental Microbiology* 73 (13): 4308-4316.
- Lynch, M., Gabriel, W. (1990). Mutation load and the survival of small populations. *Evolution* 44 (7): 1725-1737.
- Lynch, M., Burger, R., Butcher, D., Gabriel, W. (1993). The mutational meltdown in asexual populations. *Journal of Applied Microbiology* 84 (5): 339-344.
- McCutcheon, J.P., Moran, N.A. (2007). Parallel genomic evolution and metabolic interdependence in an ancient symbiosis. *PNAS* 104 (49): 19392-19397.
- Mira, A., Moran, N.A. (2002). Estimating population size and transmission bottlenecks in maternally transmitted endosymbiotic bacteria. *Microbial Ecology* 44 (2): 137-143.
- Moran, N.A. (1996). Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. *PNAS* 93 (7): 2873-2878.
- Moran, N.A., Plague, G.R. (2004). Genomic changes following host restriction in bacteria. *Current Opinion in Genetics and Development* 14 (6): 627-633.
- Nardon, P., Grenier, A.M. (1988). Genetical and biochemical interactions between the host and its endocytobionts in the weevils *Sitophilus* (Coleoptera, Curculionidae) and other related species. in Scannerini, S., Smith, D., Bonfante-Fasolo, P., Gianinazzi-Pearson, V., eds. *Cell to cell signals in plant, animal and microbial symbiosis*. Springer, Berlin.
- Nardon, P. (2006). Oogenesis and transmission of symbiotic bacteria in the weevil *Sitophilus oryzae* L. (Coleoptera: Dryophthoridae). *Annales de la Société entomologique de France* 42 (2): 129-164.
- Oliver, K.M., Russell, J.A., Moran, N.A., Hunter, M.S. (2003). Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. *PNAS* 100 (4): 1803-1807.
- Petersson, M.E., Berg, O.G. (2007). Muller's ratchet in symbiont populations. *Genetica* 130 (2): 199-211.
- Prado, S.S., Rubinoff, D., Almeida, R.P.P. (2006). Vertical transmission of a pentatomid caeca-associated symbiont. *Annals of the Entomological Society of America* 99 (3): 577-585.
- Reichl, U., King, R.C., Gilles, E.D. (1992). Effect of temperature and medium composition on mycelial growth of *Streptomyces tendae* in submerged culture. *Journal of Basic Microbiology* 32 (3): 193-200.
- Rispe, C., Moran, N.A. (2000). Accumulation of deleterious mutations in endosymbionts: Muller's ratchet with two levels of selection. *American Naturalist* 156 (4): 425-441.
- Sauer, C., Dudaczek, D., Hölldobler, B., Gross, R. (2002). Tissue localization of the endosymbiotic bacterium 'Candidatus Blochmannia floridanus' in adults and larvae of the carpenter ant *Camponotus floridanus*. *Applied and Environmental Microbiology* 68 (9): 4187-4193.
- Schröder, D., Deppisch, H., Obermayer, M., Krohne, G., Stackebrandt, E., Hölldobler, B., Goebel, W., Gross, R. (1996). Intracellular endosymbiotic: Bacteria of *Camponotus* species (carpenter ants): Systematics, evolution and ultrastructural characterization. *Molecular Microbiology* 21 (3): 479-489.
- Shahab, N., Flett, F., Oliver, S.G., Butler, P.R. (1996). Growth rate control of protein and nucleic acid content in *Streptomyces coelicolor* A3(2) and *Escherichia coli* B/r. *Microbiology* 142 (8): 1927-1935.
- Shigenobu, S., Watanabe, H., Hattori, M., Sakaki, Y., Ishikawa, H. (2000). Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. *Nature* 407 (6800): 81-86.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Tamas, I., Klasson, L., Canback, B., Naslund, A.K., Eriksson, A.S., Wernegreen, J.J., Sandstrom, J.P., Moran, N.A., Andersson, S.G.E. (2002). 50 million years of genomic stasis in endosymbiotic bacteria. *Science* 296 (5577): 2376-2379.
- Tamas, I., Andersson, S.G.E. (2003). Comparative genomics of insect endosymbionts. in Bourtzis, K., Miller, T.A., eds. *Insect symbiosis*. CRC Press, Boca Raton, Florida.
- Toh, H., Weiss, B.L., Perkin, S.A.H., Yamashita, A., Oshima, K., Hattori, M., Aksoy, S. (2006). Massive genome erosion and functional adaptations provide insights into the symbiotic lifestyle of *Sodalis glossinidius* in the tsetse host. *Genome Research* 16 (2): 149-156.
- van Ham, R.C.H.J., Kamerbeek, J., Palacios, C., Rausell, C., Abascal, F., Bastolla, U., Fernandez, J.M., Jimenez, L., Postigo, M., Silva, F.J., et al. (2003). Reductive genome evolution in *Buchnera aphidicola*. *PNAS* 100 (2): 581-586.
- Wu, D., Daugherty, S.C., van Aken, S.E., Pai, G.H., Watkins, K.L., Khouri, H., Tallon, L.J., Zaborsky, J.M., Dunbar, H.E., Tran, P., et al. (2006). Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. *PLoS Biology* 4 (6): 1079-1092.

Zientz, E., Dandekar, T., Gross, R. (2004). Metabolic interdependence of obligate intracellular bacteria and their insect hosts. *Microbiology and Molecular Biology Reviews* 68 (4): 745-770.



## CHAPTER 6

**ANTENNAL GLANDS IN FEMALE DIGGER WASPS OF THE  
GENUS *PHILANTHUS* (HYMENOPTERA, CRABRONIDAE)**

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**6.1 Summary**

Females of the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae), a solitary digger wasp, rear symbiotic bacteria that belong to the genus *Streptomyces* in specialized glands in their antennae. The glands consist of large reservoirs which are surrounded by numerous gland cells. The *Streptomyces* bacteria proliferate inside the reservoirs and probably feed on nutrients that are provided by the primary gland cell secretions. The bacteria are secreted into the subterranean brood cells where they protect the wasps' offspring from fungus infestation. Hitherto *Streptomyces* bacteria were detected with genetic methods in antennae of another 30 *Philanthus* species. However the antennal glands have been described for *P. triangulum* only. In the present study we investigated the morphology of antennal glands of 15 *Philanthus* species from Africa, Europe and the USA. We reconstructed 3D-models of the glands based on both serial semithin sections and microtomographic data sets. The comparison of the 3D-models revealed limited interspecific differences in overall shape and relative size of the antennal gland reservoirs. However, the number of the surrounding gland cell units varies among species by a factor of 12. Our results suggest that these glands are a universal feature in the genus *Philanthus* and that the ancestor of this genus already possessed complex antennal glands with reservoirs and gland cells.

## 6.2 Introduction

Females of a solitary digger wasp, the European beewolf (*Philanthus triangulum*; Hymenoptera, Crabronidae) possess unusual bacteria cultivation organs inside their antennae (Strohm and Linsenmair, 1995; Kaltenpoth et al., 2005; Goettler et al., 2007). Five segments of each of the females' antennae bear large reservoirs which are connected to numerous gland cell units, so-called acini (Goettler et al., 2007). Inside the gland reservoirs symbiotic bacteria of the genus *Streptomyces* proliferate and probably use the secretions of the gland cells as nutrients (Kaltenpoth et al., 2005; Kaltenpoth et al., 2006; Goettler et al. 2007). Possibly, there is also an uptake of substances from the hemolymph into the gland. Large amounts of the whitish mixture of bacteria and secretion droplets are delivered by the female digger wasps into their subterranean brood cells prior to oviposition (Strohm and Linsenmair, 1995). This "white substance" has two known functions. First, it serves as an orientational cue for the larvae when spinning its cocoon (Strohm and Linsenmair, 1995). Second, the bacteria are taken up by the larvae and are spun into the threads of the cocoon silk. Experiments have shown that these bacteria protect the cocoon from infestation by mould fungi (Kaltenpoth et al., 2005; Kaltenpoth et al., 2006).

The genus *Philanthus* comprises about 136 species and has a worldwide distribution with the exception of Australia and South America (Bohart and Menke, 1976; Evans and O'Neill, 1988). Using genetic methods bacteria that belong to the genus *Streptomyces* have been found in the antennae of all of the 31 *Philanthus* species from Europe, North America and Africa investigated so far (Kaltenpoth et al., 2006; Kaltenpoth, pers. comm.). Phylogenetic analyses revealed that these antennal bacteria of beewolves constitute a monophyletic group within the genus *Streptomyces* (Kaltenpoth, 2006). So far only the antennal glands of the European beewolf *P. triangulum* have been described in detail (Goettler et al., 2007). Since the gland in *P. triangulum* is rather complex and there are no known predecessors, we compared the morphology of other species of the genus *Philanthus* in order to shed light on the evolutionary path and in particular the origin of these symbiont cultivation organ.

Besides these evolutionary aspects, there are also ecological aspects that have to be taken into account. Since the rearing of the symbiotic bacteria is probably energy consuming for beewolves there should be strong selection for an optimized morphology and physiology of the antennal glands. Generally we would expect that *Philanthus* species which face a higher risk of fungal infestation bear larger reservoirs and/or more gland cells and therefore could rear *Streptomyces* bacteria at higher rates. This risk of fungal infestation for the beewolf brood could be increased in species where the nests are more humid or if more fungal spores are present in the environment.

We investigated the morphology of the antennal gland of another 15 *Philanthus* species. We reconstructed 3D-models of the glands based on either serial semithin sections or microtomographic data sets and compared details of the gland structure and the surrounding gland cells.

### 6.3 Materials and Methods

#### 6.3.1 Specimens

In total antennae of females of 15 *Philanthus* species were investigated. Seven *Philanthus* species used for semithin sectioning were collected in the Eastern and the Western Cape Province/South Africa (*P. melanderi/ rugosus*), Freiburg/Germany (*P. coronatus*), San Rafael Desert/Utah (*P. basilaris/ gloriosus/ pacificus*) and near Yellowstone National Park /Wyoming (*P. bicinctus*). The eight *Philanthus* species that were scanned with microtomography ( $\mu$ CT) came from San Rafael Desert/Utah (*P. albopilosus/ barbiger/ crotoniphilus/ parkeri/ psyche*), Salt Lake City/Utah (*P. multimaculatus/ ventilabris*) and from Würzburg/Germany (*P. triangulum*). In total we investigated four old world species, respectively two from Europe (*P. coronatus/ triangulum*) and Africa (*P. melanderi/ rugosus*) and 11 North American species (*P. albopilosus/ basilaris/ barbiger/ bicinctus/ crotoniphilus/ gloriosus/ multimaculatus/ pacifius/ parkeri/ psyche/ ventilabris*).

#### 6.3.2 Semithin sections

Preparation of female antennae was performed mainly as described in Goettler et al. (2007). In short, antennae were either fixed with Carnoy's solution (alcohol/chloroform/acetic acid, 6:3:1) or alcoholic Bouin, dehydrated in a graded ethanol series and embedded in Poly/Bed<sup>®</sup> 812 (Polysciences, Eppelheim, Germany). Serial sections of 4  $\mu$ m thickness were cut with a diamond knife on a Reichert 2040 Autocut and stained with 1% toluidine blue buffered with 1% Di-sodium-tetraborate in distilled water. Digital images of the serial sections were obtained with a Nikon DS-2Mv camera attached to a Zeiss Axioplan microscope. The images were stacked and aligned with Amira<sup>®</sup> visualization software (Mercury Computer Systems, Berlin).

#### 6.3.3 X-ray microtomography

For X-ray microtomography ( $\mu$ CT) female antennae were fixed in a solution of 4% glutardialdehyde in 0.1 M sodium-cacodylate-buffer, pH 7.2. After dehydration in a graded ethanol series and transfer to acetone the specimens were critical point dried (BAL-TEC CPD 030) and kept in an exsiccator till  $\mu$ CT scanning. The  $\mu$ CT scans were performed at the X-ray microscope at the TOMCAT beamline of the Swiss Light Source (SLS), which is part of the Paul Scherrer Institute (PSI) in Villigen, Switzerland. The dried antennae were imaged using a 20x objective, giving a field of view of 0.72 mm<sup>2</sup>. The exposure time for each projection at an energy of 9 keV was 150 ms, and a total of 1201 projections were acquired equi-angularly over a sample rotation of 180°. Each antenna was mounted



on a metal needle, attached at its proximal segment and positioned orthogonal to the X-ray beam such that it rotated about its longitudinal axis. The projections were post-processed, which included flatfield and darkfield corrections, and reconstruction of the sinograms was performed on a 32-node Linux PC farm. All voxels were isotropic with an edge length of 0.70  $\mu\text{m}$ . The resolution and contrast of the  $\mu\text{CT}$  data set was sufficient to reveal morphological details and also single cells [Figs. 6.1, 6.2].

#### 6.3.4 3D-reconstruction

Both, the serial semithin sections and the slices of the  $\mu\text{CT}$  data sets were used to reconstruct the morphology of the glands using the visualization-software Amira<sup>®</sup>. Structures of interest (gland reservoirs, acini, flap mechanisms, antennal nerves) were marked manually in every slice with different colours. Finally the software reconstructed the 3D-model of the marked structures. Whereas in the  $\mu\text{CT}$  data sets the voxels were isotropic (1x1x1 pixel), the voxels in the semithin section stacks had a size ratio of 1x1x6 due to lower resolution in the z-axis compared to the xy-plane.

#### 6.3.5 Measuring

The volume of an antennal gland reservoir is severely affected by its filling status. Reservoirs that are filled with secretion and bacteria are bulged out on one side, whereas reservoirs are completely collapsed after the female digger wasp secreted the content into the brood cell (Goettler et al., 2007). Furthermore, larvae of *P. triangulum* are supplied by their mother with 2 to 5 honey bees which results in strong variation in the size of the imago (Strohm, 1995). In order to have an estimate of the size of the gland reservoir relative to the size of the female, we calculated the surface area of the antennal gland reservoirs divided by the surface area of the respective antennomere ( $A_{\text{res}}/A_{\text{ant}}$ ). The reservoir surface area was computed with Amira<sup>®</sup> software and the outer antennomere was reasonably estimated by an approximation using the length and diameter and assuming the antennomere as an ideal cylinder ( $A_{\text{ant}} = 2r_{\text{ant}}^2 \pi + 2r_{\text{ant}} \pi l_{\text{ant}}$ ).

To reveal, whether the results obtained with this method are independent from the filling status of the reservoir we compared the ratio  $A_{\text{res}}/A_{\text{ant}}$  of 15 *P. triangulum* specimens [unpubl. data]. Our analysis showed that  $A_{\text{res}}/A_{\text{ant}}$  in *P. triangulum* does not correlate significantly with the reservoir filling status  $V_{\text{res}}/V_{\text{ant}}$  (Spearman correlation analyses: N=15, p=0.074) and can therefore be used as reliable estimate for the relative reservoir size according to female size.

## 6.4 Results

### 6.4.1 Gland reservoirs

Denomination of antennal segments followed the suggestions of Isidoro et al. (1996), starting numbering with scapus (A1) and pedicellus (A2). All *Philanthus* species that were investigated in this

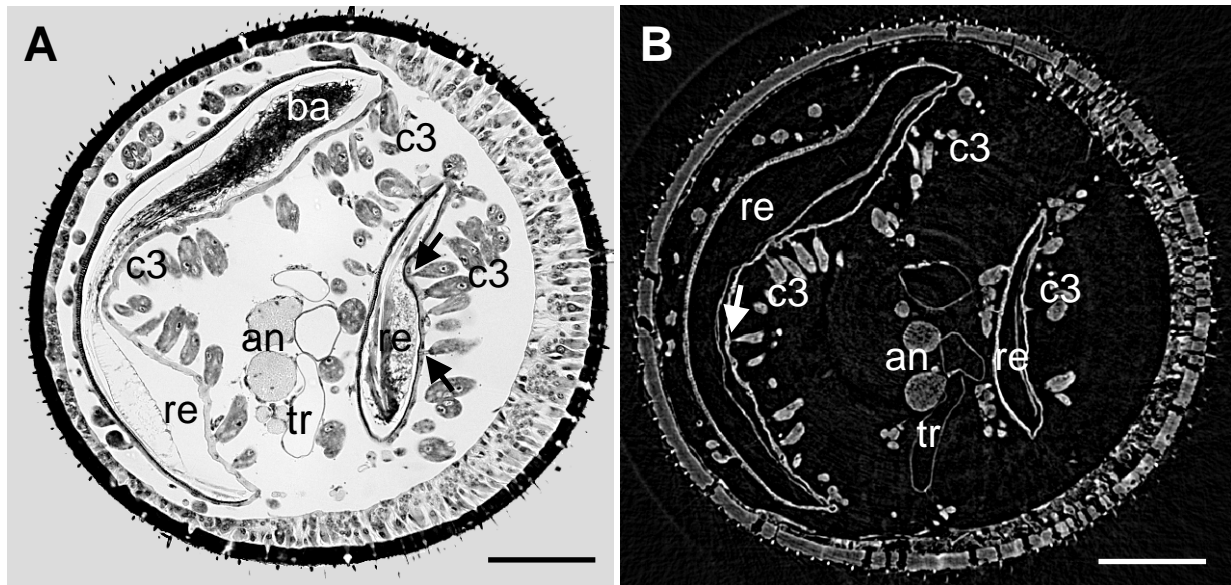


Fig. 6.1. Semithin cross section (A) and  $\mu$ CT image (B) of antennal glands of *P. triangulum* of antennomere A6. The cuticle on the left of each reservoir part is reinforced. an – antennal nerve; ba – bacteria; c3 – class 3 gland cells; re – reservoir; tr – trachea; arrows – conducting canals between acini and reservoir. scale bars = 100 $\mu$ m

study bear antennal glands in the five segments A4 to A8. We found no conspicuous differences in the overall morphology of the five single glands present in one antenna (for *P. triangulum* see Goettler et al., 2007). Therefore we used segment A6 for detailed reconstruction in all species [Figs. 6.1, 6.2]. To reveal possible variations between the 3D-reconstruction of semithin sections and  $\mu$ CT data sets we compared the 3D-model based on  $\mu$ CT data of *P. triangulum* with a reconstruction of semithin sections that has been published earlier (Goettler et al., 2007). We did not find any striking differences with regard to location and shape of organs and tissues between the two methods of 3D-reconstruction. However, in the 3D-reconstruction using  $\mu$ CT, tissues and cells showed a reduced volume, which is probably an artefact of the critical-point drying process [Fig. 6.1].

The 3D-reconstruction of the antennal gland reservoirs revealed some interspecific differences in the relative size compared to the antennomere and also in the shape of the reservoirs [Figs. 6.3, 6.4]. All reservoirs extend dorsally from the opening at the proximal side of the antennomere as more or less bent lobes [Figs. 6.3, 6.4]. The length of the glands is between  $\frac{1}{2}$  and  $\frac{3}{4}$  of the length of the respective antennomere. In all species the antennal gland reservoirs represent an invagination of the outer antenna cuticle as found in *P. triangulum* (Goettler et al., 2007) [Fig. 6.2]. The reservoir cuticle is thickened on the dorsal side and the lateral side pointing away from the body axis [Figs. 6.1, 6.2]. The cuticle at these reinforced parts shows a net-like structure in semithin sections [data not shown]. In vivo the dorsal opening of the reservoir is covered by the adjacent proximate segment and is only visible when the antenna is bent downwards [Fig. 6.2]. At the openings of the reservoir there is a membranous structure that probably acts as a flap mechanism that seals the opening when no secretion is delivered [Figs. 6.2, 6.3, 6.4].

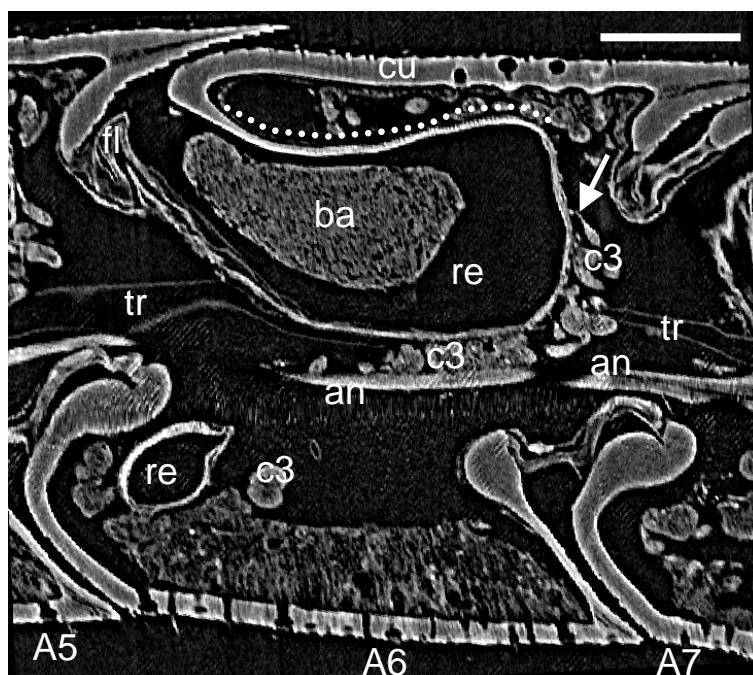


Fig. 6.2.  $\mu$ CT longitudinal section of antennomere A6 of *P. ventilabris*. an – antennal nerve; ba – bacteria; c3 – class 3 gland cells; cu – outer cuticle; fl – flap mechanism; re – reservoir; tr – trachea; arrow – conducting canal; dotted line - reinforced cuticle. scale bar = 100 $\mu$ m

All African and European species (*P. coronatus/ melanderi/ rugosus/ triangulum*) have a complex reservoir structure with two flattened lobes which are parallel to the antennomeres cuticle leaving the central part of the antennomere clear [Figs. 6.3, 6.4]. With an elongated u-shaped second lobe *P. triangulum* shows the most complex reservoir structure of all species investigated [Figs. 6.3, 6.4; see also Goettler et al., 2007]. The nearctic species (*P. albopilosus/ barbiger/ basilaris / bicinctus/ crotoniphilus/ gloriosus/ multimaculatus/ pacificus/ parkeri/ psyche/ ventilabris*) in contrast show smaller, sac-like gland reservoirs with no such prominent second lobes.

Twelve of the 15 *Philanthus* species show a relative reservoir surface between 19.5 % and 38.7 % according to the antennomere surface [Fig. 6.5]. However, three species (*P. multimaculatus, ventilabris, triangulum*) show a remarkably higher relative reservoir surface area between 60.2 % (*P. multimaculatus*) and 71.2 % (*P. triangulum*) [Fig. 6.5].

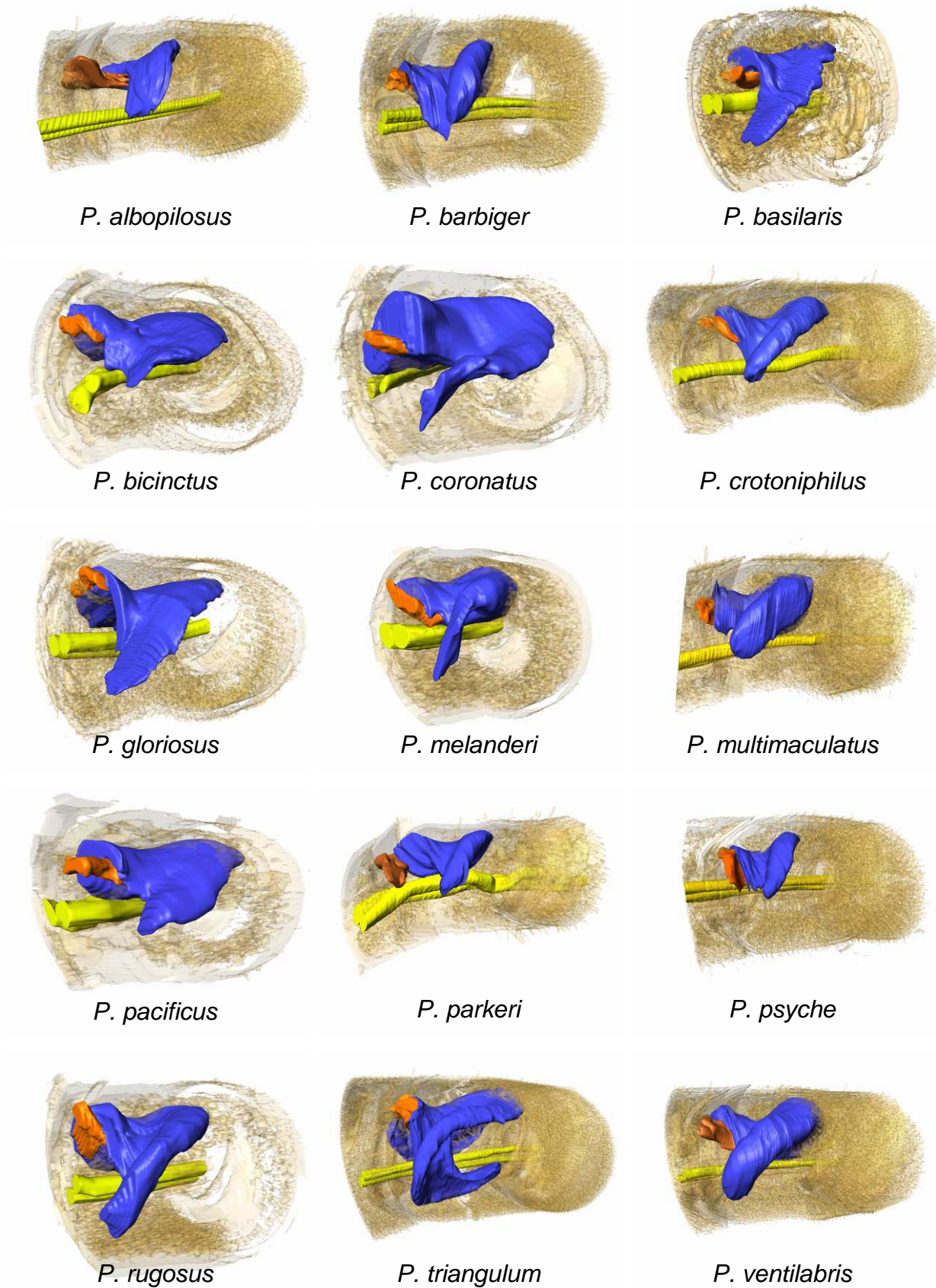


Fig. 6.3. 3D-reconstructions of antennal glands in antennomere A6 of 15 *Philanthus* species. antennal nerve (yellow); flap mechanism (orange); gland reservoir (blue); the outer antennomere cuticle is displayed half transparent



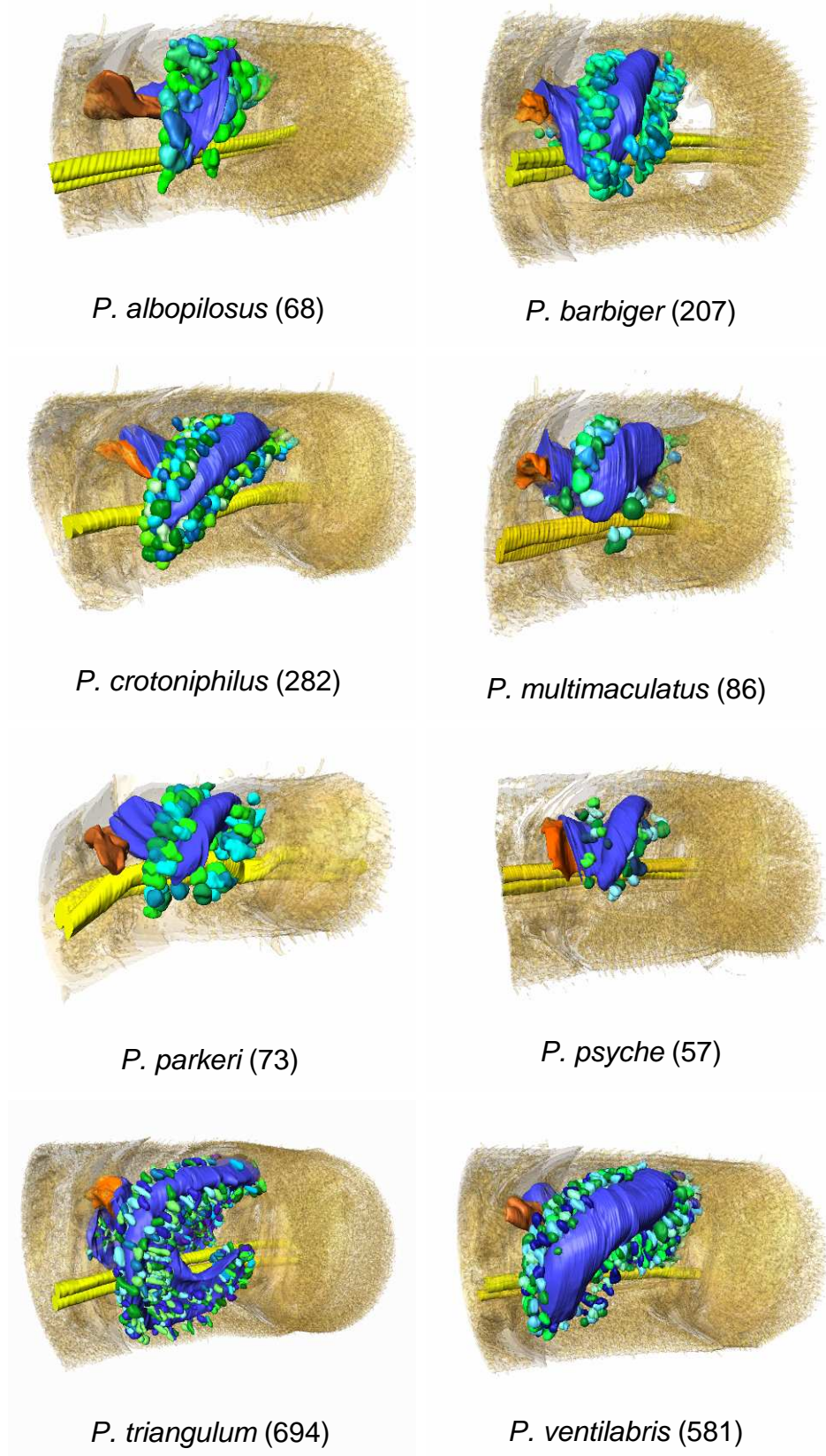


Fig. 6.4. 3D-reconstructions based on  $\mu$ CT data sets of antennal glands in antennomere A6 of 8 *Philanthus* species with surrounding gland cell units (total number of acini in brackets). acini (green-blue); antennal nerve (yellow); flap mechanism (orange); gland reservoir (blue); the outer antennomere cuticle is displayed half transparent

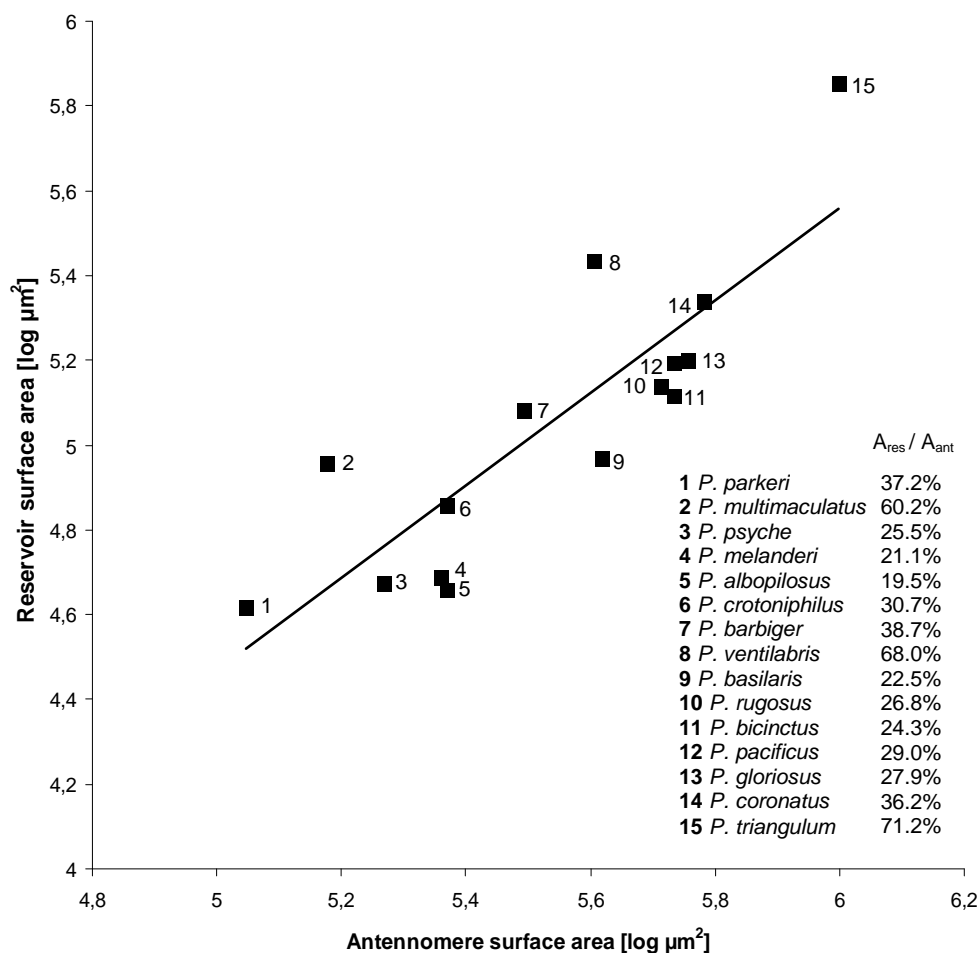


Fig. 6.5. Reservoir surface area (y-axis) against antennomere surface area (x-axis) [both log scale] of 15 *Philanthus* species. Inset shows ratio of reservoir to antennomere surface area in percent. ( $y = 1.0892x - 0.9765$ ;  $R^2 = 0.7215$ )

#### 6.4.2 Gland cells

In all species under study the antennal gland reservoirs are surrounded by class 3 gland cells (according to Noirot and Quenedey, 1974) that are clustered in spherical or drop-shaped acini. The acini consist of 2 to 9 single gland cells which are connected to the reservoir through conducting canal cells. In all species investigated with  $\mu$ CT the acini form a more or less dense belt around the reservoir [Fig. 6.4]. Only in *P. triangulum* the acini are more evenly distributed over the reservoirs' surface. The total number of the acini could be exactly estimated in the  $\mu$ CT data sets and varies between 57 (*P. psyche*) and 694 (*P. triangulum*) [Fig. 6.4].

### 6.5 Discussion

All the 15 *Philanthus* species under study possess antennal glands in five of their antennomeres (A4 to A8). Each gland consists of a reservoir with surrounding acini that are connected to the reservoir

through conducting canals. Inside the gland reservoirs closely related bacteria of the genus *Streptomyces* have been found (Kaltenpoth et al., 2005, 2006; Goettler et al., 2007).

The use of  $\mu$ CT scans in this study turned out to be a suitable method to investigate insect anatomy. Specimen preparation for  $\mu$ CT is comparatively easy and the non-destructive scans reveal anatomical details without the risk of losing slices or inadequate staining as it appears in semithin sectioning. The lower resolution in the xy-plane of the  $\mu$ CT-scans compared to semithin sections is sufficiently compensated by a high z-axis resolution and the fact, that the microtomographic data sets need no additional manual alignment. However, we recognized tissue shrinkage in the critical-point dried specimens which is probably due to the preparation and not to the  $\mu$ CT scanning method.

We proposed a hypothetical evolutionary scenario for the evolution of the antennal glands in the genus *Philanthus* (Goettler et al., 2007). The ancestors of *Philanthus* may have produced a secretion only as an orientational cue (Strohm and Linsenmair, 1995) and had no reservoir yet. In a second step streptomycetes invaded the glands, e.g. as commensals and somehow enhanced the survival of the digger wasps' offspring. This generated a positive feedback and in the third step the antennal glands morphology changed to complex bacteria cultivation organs with reservoirs, flap mechanisms and numerous class 3 gland cells. However, since all species showed more or less complex glands, only the third step of the scenario is supported by this study.

The four investigated *Philanthus* species from Africa and Europe show a slightly more complex structure of their gland reservoirs compared to the 11 nearctic species. This suggests that nearctic and old-world species represent distinct subgroups within the genus. However, this hypothesis has to be further tested. It is unclear whether all extant *Philanthus* species use their antennal gland secretion in the same way as *P. triangulum* for both orientation and as a means against fungi. However this is most likely the case, since they are all confronted with the similar problems of finding their way out of the subterranean nests and protecting the offspring against pathogenic fungi.

In all aspects of gland morphology like relative reservoir size, shape, and number of gland cell units the European beewolf, *P. triangulum* shows the most derived status. However, it is problematic to draw conclusions about the amount of streptomycetes produced based on morphological characters like relative reservoir volume or number of gland cells. We have estimated the growth rate of the bacteria in *P. triangulum* (Kaltenpoth et al., in prep.) but it is not clear whether these results can be transferred to other species. However, everything else being equal, species with relatively large reservoirs and many associated gland cells might produce more bacteria, probably because they experience a more severe threat by mould fungi.

A comparable system of exocrine glands and symbiotic bacteria is known from attine ants (Formicidae) (e.g. Currie et al., 1999, 2003a,b; 2006; Poulsen et al., 2003; Cafaro and Currie, 2005). The fungus-growing ants show a mutualistic relationship with bacteria of the genus *Pseudonocardia* that protect the ants' fungus-gardens from parasitic *Escovopsis* fungus (Currie et al., 1999, 2003b). The ants exhibit glands which secrete on their outer cuticle, either on distinct areas on their propleural plates or on the whole body surface. The symbiotic *Pseudonocardia* bacteria show an increased growth on the cuticle areas with glands where the bacteria probably use the gland secretions as nutrition basis. In some ant taxa the gland cells secrete into cuticular crypts and cavities in which the bacteria proliferate in (Currie et al., 2006). Comparative morphological investigations suggest that the complexity of glandular structures increased during the evolution of attine ants, developing from simple gland cells at distinct areas of the cuticle to complex cuticular differentiations on the whole body surface (Currie et al., 2006).

All 31 *Philanthus* species investigated so far with genetic methods bear bacteria in their antennae that form a monophyletic group within the *Streptomyces* (Kaltenpoth 2006, Kaltenpoth pers. comm.) and all species in the present study possess well developed antennal glands in the same five antennomeres and show only limited differences in morphology. This suggests that antennal glands with reservoirs and streptomycetes were already present in the common ancestor of the genus *Philanthus*. However, we do not know whether symbiotic bacteria and antennal glands are an apomorphy in the genus *Philanthus*. To clear this point other taxa of the subfamily Philanthinae have to be investigated, especially the sister genus *Trachypus* (for the phylogeny of the subfamily Philanthinae see Alexander, 1992).

In conclusion, our results on the morphology of the antennal glands together with earlier studies on the phylogeny of the bacterial symbionts (Kaltenpoth et al. 2005, 2006) suggest that these unique glands are a common feature in the genus *Philanthus*. Whether there are species or related genera that possess ancestral glands with less elaborate gland morphology and have not yet established a symbiosis with bacteria has to be analysed by a more extensive analysis of the subfamily Philanthinae.

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## 6.6 References

- Alexander, B.A. (1992). A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 17: 91-108.
- Bohart, R.M., Menke, A.S. (1976). Sphecid wasps of the world. University of California Press, Berkeley and Los Angeles.
- Cafaro, M.J., Currie, C.R. (2005). Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Canadian Journal of Microbiology* 51 (6): 441-446.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (1999). Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398 (6729): 701-704.
- Currie, C.R., Bot, A.N.M., Boomsma, J.J. (2003a). Experimental evidence of a tripartite mutualism: bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101 (1): 91-102.
- Currie, C.R., Scott, J.A., Summerbell, R.C., Malloch, D. (2003b). corrigendum: Fungus-growing ants use antibiotic-producing bacteria to control garden parasites (vol 398, pg 701, 1999). *Nature* 423 (6938): 461-461.
- Evans, H.E., O'Neill, K.M. (1988). The Natural History and Behavior of North American Beewolves. Cornell University Press, Ithaca, New York.
- Goettler, W., Kaltenpoth, M., Herzner, G., Strohm, E. (2007). Morphology and ultrastructure of a bacteria cultivation organ: The antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36: 1-9.
- Kaltenpoth, M., Goettler, W., Herzner, G., Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479.
- 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: 1403-1411.
- Poulsen, M., Bot, A.N.M., Currie, C.R., Nielsen, M.G., Boomsma, J.J. (2003). Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. *Functional Ecology* 17: 260-269.
- Strohm, E. (1995). Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). *Wissenschaftliche Schriftenreihe Biologie* 6.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.

## CHAPTER 7

**A ‘SOCIAL’ GLAND IN A SOLITARY WASP? THE POSTPHARYNGEAL GLAND OF FEMALE EUROPEAN BEEWOLVES (HYMENOPTERA, CRABRONIDAE)**

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### 7.1 Summary

Exocrine glands play an important role in maintaining the integrity of colonies of social Hymenoptera. The postpharyngeal gland (PPG) of ants is crucial for the generation of a nest odour that enables nestmate recognition. The evolutionary history of this gland is unknown and it was thought to be restricted to ants. Here we describe an exocrine head gland in females of a solitary crabronid wasp, the European beewolf, *Philanthus triangulum*, that resembles the PPG of ants in many respects. The newly described gland has the same location and the same glove like shape as in ants, and it also has a monolayered epithelium with similar ultrastructure. Unlike in ants, the epithelium bears hairs that reach into the lumen of the gland. Although the PPG of beewolves serves a completely different function it is also associated to an allogrooming behaviour as in ants. Based on these morphological and behavioural similarities as well as similarities in the chemical composition of the content of the PPG of both taxa we hypothesise that the PPGs of ants and beewolves have a common evolutionary origin. Thus, our results suggest that the PPG in ants might not have evolved in response to social requirements but might have already existed in solitary predecessors.

## 7.2 Introduction

Social Hymenoptera possess of a huge variety of exocrine glands that serve different functions in communication among nestmates and colony defence (e.g. Hölldobler and Wilson, 1990; Downing, 1991). One type of gland that plays a key role for the organisation and integrity of a eusocial group is the postpharyngeal gland (PPG) of ants. It has been shown that in several ant species workers take up as well as deliver cuticular hydrocarbons during allogrooming of nestmates and/or trophallaxis. These compounds are stored in the PPG (Vienne et al., 1995; Lenoir et al., 2001). Additionally, hydrocarbons that are synthesized in the fat body are sequestered by the PPG from the hemolymph (Soroker and Hefetz, 2000). Thus, the hydrocarbons of different nestmates are stored and mixed in the PPG, and continuously spread among the members of the colony (Bagnères and Morgan, 1991; Hefetz et al., 1992, Soroker et al., 1994; 1995; 1998). This mechanism generates the specific “Gestalt” odour of the colony (Crozier and Dix 1979; Soroker et al., 1994, Hefetz et al., 1992; Dahbi and Lenoir, 1998; Lenoir et al., 1999; Oldham et al., 1999). In some ant species workers probably feed the content of the PPG to young larvae (reviewed in Eelen et al., 2006). Moreover, in ant queens the PPG may contain pheromones that signal her identity or fertility (Vargo and Hulsey, 2000; Dietemann et al., 2003) and the lipids stored in the PPG might be used for egg production (Eelen et al., 2006). Since several other taxa of Hymenoptera (bees, paper wasps) do not possess such glands, the PPG was assumed to occur only in ants (Hölldobler and Wilson, 1990; Schoeters and Billen, 1997; Lenoir et al., 1999; Eelen et al., 2006).

The hitherto assumed exclusive occurrence of the PPG in ants suggested that it might have evolved *de novo* in response to the necessity of having an organ for the uptake, storage, and mixing of cuticular hydrocarbons of nestmates to create the chemical colony badge (Crozier and Dix 1979, Lenoir et al. 1999). An alternative is, however, that the gland might have already existed in ancient solitary predecessors of ants but with a different function (Lenoir et al. 1999). Since PPGs are not known to occur in bees and vespid wasps, this scenario implies that the PPG was lost in all other taxa of the Hymenoptera, making this second scenario *a priori* less plausible. However, there is no extensive knowledge of the occurrence of PPGs in taxa other than ants. If there were other Hymenoptera that possess a PPG, the second scenario would become a valid alternative to the first. Moreover, such a finding might provide new insights into the evolution of this gland and its important function in nestmate recognition.

Here, we describe a cephalic gland in females of a solitary digger wasp (Crabronidae, formerly Sphecidae; Melo, 1999), the European beewolf, *Philanthus triangulum* that closely resembles the PPG of ants in morphological details. We discuss our findings of this presumptive PPG in this solitary wasp with regard to the evolutionary origin of the 'social' PPG in ants.

### 7.3 Materials and Methods

Adult female European beeswolves were taken from a breeding population in the field in Central Germany or from a laboratory population (see e.g. Strohm and Linsenmair, 1997). Heads were dissected under a stereomicroscope. Alternatively, entire glands were obtained by removing the maxillae of beeswolf females, holding the hypopharyngeal plate with tweezers and gently pulling it out through the mouth opening. For investigation of the fresh glands they were transferred to microscope slides and examined under a compound microscope. For comparison, we dissected PPGs from workers of *Camponotus floridanus* (Formicidae) in a similar manner.

Histological investigations of female heads as well as of the heads of *C. floridanus* workers were conducted using standard histological methods (see e.g. Strohm and Linsenmair, 1995). To enable the embedding medium to soak into the head capsules of the specimens, small pieces of the head cuticle were cut off laterally using razor blades prior to embedding. Semithin sections (1-4  $\mu\text{m}$ ) were cut on an ultramicrotome (Reichert Ultracut) using diamond knives (Diatome). The sections were either stained with methylene blue/ Azur II or with the Azan staining after Heidenhain (Böck, 1989).

To reconstruct the overall shape and localisation of the gland in the head, we took digital pictures (Nikon Coolpix 990) of a series of semithin sections and manually marked the outer margins of the gland as well as the brain, mandibles, and eyes using the 3D-visualization-software Amira<sup>®</sup> (Indeed-Visual Concepts, Berlin, Germany). Then the consecutive pictures were manually aligned with regard to each other and combined to yield a 3D view of the gland and other organs of the head, with the different parts in different colours. Since we had to remove the lateral part of the head capsule to allow the embedding medium to penetrate into the head, the respective parts are missing in the reconstruction.

To observe the structure of the inner side of the epithelium, dissected glands were fixed in alcoholic Bouin (Böck, 1989) for 3 hours, and cut in pieces with a razor blade. The specimens were washed in 70% ethanol (2x), and dehydrated in a graded acetone series. The objects were then critical point dried (BAL-TEC CPD 030), sputtered with Pt/Pd (BAL-TEC SCD 005) and viewed under a Zeiss DSM 1962 at 15 kV. Pictures were taken on Ilford FP4 film (24x36 mm). For ultrastructural investigations of the tissue, dissected glands were fixed in a solution of 2% formalin, 2.5% glutardialdehyde, and 5% sucrose in PBS. After postfixation in 2% OsO<sub>4</sub> in PBS and dehydration in a graded ethanol series, the specimens were embedded in Epon 812. Ultrathin sections (about 50nm, Reichert Ultracut E microtome / 45° diamond knife, stained with 2% uranyl acetate and Reynold's lead citrate) were investigated using a Zeiss EM10 transmission electron microscope at 80 kV (TEM). Pictures were taken on Agfa Scientia film (6x9 cm).

## 7.4 Results

### 7.4.1 Overall appearance

Beewolf females possess a large gland reservoir that originates from the posterior part of the hypopharyngeal plate. The H-shaped sclerotised (light to dark brown) hypopharynx is about 2.3-2.6 mm long and 0.35-0.4 mm wide (the head width of females is about 3.8-4.6mm). Immersing the isolated gland in water spreads out the gland and reveals an overall glove-like structure [Fig. 7.1A]. The gland reservoir consists of paired lateral evaginations of the pharynx and an additional median (unpaired) sac-like evagination that extends ventrally of the pharynx. The paired sclerotised (dark brown) elongations forming the hypopharyngeal suspensorium are slightly bowed outwards and form the anterior rim of the glove-like parts of the gland reservoir. The glove-like part on each side consists of a main branch and 10–15 ‘fingers’. These ‘fingers’ are 0.6-1 mm long and 0.2-0.25 mm wide. The sac-like part of the reservoir is nearly spherical with a diameter of about 0.4 –0.5 mm. On the basis of these dimensions the maximum volume of the filled gland reservoir can be calculated to be about 3-4  $\mu\text{l}$ . The walls of the gland reservoir are thin and translucent. The content of the gland is whitish in some individuals but mostly yellowish and oily.

### 7.4.2 3D-reconstruction

The 3D-reconstruction [Fig. 7.2] visualizes the overall location and arrangement of the gland in the head capsule of beewolf females. Similar to the immersed dissected gland, the fingers of the lateral part of the reservoir diverge somewhat from the main branch. The gland is located in the forehead area in the upper two thirds of the head capsule and extends horizontally between the anterior margins of the eyes and vertically up to the position of the median ocellus. A layer of air sacs separates the gland from the brain.

### 7.4.3 Light microscopy

Semithin sections confirmed that the reservoir has two different parts. The unpaired sac-like part is located ventrally of the pharynx whereas the two branches of the glove-like part are located laterally somewhat above the pharynx, with the main branches extending laterally and the ‘fingers’ extending dorsally. Some of the ‘fingers’ of the gland are located directly subjacent to the front cuticle and cuticular epithelium of the upper head and are separated from the brain by air sacs. The wall of the gland reservoir is formed by a monolayered epithelium [Fig. 7.3A,B,C]. The cells are 15-30  $\mu\text{m}$  wide and 10-25  $\mu\text{m}$  high. The shape of the cells varies considerably within the epithelium; they are either flattened quadrangular, pentangular or nearly triangular with the tip pointing towards the lumen of the reservoir. Noteworthy, the inner sides of these cells bear several (Fig. 7.3A,B,C, apparently about 2-5 in the semi-thin sections) hairs (in the triangular and pentangular cells the hairs are located on the ‘tips’). These hairs occur in both, the sac-like and the glove-like parts of the reservoir and resemble the

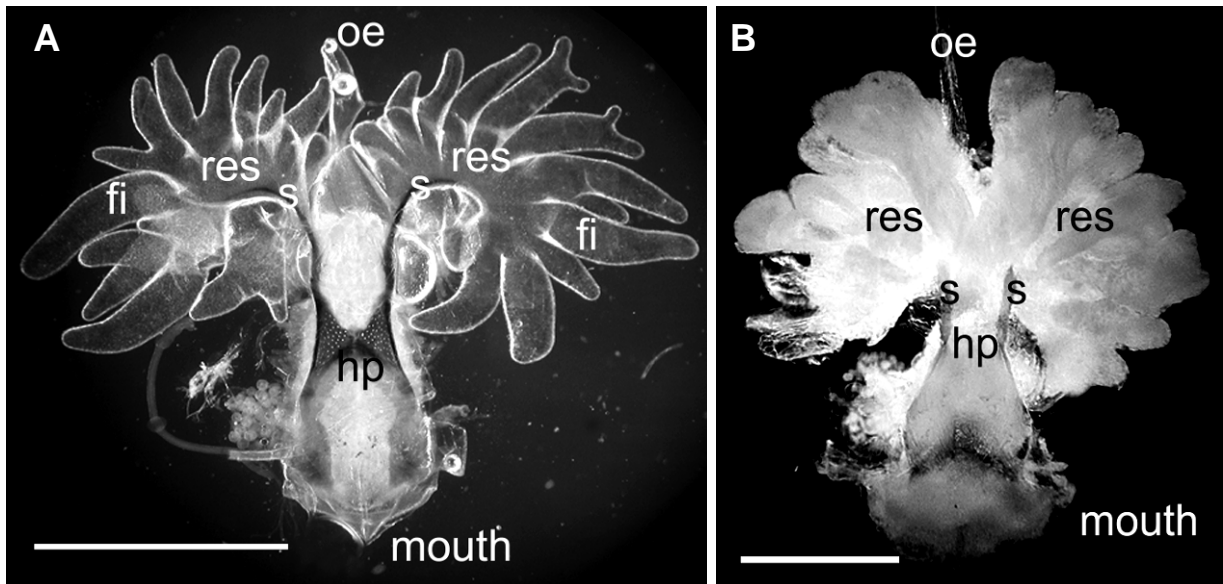


Fig. 7.1. Dissected postpharyngeal gland of **A**: a female European beewolf, scale bar = 1 mm, and **B**: a worker of the ant species *Camponotus floridanus* scale bar = 0.25 mm. The basic similarities of the glove-like gland reservoir and the location relative to the hypopharynx are obvious. fi, finger; hp, hypopharyngeal plate; oe, oesophagus ; res, reservoir; s, suspensorium.

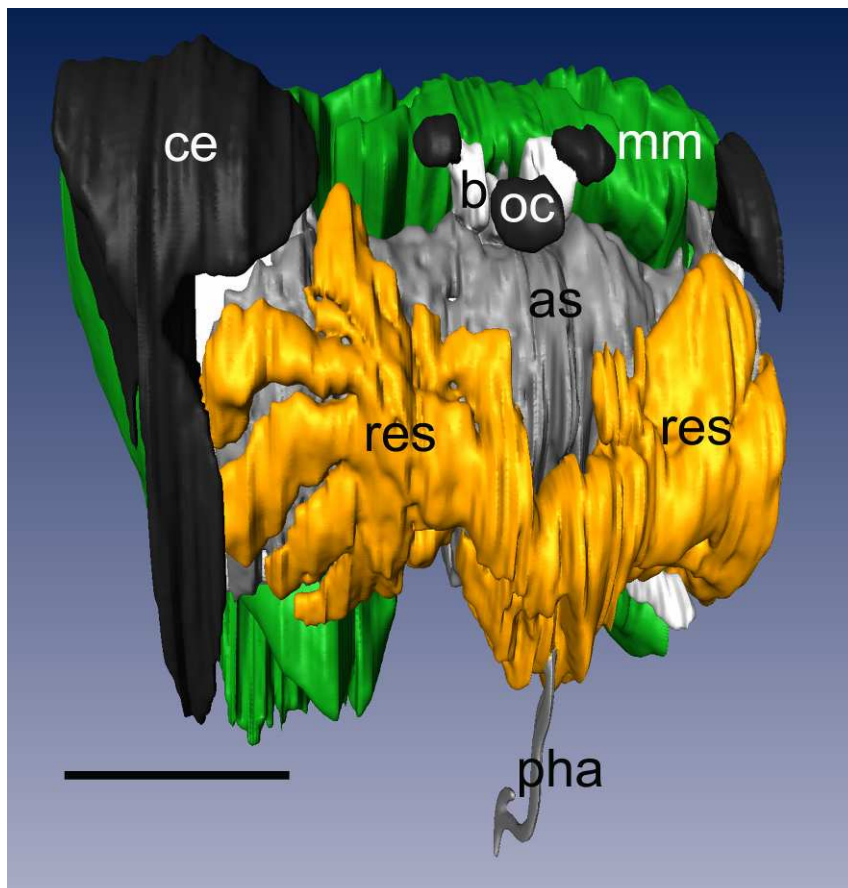


Fig. 7.2. 3D-reconstruction of the PPG in the head of a female of the European beewolf (frontal view). The glove-like part of the gland (yellow) extends dorso-laterally from the hind-pharynx to the inner border of the compound eyes. The sac-like part that extends ventrally from the pharynx is not visible in this view (see supporting online material, movie 1). (Note: due to cutting off of the outer parts of the head, these parts could not be reconstructed). as, air sac (grey); b, brain and nerves (white); ce, compound eye (black); mm, mandibular muscles (green); oc, ocelli (black); pha, pharynx (grey); res, reservoir of the PPG (yellow); scale bar = 1 mm.

hairs on the inner walls of the pharynx. However, in the pharynx the hairs are longer (about 1.5 x), more regularly arranged, and are associated with a thick layer of connective tissue.

No typical class III gland cells with duct cells (Noirot and Quennedey, 1974; Quennedey, 1998) occur in the epithelium and no such cells are connected to the gland reservoir [Fig. 7.3A]. Furthermore, some epithelial cells contain a few conspicuous large vesicles. The nuclei are oval and large (8-10  $\mu\text{m}$ ) in relation to the amount of cytoplasm. The epithelium of the gland reservoir mostly borders on air sacs that resemble the gland reservoir when viewed at low magnifications. At higher magnifications air sacs and the gland reservoir can be easily distinguished since the former show the typical taenidia on the inner surface, whereas the latter bears the already mentioned hairs on the inner surface. In some regions the epithelium borders a tissue of large rounded or slightly oval fat cells (diameter: 28-35  $\mu\text{m}$ ) some of which contain large numbers of colourless (and rarely yellowish) vesicles [Fig. 7.3A].

The opening of the ventral sac-like part of the reservoir to the pharynx bears a fringe that contains a ring muscle [Fig. 7.3B,C]. This muscle is more pronounced in the anterior part of the fringe (about 50 muscle cells about 150-200  $\mu\text{m}$  long, 80-100  $\mu\text{m}$  wide) compared to the posterior part (about 10 muscle cells, 20-25  $\mu\text{m}$  long, 8-10  $\mu\text{m}$  wide). Furthermore, the anterior part of the opening is terminated by a brim (50-150  $\mu\text{m}$  long, 25-50  $\mu\text{m}$  wide, Fig. 7.3C). Possibly, the sac like part of the reservoir can be closed and opened by contraction and relaxation of this ring muscle. The anterior brim might cover the remaining opening when the ring muscles are contracted.

The two branches of the glove-like part of the reservoir open to the pharynx laterally and dorsally to the middle axis of the pharynx. These openings also show thin brims both at their anterior and their posterior side but no ring muscle is visible. However, there are several muscles connected to the hypopharynx. A set of muscles that runs upwards from the sclerotised elongations of the hypopharyngeal suspensorium to the cuticle of the head capsule might close the gland when the fibres contract. Another set of muscles that runs more or less downwards to the cuticle of the head capsule might be involved in the closing of the gland. Several other muscles that run transversally might also contribute to the control of the gland opening. However, since these muscles probably have additional other functions and might interact in a complex way, we cannot yet establish how the opening and closing of the gland is accomplished.



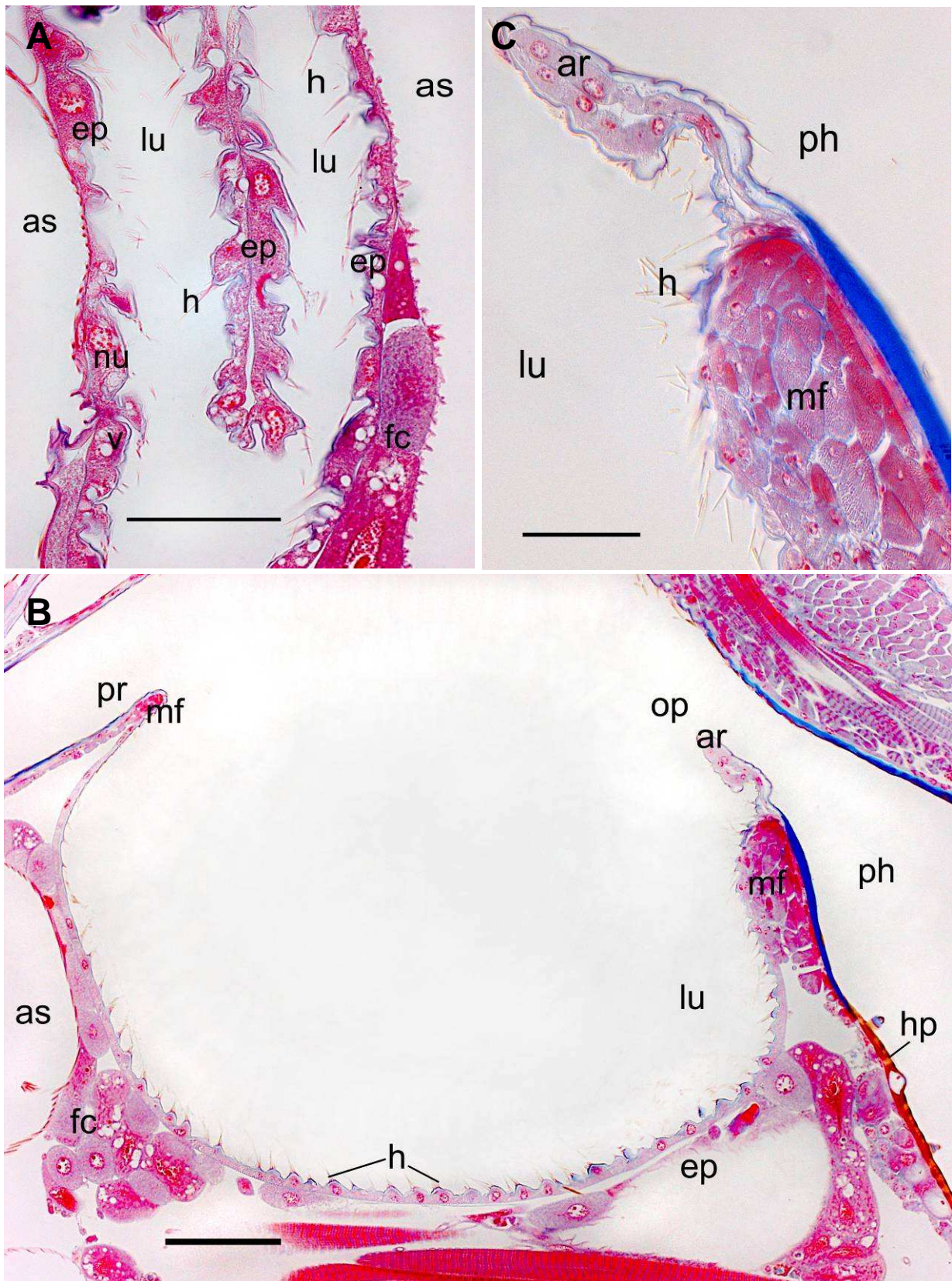


Fig. 7.3. **A** Semithin section of the glove like part of the PPG of female beewolves showing a branching point of two fingers surrounded by air sacs. scale bar = 50  $\mu\text{m}$ . **B** Semithin section of the lower part of the PPG of a female European beewolf and surrounding tissue. Note the nearly triangular shape of the cells of the epithelium and their apical hairs. scale bar = 150  $\mu\text{m}$ . **C** anterior rim of the opening of the lower part of the PPG with muscle fibres. scale bar 50  $\mu\text{m}$ . ar, anterior rim of the opening of the lower part formed by a brim; as, air sac; ep, epithelium of the PPG; h, hairs; hp, hypopharynx; fc, fat cell; lu, lumen of the PPG; mf, muscle fibres; nu, nucleus; op, opening; ph, pharynx; pr, posterior rim of the lower part. scale bar = 50  $\mu\text{m}$ .



#### 7.4.4 Ultrastructure

SEM investigation of dissected glands revealed that the outer wall has a reticulate surface [Fig. 7.4A] that apparently enlarges the surface area. The inner walls bear the hairs that were already visible in the semithin sections [Fig. 4B]. The length and structure of the hairs varies somewhat within the reservoir. Near the opening the hairs are about 30  $\mu\text{m}$  long, 2  $\mu\text{m}$  thick and their tips are spliced. In the more distal parts of the glove-like part, the hairs are located on more or less regularly spaced scale like structures in groups of 15-20 [Fig. 7.4B] and are about 20-40  $\mu\text{m}$  long.

Ultrathin sections revealed the typical organisation of a cuticle lining on the inner side of the reservoir [Fig. 7.5A], showing that the reservoir represents an exocrine gland. The scales that bear the hairs are extensions of the cells of the epithelium. The cells of the epithelium showed well developed smooth endoplasmatic reticulum, Golgi-apparatus, and some mitochondria. Microvilli appear in the apical region of the cells. Some conspicuous multilamellar bodies of different sizes are visible and most of them are localized in the apical region, associated with

the microvilli. Both the microvilli and the multilamellar bodies are not evenly distributed over the epithelium but seem to be concentrated in some areas. Furthermore, the microvilli are not as numerous and not as regularly shaped as in the epithelium of the PPG of *C. floridanus* workers and as reported in some earlier publications of the PPG of ants (e.g. Fig. 9 in Billen, 1991). Some electrolucent vesicles can be seen in the epithelium. Furthermore, septate desmosomes are visible. The basal part of the epithelium shows numerous invaginations [Fig. 7.5B] some of which seem to have extensions into the cell. At some places there are large rounded cells filled with numerous large electrolucent vesicles adjacent to the epithelium. These are the fat cells that can also be seen in the semithin sections.

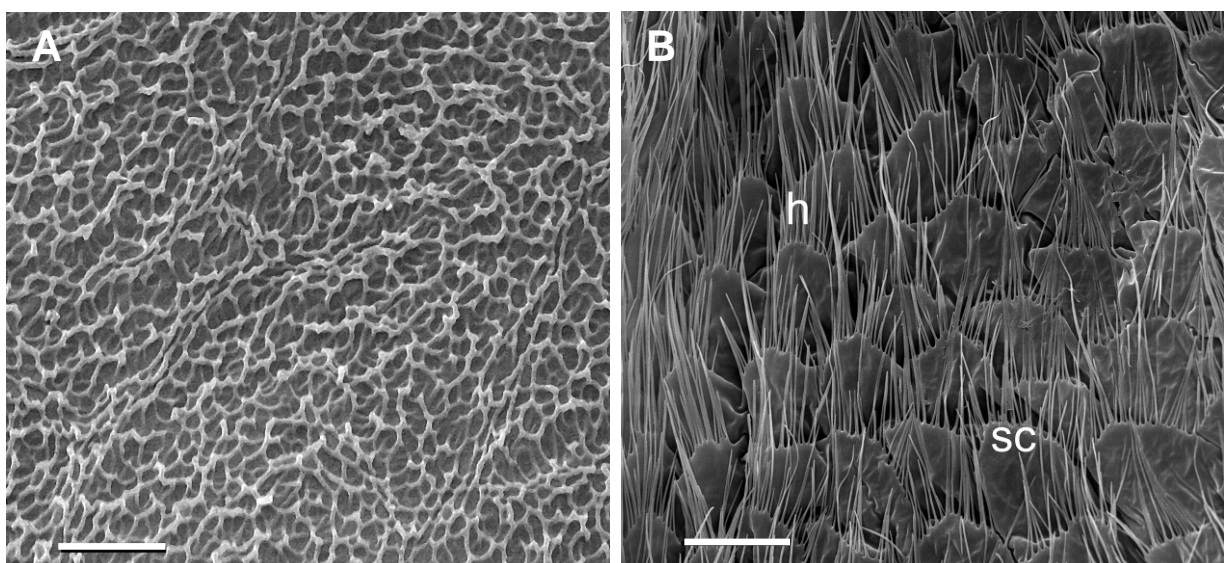


Fig. 7.4. **A** SEM picture of the outer wall of a dissected PPG of a female European beewolf showing the reticulate surface structure. scale bar = 2  $\mu\text{m}$ . **B** SEM picture showing the inner surface of the reservoir of a dissected PPG of a female European beewolf showing a regular arrangement of scale like structures bearing a number of hairs. h, hair; sc, scale; scale bar = 20  $\mu\text{m}$ .

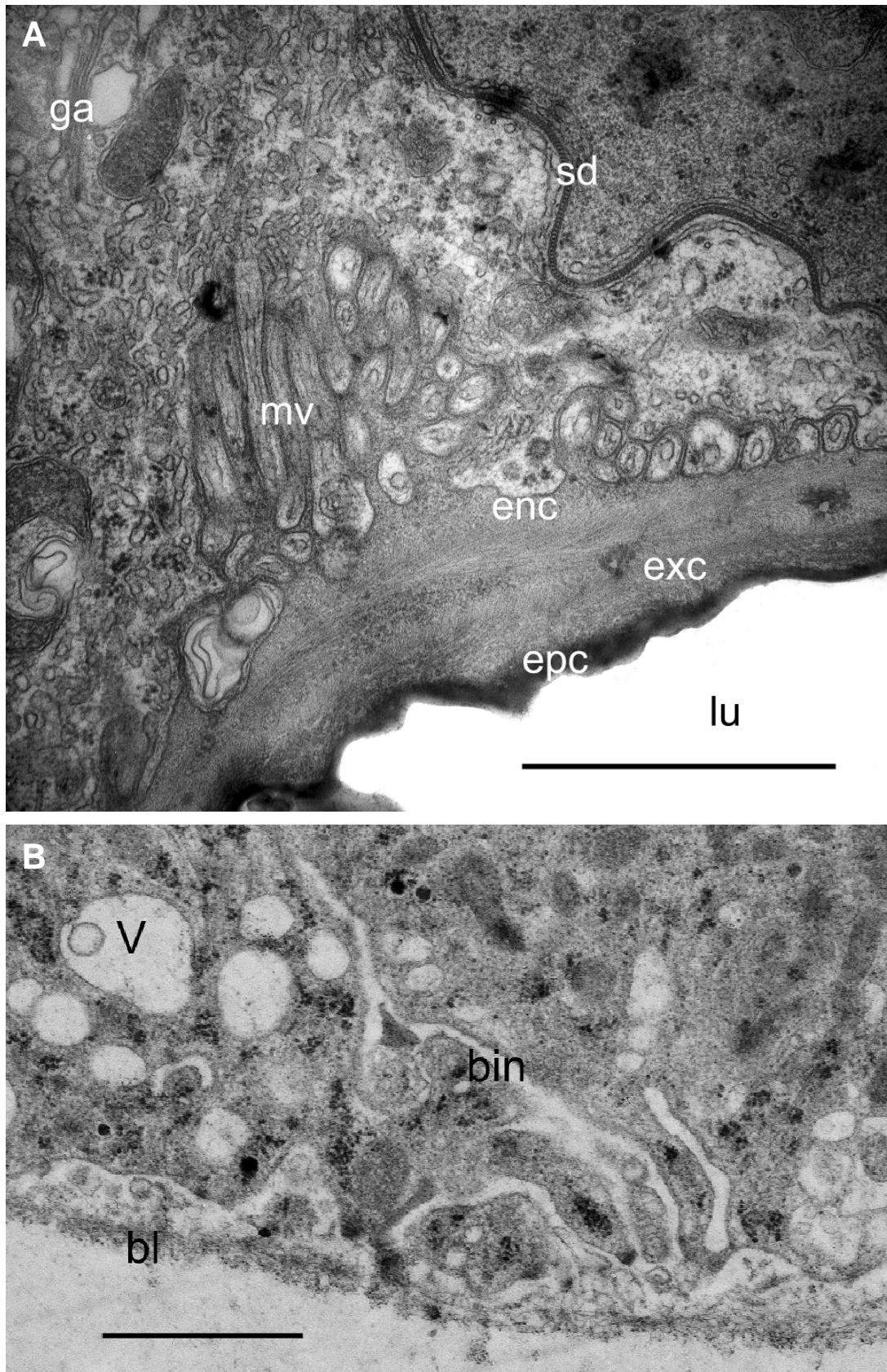


Fig. 7.5. **A** TEM picture of the apical region of the epithelium of the PPG of a female European beewolf, showing the well developed cuticle that lines the lumen of the gland. scale bar = 1  $\mu$ m. **B** TEM picture of the basal region of the epithelium, showing multiple invaginations and electrolucent vesicles; scale bar = 1  $\mu$ m. bin, basal invaginations; bl, basal lamina; enc, endocuticle; epc, epicuticle; exc, exocuticle; ga, Golgi apparatus; lu, lumen of the gland; M, mitochondria; mv, microvilli; sd, septate desmosome; sER, smooth endoplasmatic reticulum; v, vesicle.

#### 7.4.5 Comparison with *Camponotus floridanus*

The PPG of *C. floridanus* workers has a very similar general appearance as the described gland reservoir of beewolf females [Fig. 7.1B]. Dissected glands show a larger number of ‘fingers’, a shorter hypopharyngeal suspensorium, and the whole gland is more opaque. Semi-thin sections show that the location of the PPG is similar to the described reservoir of beewolf females. However, *C. floridanus* workers lack the ventral sac-like part of the gland in beewolves. The epithelium of the reservoir is also monolayered; the epithelial cells are mostly quadrangular and less variable in their shape than in beewolf females. Furthermore, in contrast to beewolves they contain numerous large vesicles that are possibly responsible for the opaque appearance of the gland. The most conspicuous difference is the lack of any hairs on the inner surface of the reservoir. The ultrastructure of the *C. floridanus* PPG also largely resembles the reservoir of beewolf females with conspicuous basal invaginations, but the layer of microvilli just beneath the cuticle is more regularly arranged. Other components of the epithelial cells of the PPG of *C. floridanus* do not differ strikingly from the sections of the reservoir of beewolf females.

### 7.5 Discussion

Females of the European beewolf possess a large gland reservoir in their heads that closely resembles the PPG of ants. The location of the glands is identical in both taxa as is their overall appearance, at least with regard to the glove like part of the reservoir in beewolf females. The structure of the walls of the glands of beewolves and ants (Pergrine et al., 1973; Soroker et al., 1995) appears to be largely similar (noteworthy, the PPG of some ant taxa has a deviating overall structure, Schoeters and Billen, 1997). The epithelium that forms the gland reservoir is monolayered in both taxa and there are apparently no secretory gland cells associated with the reservoir. The ultrastructure of the epithelium also shows similarities with regard to the well developed smooth endoplasmic reticulum and the microvilli (Billen, 1991) as well as the basal invaginations and the concentration of mitochondria in the apical (inner) region of the epithelial cells (Eelen et al., 2006).

However, there are also some differences in gland anatomy between beewolves and ants. Ants seem to completely lack the sac-like, ventral part of the reservoir that occurs in beewolves. The cells of the epithelium differ somewhat in shape and they contain more and larger lipid vesicles in ants (Peregrine et al., 1973; Soroker et al., 1995; Schoeters and Billen, 1997). Furthermore, the PPG of ants lack the hairs found on the inner walls of the gland in *P. triangulum*. The function of these hairs is not yet known. Since the hairs are not connected to muscles it is unlikely that they move to mix the content of the gland. Possibly, the hairs prevent that the gland collapses when nearly empty. Another difference is that the microvilli in the apical region of the epithelium are more abundant and more regularly

arranged in ants compared to beewolves. Nevertheless, the striking similarities of the described gland in beewolf females with the PPG of ants suggest that the former is also a PPG.

The occurrence of a PPG in beewolves is remarkable since it has been assumed that this gland is idiosyncratic to ants (Hölldobler and Wilson, 1990; Schoeters and Billen, 1997, Lenoir et al., 1999; Eelen et al., 2006). Ants are not closely related to crabronids (Brothers, 1999). Thus, the PPG exhibits a peculiar phylogenetic pattern.

Interestingly, the PPG of beewolves and ants contains similar classes of chemicals. Mainly hydrocarbons have been found both in ants (e.g. Hefetz et al., 1992; Soroker et al., 1995a,b) and in beewolf females (E. Strohm, G. Herzner, T. Schmitt, unpubl. data, see also Herzner et al., 2007; Kroiss et al., 2006; for the morphology and chemistry of the respective gland in male beewolves). In ants, the gland has a function in communication among nestmates (e.g., Hefetz et al., 1992; Soroker et al., 1994; 1995b; 1998; Vienne et al., 1995). There is evidence that in ants the content of the PPG is sequestered from the own cuticle and/or hemolymph and/or from nestmates during allogrooming. These compounds are then mixed and applied to the own cuticle and delivered to nestmates during repeated allogrooming (Hefetz et al., 1992). This process results in a mixing and unification of the individual odours and provides a mechanism for the formation of a chemical colony badge (for possible other functions see e.g., Schoeters and Billen, 1997, Eelen et al., 2006). Thus, the PPG is associated with (allo-) grooming behaviour in ants.

Beewolf females might also spread the content of the PPG over their own bodies during grooming. This could be the initial function of the gland in beewolves. However, the current main function of the gland in beewolves seems to be the protection of the prey against fungus (Strohm and Linsenmair, 2001). Prior to provisioning of the brood cells, beewolf females extensively lick the whole surface of the paralysed honeybees that serve as larval food, a behaviour that, although it is directed to a prey, might be considered as allogrooming (Strohm and Linsenmair, 2001). During this treatment, females apply chemicals from the PPG onto the cuticle of the prey (G. Herzner, T. Schmitt, E. Strohm, unpubl. data).

Assuming that the large reservoir in beewolf females is a PPG, its evolutionary origin might be homologous to the one in ants. Alternatively, it may have evolved independently in both taxa. Despite the considerable similarity, a convergent evolution of the PPGs in two unrelated taxa cannot be excluded. Hymenoptera show extremely diverse glands and some taxa have glands that do not occur in any other Hymenoptera (e.g. Isodoro et al., 1999; 2000; Gobin et al., 2003). There is, for example, a unique gland in the antennae of beewolves (Strohm and Linsenmair, 1995; Kaltenpoth et al., 2005, Goettler et al., 2007). Thus, the evolution of new gland systems seems to occur frequently in

Hymenoptera. Therefore, the similarities might have evolved due to shared selection pressures. For example, the overall glove like shape of the PPG with several ‘fingers’ both in ants and beewolves might reflect the need for an enlarged surface to increase the rate of uptake of hydrocarbons from the hemolymph. Remarkably, in ants the rate of sequestration of the PPG content is probably much lower than in beewolves who have to embalm several prey items per day with the secretion. We have estimated that beewolf females have to apply a mean of about 300  $\mu\text{g}$  of PPG secretion per day (G. Herzner, T. Schmitt, E. Strohm unpubl. data). The location of the reservoir at the proximal end of the pharynx might be ideal for the delivery of long chain hydrocarbons through the mouth. However, the evolutionary starting points in ants and beewolves are different and the primary selection pressures that have promoted the evolution of the PPG also differ considerably. Noteworthy, the labial gland in honeybees might serve a function that is similar to that of the PPG of ants (Katzav-Gozansky et al., 2001). Thus, the generation of a nest odour, might just as well be accomplished by a different kind of gland. Thus, the hypothesis that such similar PPGs have evolved by chance in ants and beewolves despite the differences in evolutionary background might be considered equivocal at least.

Is there any evidence supporting the hypothesis that the PPG of ants and beewolves are homologous? One concordance with regard to the ecology is that both taxa spend most of their time below ground. This might make an effective protection against the multitude of microbial pathogens that occur in the soil more important than for species that nest above ground. Thus, one possible scenario for the evolution of the PPG is that it evolved in a common ancestor that was a solitary, soil nesting species that may have used the PPG to store compounds for the protection of its own body or of its prey against microbial attack. The PPG of beewolves and ants would then be homologous. That this gland has so far only been described from ants and the European beewolf does not necessarily mean that it does not exist in other aculeate Hymenoptera, since not all groups were carefully screened for the occurrence of a PPG. Noteworthy, males of the European beewolf also possess a PPG that was very hard to discover due to its similarity with air sacs (Herzner et al., 2007). A total absence of PPGs in all aculeate Hymenoptera except beewolves and ants would make a homologous origin questionable though not impossible.

It is interesting to consult recent phylogenetic trees to assess the plausibility of the homology hypothesis. Using the phylogenetic reconstruction provided by Brothers (1999), an evolution of a PPG in early aculeates and a subsequent loss in all taxa but the genus *Philanthus* (preliminary investigations show that other species of the subfamily Philanthinae have the gland, but other crabronids and sphecids do not, E. Strohm, G. Herzner, unpubl. data) and the family Formicidae would require at least 8-10 evolutionary steps (losses). Additional consideration of the phylogenetic relationships within the digger wasps (*sensu lato*) indicates that there are an additional 7 (Brothers 1999) or 11 (Melo 1999) losses necessary. In contrast, assuming that the PPGs of beewolves and ants

have evolved independently only two evolutionary changes (gains) would be necessary. However, these calculations are based on the assumption that no other taxa possess a PPG. The fact that the European beewolf is one of the best studied solitary aculeates (Tinbergen, 1932; 1935; Rathmayer, 1962; Strohm, 2000; Strohm and Lechner, 2000; Strohm and Linsenmair, 1995; 1997; 1999; 2000; 2001) but its PPG has only been described now suggests that there might be PPGs awaiting discovery in hitherto neglected taxa. Furthermore, in such calculations, losses and gains of traits are considered to be equivalent evolutionary steps. However, the loss of a structure might involve fewer microevolutionary steps than the *de novo* evolution of a complex structure. Therefore, the overall probability for a loss and an acquisition might differ considerably (Felsenstein, 1983; Wiens et al., 2001). As a consequence, a phylogeny with many losses might still be more likely than a competing hypothetical phylogeny with few gains.

In conclusion, the discovery of the PPG in beewolves and the investigation of its functional significance may provide new insights into the evolution of a gland that is known to play an important role for communication and colony organisation in the highly eusocial ants.

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## 7.6 References

- Bagnères, A.G., Morgan, E.D. (1991). The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 47: 106-111.
- Billen, J. (1990). Phylogenetic aspects of exocrine gland development in the Formicidae. Pages 317-318 in Veeresh, G.K., Mallik, B., Viraktamath, C.A., eds. *Social insects and environment*. Oxford and IBH Publishing, New Delhi, Bombay, Calcutta.
- Billen, J. (1991). Ultrastructural organization of the exocrine glands in ants. *Ethology Ecology & Evolution* (1): 67-73.
- Böck, P. (1989). *Romeis Mikroskopische Technik*. Urban & Schwarzenberg, München, Wien, Baltimore.
- Brothers, D.J. (1999). Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). *Zoologica Scripta* 28 (1-2): 233-249.
- Crozier, R.H., Dix, M.W. (1979). Analysis of two genetic models for the innate components of colony odour in social Hymenoptera. *Behavioral Ecology and Sociobiology* 4: 217-224.
- Dahbi, A., Cerda, X., Lenoir, A. (1998). Ontogeny of colonial hydrocarbon label in callow workers of the ant *Cataglyphis iberica*. *Comptes Rendus de L'Academie des Sciences - Series III - Sciences de la Vie* 321: 395-402.
- Dietemann, V., Peeters, C., Liebig, J., Thivet, V., Hölldobler, B. (2003). Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. *PNAS* 100: 10341-10346.
- Downing, H.A. (1991). The function and evolution of exocrine glands. Pages 540-569 in Ross, K.G., Matthews, R.W., eds. *The social biology of wasps*. Cornstock, Ithaca.
- Eelen, D., Borgesen, L., Billen, J. (2006). Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 87: 101-111.
- Felsenstein, J. (1983). Parsimony in systematics: biological and statistical issues. *Annual Review of Ecology and Systematics* 14: 313-333.
- Gobin, B., Ito, F., Billen, J. (2003). The subepithelial gland in ants: a novel exocrine gland closely associated with the cuticle surface. *Acta Zoologica* 84: 285-291.
- Goettler, W., Kaltenpoth, M., Herzner, G., Strohm, E. (2007). Morphology and ultrastructure of a bacteria cultivation organ: The antennal glands of female European beewolves, *Philanthus triangulum* (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36: 1-9.
- Hefetz, A., Errard, C., Cojocar, M. (1992). The occurrence of heterospecific substances in the postpharyngeal gland secretion of ants reared in mixed species colonies (Hymenoptera: Formicidae). *Naturwissenschaften* 79: 417-420.
- Herzner, G., Goettler, W., Kroiss, J., Pura, A., Webb, A.G., Jakob, P.M., Rössler, W., Strohm, E. (2007). Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 36 (2): 123-133.
- Hölldobler, B., Wilson, E.O. (1990). *The ants*. Harvard University Press, Cambridge.
- Isidoro, N., Bin, F., Romani, R., Pujade-Villar, J., Ros-Farre, P. (1999). Diversity and function of male antennal glands in Cynipoidea (Hymenoptera). *Zoologica Scripta* 28 (1-2): 165-174.
- Isidoro, N., Romani, R., Velasquez, D., Renthal, R., Bin, F., Vinson, S.B. (2000). Antennal glands in queen and worker of the fire ant, *Solenopsis invicta* Buren: first report in female social Aculeata (Hymenoptera, Formicidae). *Insectes Sociaux* 47 (3): 236-240.
- Kaltenpoth, M., Goettler, W., Herzner, G., Strohm, E. (2005). Symbiotic bacteria protect wasp larvae from fungal infestation. *Current Biology* 15: 475-479.
- Katzav-Gozansky, T., Soroker, V., Ibarra, F., Francke, W., Hefetz, A. (2001). Dufour's gland secretion of the queen honeybee (*Apis mellifera*): an egg discriminator pheromone or a queen signal? *Behavioral Ecology and Sociobiology* 51: 76-86.
- Kroiss, J., Schmitt, T., Schreier, P., Strohm, E., Herzner, G. (2006). A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 32: 2763-2776.
- Lenoir, A., Fresneau, D., Errard, C., Hefetz, A. (1999). Individuality and colonial identity in ants: the emergence of the social representation concept. in Detrain, C., Deneubourg, J.L., Pasteels, J.M., eds. *Information processing in social insects*. Birkhäuser, Basel.
- Lenoir, A., Hefetz, A., Simon, T., Soroker, V. (2001). Comparative dynamics of gestalt odour formation in two ant species *Camponotus fellah* and *Aphaenogaster senilis* (Hymenoptera: Formicidae). *Physiological Entomology* 26: 275-283.

- Melo, G.A.R. (1999). Phylogenetic relationships and classification of the major lineages of apoidea (Hymenoptera), with emphasis on the crabronid wasps. *Scientific Papers, The Natural History Museum of University of Kansas* 14: 1-55.
- Noirot, C., Quennedey, A. (1974). Fine-structure of insect epidermal glands. *Annual Review of Entomology* 19: 61-80.
- Oldham, N.J., Morgan, E.D., Agosti, D., Wehner, R. (1999). Species recognition from postpharyngeal gland contents of ants of the *Cataglyphis bicolor* group. *Journal of Chemical Ecology* 25 (6): 1383-1393.
- Peregrine, D.J., Mudd, A., Cherrett, J.M. (1973). Anatomy and preliminary chemical analysis of postpharyngeal glands of leaf-cutting ant, *Acromyrmex octospinosus* (Reich) (Hym, Formicidae). *Insectes Sociaux* 20 (4): 355-363.
- Quennedey, A. (1998). Insect epidermal gland cells: ultrastructure and morphogenesis.
- Rathmayer, W. (1962). Das Paralysisierungsproblem beim Bienenwolf *Philanthus triangulum* F. (Hym. Sphec.). *Zeitschrift für vergleichende Physiologie* 6: 105-122.
- Schoeters, E., Billen, J. (1997). The post-pharyngeal gland in Dinoponera ants (Hymenoptera: Formicidae): Unusual morphology and changes during the secretory process. *International Journal of Insect Morphology & Embryology* 25 (4): 443-447.
- Soroker, V., Vienne, C., Hefetz, A. (1994). The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 81 (11): 510-513.
- Soroker, V., Hefetz, A., Cojocar, M., Billen, J., Franke, S., Francke, W. (1995a). Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 20 (4): 323-329.
- Soroker, V., Vienne, C., Hefetz, A. (1995b). Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera: Formicidae). *Journal of Chemical Ecology* 21: 365-378.
- Soroker, V., Fresneau, D., Hefetz, A. (1998). Formation of colony odor in Ponerine ant *Pachycondyla apicalis*. *Journal of Chemical Ecology* 24: 1077-1090.
- Soroker, V., Hefetz, A. (2000). Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. *Journal of Insect Physiology* 46: 1097-1102.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (1997). Low resource availability causes extremely male-biased investment ratios in the European beewolf, *Philanthus triangulum* F (Hymenoptera, Sphecidae). *Proceedings of the Royal Society of London Series B* 264 (1380): 423-429.
- Strohm, E., Linsenmair, K.E. (1999). Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behavioral Ecology and Sociobiology* 47: 76-88.
- Strohm, E. (2000). Factors affecting body size and fat content in a digger wasp. *Oecologia* 123: 184-191.
- Strohm, E., Lechner, K. (2000). Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 59: 183-191.
- Strohm, E., Linsenmair, K.E. (2000). Allocation of parental investment among individual offspring in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Biological Journal of the Linnean Society* 69 (2): 173-192.
- Strohm, E., Linsenmair, K.E. (2001). Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 26 (2): 198-203.
- Tinbergen, N. (1932). Über die Orientierung des Bienenwolfes (*Philanthus triangulum* Fabr.). *Zeitschrift für vergleichende Physiologie* 16: 305-335.
- Tinbergen, N. (1935). Über die Orientierung des Bienenwolfes II. Die Bienenjagd. *Zeitschrift für vergleichende Physiologie* 16: 305-355.
- Vargo, E.L., Hulsey, C. (2000). Multiple glandular origins of queen pheromones in the fire ant *Solenopsis invicta*. *Journal of Insect Physiology* 46 (1151-1159).
- Vienne, C., Soroker, V., Hefetz, A. (1995). Congruency of hydrocarbon patterns in heterospecific groups of ants - transfer and/or biosynthesis. *Insectes Sociaux* 42 (3): 267-277.
- Wiens, J.J. (2001). Widespread loss of sexually selected traits: how the peacock lost its spots. *Trends in Ecology and Evolution* 16: 517.





## CHAPTER 8

## MALES OF A SOLITARY WASP POSSESS A POSTPHARYNGEAL GLAND

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### 8.1 Summary

The postpharyngeal gland has long been thought to occur only in ants. Here we characterize, by use of light and electron microscopy as well as 3D reconstruction based on nuclear magnetic resonance (NMR) imaging data, a large cephalic gland reservoir of males of a solitary digger wasp, the European beewolf, *Philanthus triangulum*. Several lines of evidence suggest that this reservoir is a postpharyngeal gland. The gland reservoir originates from the posterior part of the pharynx and consists of two pairs of unbranched tubular structures that occupy a large portion of the head capsule. Its wall is composed of a unicellular epithelium that is lined by a cuticle. The gland contains a blend of hydrocarbons and compounds with functional groups, and we show that the hydrocarbon fraction of the pheromone is congruent with the hydrocarbons on the cuticle. We discuss the implications of our findings for the evolution of the postpharyngeal gland in ants.

## 8.2 Introduction

The postpharyngeal gland (PPG) has long been thought to be idiosyncratic to the Formicidae (Lenoir et al., 1999; Schoeters and Billen, 1997; Soroker et al., 1995a; Jackson and Morgan, 1993; Hölldobler and Wilson, 1990), where it is involved in the formation and distribution of a nest-specific chemical signature (the ‘gestalt odor’, Crozier and Dix, 1979) that mediates nestmate recognition (Lenoir et al., 1999; Soroker et al., 1998, 1995a,b, 1994; Vienne et al., 1995; Hefetz et al., 1992). It usually contains a multi-component blend of hydrocarbons (HCs) (Cabrera et al., 2004; Lucas et al., 2004; Soroker et al., 1995a; Hefetz et al., 1992; Vander Meer et al., 1982) that shows a high chemical congruency with the epicuticular chemical profile (Lucas et al., 2004; Soroker et al., 1995a; Do Nascimento et al., 1993; Bagnères and Morgan, 1991). In ant queens the PPG may contain some kind of queen pheromone (Dietemann et al., 2003; Vargo and Hulsey, 2000). For a review of additional hypothesis on the function of the PPG in ants see Eelen et al. (2006).

Recently, a PPG was found in females of a solitary hunting wasp, the European beewolf *Philanthus triangulum* F. (Hymenoptera, Crabronidae; formerly Sphecidae) (Strohm et al., 2007). While the structure, location, and chemical content of the PPG of beewolf females are similar to those of ants (Strohm et al., 2007; E. Strohm, G. Herzner, T. Schmitt, unpublished), its function is very distinct. Female *P. triangulum* hunt honeybees as food for their larvae and store the paralyzed bees in their subterranean nests (Herzner et al., 2005; Strohm, 2000, 1995; Strohm and Linsenmair, 2000, 1999). To preserve their prey from microbial degradation, beewolf females apply the secretion of their PPG to the surface of their prey (Herzner et al., unpublished). The PPG secretion prevents fungus growth on the larval provisions (Strohm and Linsenmair, 2001) and in this way enhances the survival probabilities of the offspring.

It is known that PPGs also occur in male ants (Phillips and Vinson 1980). Therefore we investigated males of the European beewolf in order to extend the knowledge on the distribution and possible functional variety of the PPG among Hymenoptera. Male beewolves establish and scent mark territories to attract conspecific females (Schmitt et al., 2003; Strohm and Lechner, 2000; Strohm, 1995; Evans and O’Neill, 1988; Simon Thomas and Poorter, 1972). In an accompanying study we show that this marking pheromone is stored in an extraordinary large cephalic reservoir (Kroiss et al., 2006). The aim of the current study is to assess by virtue of morphological and chemical analyses, whether this reservoir for the marking pheromone of male *P. triangulum* is a PPG.

To qualify as a PPG the reservoir has to meet the following criteria. First, its location should correspond to the PPG of ants and beewolf females, i.e. it should extend from the posterior part of the pharynx along the proximal spines of the suspensorium. Second, the wall of the reservoir should be formed by a unicellular epithelium and lined by a cuticle (Schoeters and Billen, 1997; Soroker et al.,

1995a; Peregrine et al., 1973). Third, in female *P. triangulum*, as in ants, the substances that are present in the PPG also comprise the cuticular chemical profile of the animals (E. Strohm, G. Herzner, T. Schmitt, unpublished). To test for a similar chemical congruency in male *P. triangulum* we compared the chemical profiles of the PPG content and the cuticle.

### 8.3 Material and Methods

#### 8.3.1. Specimens/Histological investigation

Adult male beeswolves were obtained from a field population in Würzburg or from a laboratory population (see e.g. Strohm and Linsenmair, 1997). They were anaesthetized with CO<sub>2</sub> and decapitated. The heads were then dissected in cold physiological saline (130 mM NaCl/5 mM KCl/4 mM MgCl<sub>2</sub>/5 mM CaCl<sub>2</sub>/15 mM HEPES/25 mM glucose/160 mM sucrose, pH 7.2) under a stereomicroscope. The first cut was performed horizontally close to the toruli. The second and third cuts were made vertically just medial to the eyes. The last cut was made horizontally from eye to eye between the mid ocellus and the lateral ocelli. The greatest care was taken to cut only through the cuticle but no subjacent structures. The cuticular flap and the subjacent air sacs were then carefully removed. The cuticular opening was carefully enlarged step by step so that finally the delicate glands could be removed from the head unharmed. The glands were then transferred onto microscope slides, immersed in physiological saline and examined under stereo- and compound microscopes.

Histological investigations of male heads were conducted using light microscopy following standard histological methods (see e.g. Strohm and Linsenmair, 1995). In brief, heads were fixed in alcoholic Bouin, then rinsed in 70% ethanol, dehydrated in a graded ethanol series and propylene oxide and embedded in Durcupan (Fluka, Deisenhofen, Germany). To enable the embedding medium to soak into the head capsules of the specimens, one or both eyes were cut off the head capsule using razor blades. Semithin sections (4 µm) were cut on a steel-blade microtome and stained with Methylene-Blue-Azur II (Böck, 1989). Specimens were viewed under a Zeiss Axioskop. Due to the partly low contrast of some delicate structures despite staining, we used phase contrast as well as differential interference contrast. Photographs were taken using a Zeiss AxioCam HRc digital camera and Zeiss AxioCam software (Carl Zeiss, Germany).

#### 8.3.2 Scanning electron microscopy

For scanning electron microscopy (SEM), specimens were fixed in alcoholic Bouin for 3 hours, washed in 70% ethanol twice, and dehydrated in a graded acetone series. The objects were then critical point dried (BAL-TEC CPD 030), sputtered with Pt/Pd (BAL-TEC SCD 005) and examined through a digital Zeiss DSM 962.

### 8.3.3 Transmission electron microscopy

Objects for transmission electron microscopy (TEM) were fixed overnight at 4°C in a solution of 2.5% glutardialdehyde and 2% formaldehyde in Sörensen phosphate buffer. After postfixation in 2% OsO<sub>4</sub> in Sörensen phosphate buffer and dehydration in a graded ethanol series, the specimens were embedded in Epon 812. The ultrathin sections of about 70 nm thickness (Reichert Ultracut E microtome / 45° diamond knife) were stained with 2% uranyl acetate and Reynold's lead citrate. The sections were examined with a Zeiss EM10 at 80 kV. Unfortunately, the fine structure of the cells of the epithelium that forms the wall of the reservoir was not optimally conserved in all regions of the reservoir, although – besides the ones described - we tried several different fixatives and conditions during fixation. Thus, some very fine structures might have been lost. We nevertheless provide the results here, since some important evidence could be gained.

### 8.3.4 Nuclear magnetic resonance imaging and 3D-reconstruction

Since the dissections did not fully reveal the position of the glands in the head, we reconstructed the cephalic structures in the head by use of nuclear magnetic resonance (NMR) imaging. A male beewolf (head width 3.3 mm) was anaesthetized with CO<sub>2</sub> and killed with diethyl ether. After decapitation the head was immersed in 100% ethanol and kept for 1 hour in an exsiccator with water jet vacuum. In this manner the air sacs were filled with liquid so that air could not cause susceptibility artifacts in the imaging experiment. The head was transferred to a 5 mm NMR tube filled with Gadovist (5 mM) (Schering, Berlin, Germany) as a contrast agent and evacuated for another 30 minutes. NMR imaging was carried out on a 17.6 T (750 MHz) widebore magnet using AVANCE console, Micro2.5 microimaging gradients capable of 1 T/m maximum gradient strength and a 5 mm birdcage coil (Bruker Analytic, Rheinstetten, Germany). Three-dimensional data sets were obtained using a 3D FLASH sequence. Data acquisition parameters were TR 40 ms, TE 3.0 ms, number of averages 6, a data matrix of 256 x 256 x 256 points and an isotropic spatial resolution of (20 µm)<sup>3</sup>. Total data acquisition time was 4.4 hours.

3D-reconstruction based on the NMR-data was conducted using the 3D-visualization-software AMIRA (Indeed-Visual Concepts, Berlin, Germany). Different structures in the head were manually marked with different colors, so that finally these structures could be visualized in their natural arrangement. The results were in perfect accordance with the structure that could be inferred from the dissections and from histological analyses. Thus, there is no evidence for any significant artifact caused by the treatment or the NMR experiment. The volumes of the head capsule and of the different parts of the gland reservoir were determined in the reconstructed head.

### 8.3.5 Extracts

Newly emerged males of our laboratory population were individually marked and released into an environmental chamber (240x180x210 cm; 26/22°C day/night and 14h/10h light/dark cycle) containing sand-filled buckets for nesting and artificial territories. The animals were provided with honey *ad libitum*. These conditions induce males to establish and scent mark territories (Strohm, 1995). Since pheromone composition is age dependent (Kaltenpoth and Strohm, 2006), all males used for the analyses were about the same age. Twelve to fourteen days after emergence, males were caught and stored individually in small polystyrene vials (35mm diameter, 82mm length, filled with 2 cm moist sand) with rubber foam plugs for two days, so that they could replenish their glands. They were then individually frozen at -18°C. For chemical analyses, 14 males were thawed, their heads were cut off, affixed by an insect needle and dissected under a stereo microscope as described above (but without the saline). Dissection was carried out on sheets of filter paper that were renewed for each male. Instead of removing the gland from the head, we took a sample of the pure PPG content by inserting a fine Pasteur pipette directly into the gland. The secretion was automatically sucked into the pipette by capillary forces. The sample was then dissolved in re-distilled hexane. All dissection instruments were cleaned in re-distilled hexane prior to the handling of the next specimen. The remaining thoraces and abdomens were (both parts combined but for each male individually) extracted in distilled hexane for five minutes (surface washes). The volumes of the extracts were reduced to approximately 100 µl by a stream of nitrogen at ambient temperature. An aliquot of 1µl of each sample was analyzed by combined gas chromatography - mass spectrometry.

### 8.3.6 Gas chromatography – mass spectrometry

GC-MS analysis was performed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with an RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID;  $df = 0.25\mu\text{m}$ ), and the temperature program ramped from 60°C to 300°C with 5°C/min. The temperature was held constant in the beginning at 60°C for 1 min and at the end at 300°C for 10 min. Helium was used as carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250°C and in the splitless mode for 60 sec. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70 eV, a source temperature of 230°C and an interface temperature of 315°C. The software MSD ChemStation (Agilent Technologies, Palo Alto, CA, U.S.A.) for Windows was used for data acquisition. The identification of the PPG content and the cuticular substances was accomplished by comparing retention times and mass spectra with data from earlier analyses (Schmitt et al., 2003; Kroiss et al., 2006; E. Strohm, G. Herzner, T. Schmitt, unpublished) and with data from a commercial library (NIST, Gathersburg, MD, USA).

### 8.3.7 Statistics

Peak areas were obtained by manual integration and total peak area of each individual extract was standardized to 100%. Because relative peak areas represent compositional data, the areas were transformed to logcontrasts (Reyment, 1989; Aitchison, 1986) prior to analysis. The means of all peaks for the 14 individual males were calculated and subsequently normalized by log-transformation to allow for parametric testing. To test for a chemical congruency between the HCs found in the gland reservoirs and on the cuticles of beewolf males, we conducted a correlation and regression analysis between the mean proportions of components (Aitchison- and log-transformed) in the reservoir and on the cuticle. Since both variables are GC-MS measurements and thus have the same measurement error we used a reduced major axis 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 State University, U.S.A.; freely available at <http://www.bio.sdsu.edu/pub/andy/RMA.html>). To assess the chemical similarity between the reservoir and the cuticle, we tested whether there was a 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.

## 8.4 Results

### 8.4.1 Morphology

Dissection of the heads revealed that males of the European beewolf possess, besides the already known mandibular gland (Evans and O'Neill 1988, E. Strohm and W. Goettler, unpublished), a large multi-compartment gland reservoir. The reservoir consists of two pairs of tube-like evaginations of the pharynx that branch off the pharynx at the posterior part of the hypopharyngeal plate [Fig. 8.1]. The upper pair of evaginations extends dorsally subjacent to the frontal cuticle of the head, winds around the brain to proceed ventrally, again subjacent to the cuticle, and ends slightly above the base of the mandibles. The basal parts of these tubes are supported by the suspensorium. The lower pair of evaginations extends slightly ventrally and then proximally towards the rear end of the head capsule and is considerably smaller. The walls of the gland reservoir appear to be very thin and translucent and are very easily damaged during dissection. The content of the reservoir was clear and oily.

The semithin sections and the 3D reconstruction based on the NMR data confirmed that the reservoir has two main parts [Fig. 8.2]. The larger (longer) part originates above the pharynx, virtually embraces the brain and takes up a considerable amount of space within the head capsule (2.12  $\mu\text{l}$  in a male with 3.3 mm head width and 9.3  $\mu\text{l}$  head capsule volume). The lower part (0.41  $\mu\text{l}$ ) originates below the pharynx and extends straight to the rear wall of the head capsule. The combined volume (2.54  $\mu\text{l}$ ) makes up about one quarter of the head capsule volume.

8.1

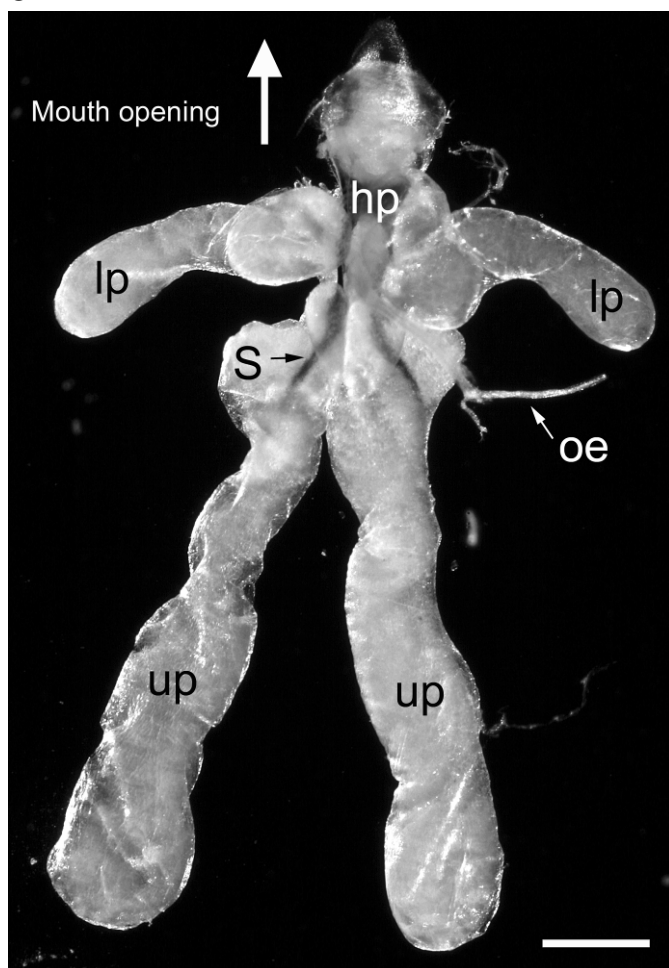


Fig. 8.1. (left). Dissected postpharyngeal gland of a male *P. triangulum*. The two pairs of symmetrical tubes are clearly visible (scale bar 0.5 mm; the corresponding male had a head capsule width of 3.1 mm). hp, hypopharyngeal plate; lp, lower part of PPG; oe, oesophagus; S, suspensorium (shining through the gland); up, upper part of PPG.

8.2

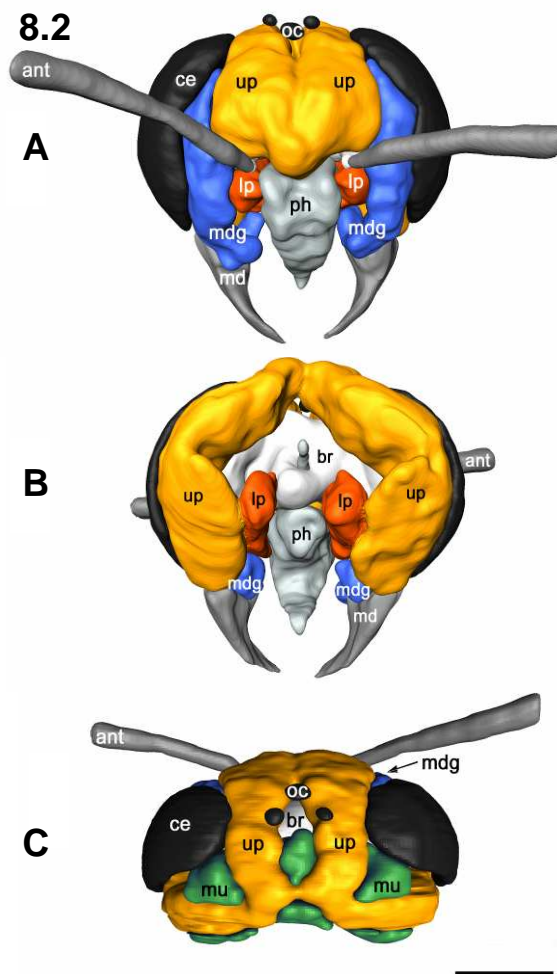


Fig. 8.2. (right). 3D reconstruction of the postpharyngeal gland of a male *P. triangulum*. **A** Frontal view. **B** Rear view. **C** Top view (scale bar 1 mm). Please note that all four tubes of the PPG are entirely separated from each other and not connected as might appear to be the case due to the constraints of contrast and resolution of the illustration. ant, antenna; br, brain; ce, compound eye; lp, lower part of PPG; md, mandible; mdg, mandibular gland; mu, mandibular muscles; oc, ocellus; ph, pharynx; up, upper part of PPG.

Light microscopy of semi-thin sections [Fig. 8.3] showed that the gland reservoir is formed by a monolayered epithelium. The cells of the epithelium are of an irregular more or less triangular shape with the tip pointing towards the lumen. These cells bear long hairs that range into the lumen [Fig. 8.3A]. There are no ducts of class 3 gland cells (classification after Noirot and Quennedey, 1974) opening into the lumen although in the immediate vicinity of the walls of the reservoir there are numerous acini of class 3 gland cells that belong to the mandibular gland [Fig. 8.3A; E. Strohm and W. Goettler, unpublished]. The nuclei are small compared to those of the gland cells of the mandibular gland. There are no visible remains of the gland content in the semithin sections, the lumen looks entirely clear. The lower and upper parts of the gland reservoir look basically very similar in all these respects.



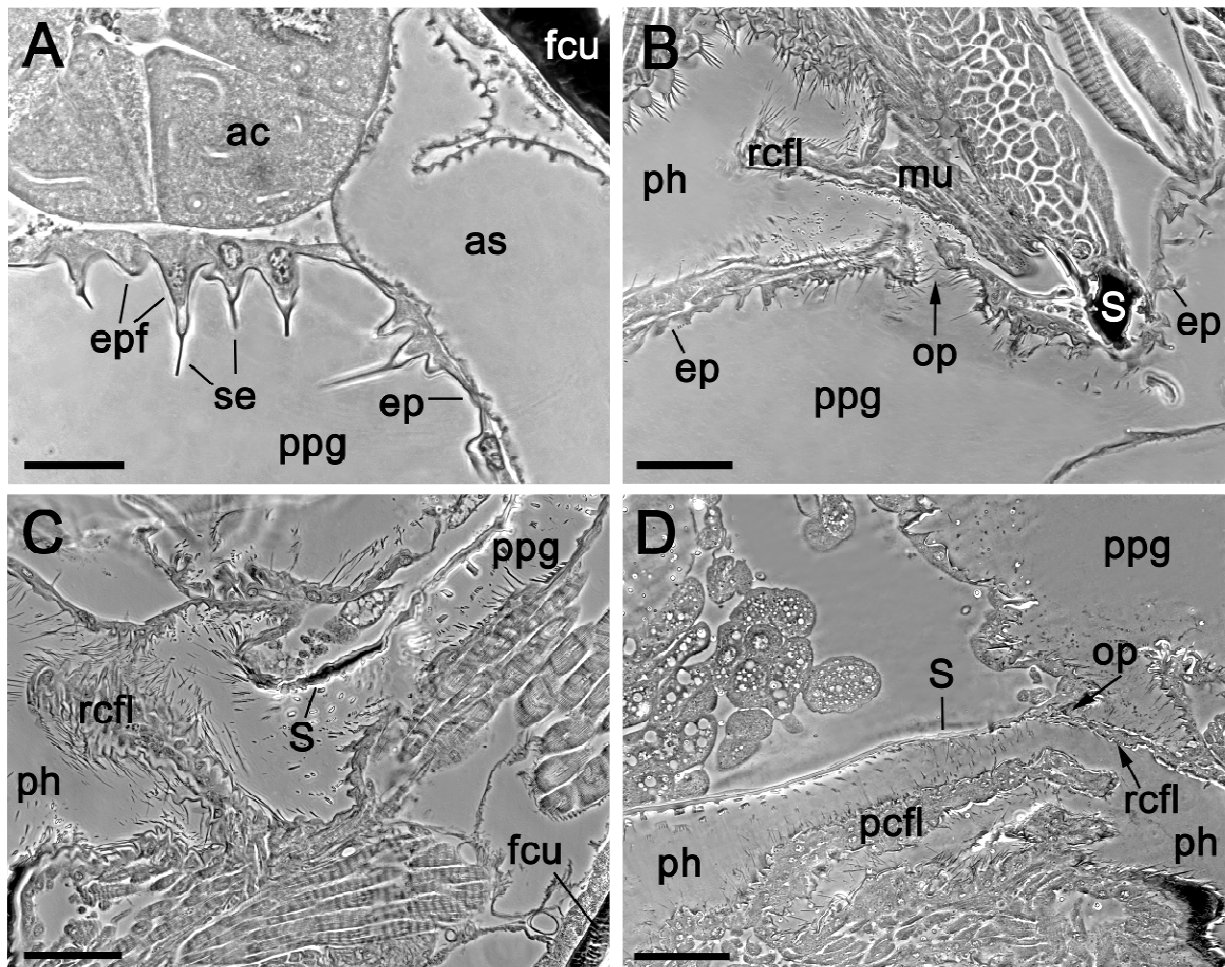


Fig. 8.3: Semithin sagittal (A-C) and cross sections (D) through the head of a beewolf male. A. Postpharyngeal gland with air sac and an acinus that belongs to the adjacent mandibular gland (scale bar 20  $\mu\text{m}$ ). B. Opening of the lower part of the PPG with reservoir closing flap, mouth opening is on the right side (scale bar 50  $\mu\text{m}$ ). C. Opening of the upper part of the PPG with reservoir closing flap; mouth opening is on the bottom side (scale bar 50  $\mu\text{m}$ ). D. Opening of the lower part of the PPG with pharynx closing flap and PPG reservoir closing flap; mouth opening is on the right side (scale bar 50  $\mu\text{m}$ ). See text for details. ac, acinus of the mandibular gland; as, air sac; ep, epithelium of the PPG; epf, epithelial folds; fcu, frontal (facial) cuticle; h, hairs; mu, muscles; op, opening of the PPG; pcfl, pharynx closing flap; ph, pharynx; ppg, lumen of the PPG; rcfl, (PPG) reservoir closing flap; S, suspensorium.

Both parts of the reservoir show paired openings to the pharynx at the cuticular spines that form the suspensorium [Fig. 8.3B-D]. The openings of the upper part are located more proximally than those of the lower part. The interconnection between the gland and the alimentary tract entails some advantages for the delivery of the secretion but also requires specialized mechanisms to control the routes of secretion and food.

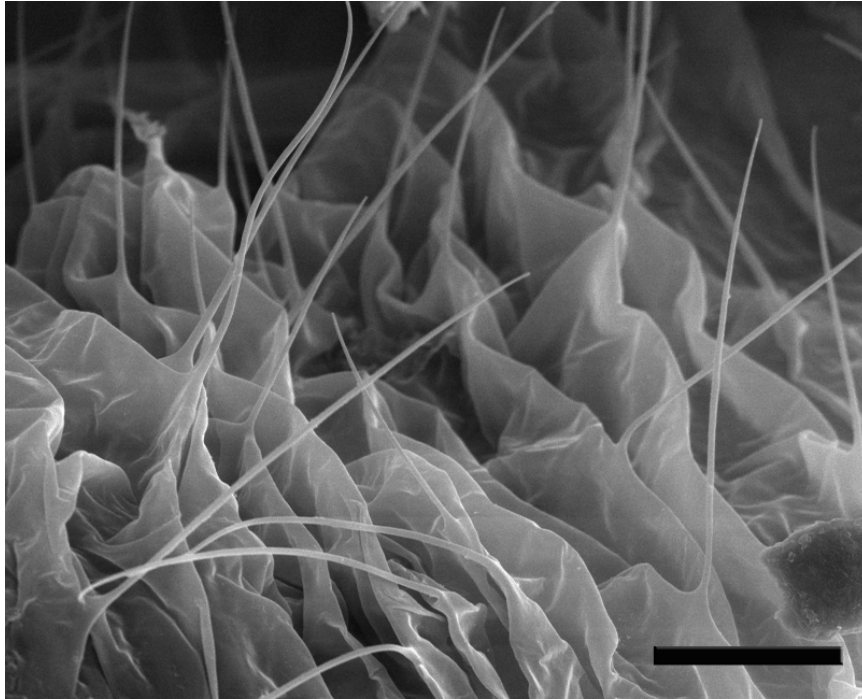


Fig. 8.4. Scanning electron micrograph of the inner surface of the postpharyngeal gland of male *P. triangulum* (scale bar 20  $\mu\text{m}$ ). Note the epithelial folds and hairs.

Male *P. triangulum* apply the secretion from the reservoir, i.e. their marking pheromone, to the territories with a clypeal brush (e.g. Evans and O'Neill, 1988; E. Strohm, G. Herzner, J. Kroiss, unpublished). The pheromone from the reservoir can easily reach this brush through the pharynx and the mouth opening. The intake of food into the gland has to be impeded, however. In the semi-thin sections we found two different types of closing flaps that might control the flow of secretion and food. All openings of the gland into the pharynx have a similar basic organization. Each one is covered by a flattened multicellular flap (reservoir closing flaps)[Fig. 8.3B-D]. The flaps are fixed at their distal sides and not attached at their proximal sides and we propose that they cover the gland reservoir openings when food is swallowed. This closing mechanism could thus prevent the uptake of food into the reservoirs.

How the reservoirs are opened and their content released is less clear yet. In the flap covering the opening of the lower part small muscle fibers are visible [Fig. 8.3B]. These might be able to retract the flap and open the reservoir. A simultaneous contraction of other muscles or an increase of the hemolymph pressure in the head might then press out the content.

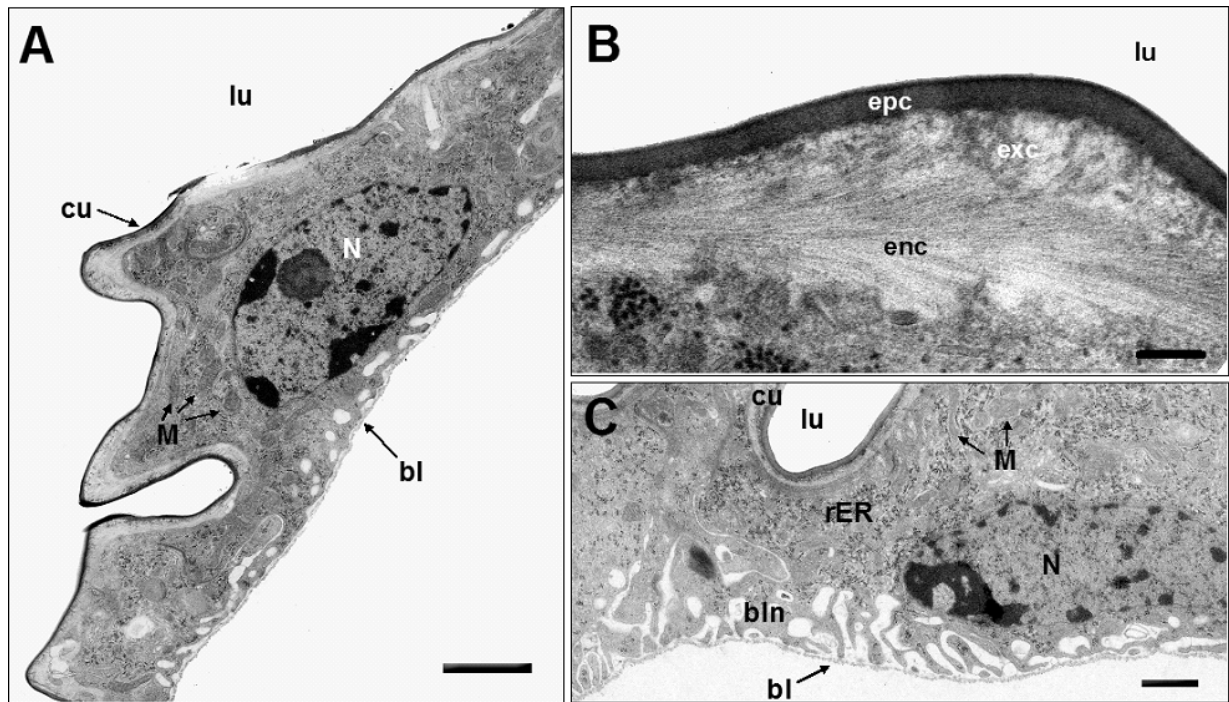


Fig. 8.5. Transmission electron micrographs of the postpharyngeal gland epithelium (A, scale bar 4  $\mu\text{m}$  and C, scale bar 2  $\mu\text{m}$ ) and cuticle (B, scale bar 0.2  $\mu\text{m}$ ) of male *P. triangulum*. The basal invaginations of the epithelium suggest an uptake of substances from the hemolymph. bl, basal lamina; bln, basal invaginations; cu, cuticle; enc, endocuticle; epc, epicuticle; exc, exocuticle; lu, lumen of the PPG; M, mitochondria; N, nucleus; rER, rough endoplasmic reticulum.

In cross sections a second set of flaps with similar structure is visible [Fig. 8.3D]. These flaps are fixed proximally to the wall of the pharynx and extend with their anterior part to the openings of the respective parts of the gland. From their location, dimension and arrangement these flaps seem to be able to close the pharynx just proximally to the opening of the reservoir (pharynx closing flaps in Fig. 8.3D). Therefore, they might facilitate the transfer of secretion between the gland and the mouth opening. This scenario is hypothetical, however, and the exact assignment and functioning of the flaps remain to be shown.

The SEM investigations confirmed the results of the histological investigations and revealed that the cells of the epithelium form dense foldings into the lumen of the reservoir [Fig. 8.4]. The rims of these folds bear numerous thin hairs that extend into the lumen of the reservoir and are variable in length (approx. 10-60  $\mu\text{m}$ ). The function of these hairs is not yet known. The walls of the pharynx show similar but more regular foldings and the hairs are shorter (not shown).

The TEM analysis confirmed the finding that the wall of the gland reservoir is formed by a monolayered epithelium lined by an inner intima that shows the typical ultrastructure of an insect cuticle [Fig. 8.5]. Owing to the epithelial foldings and hairlike extensions the epithelial thickness is very variable. The TEM investigation did not reveal any gland ducts that might discharge into the

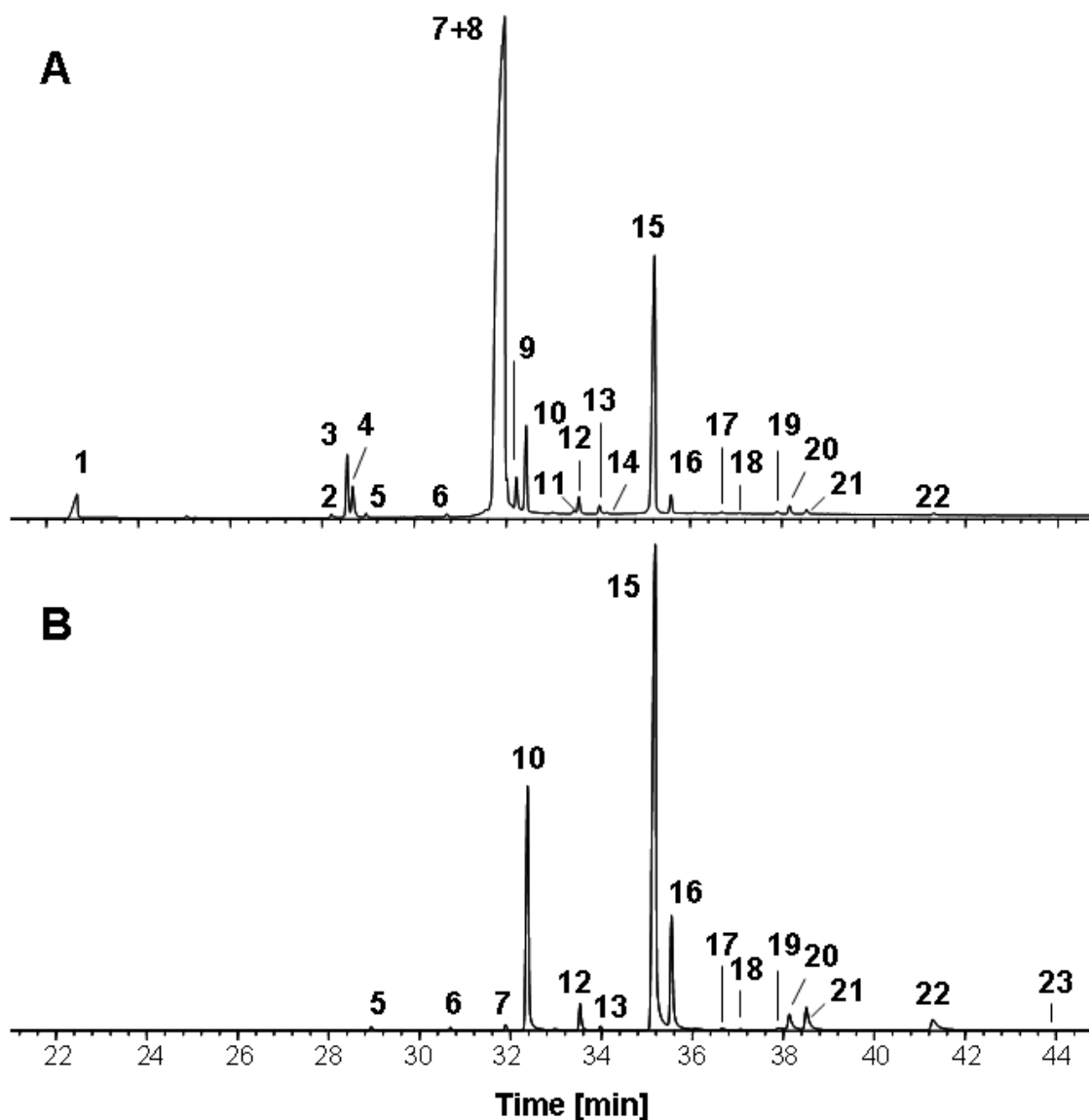


Fig. 8.6. Gas chromatograms of hexane extracts of an individual male *P. triangulum*. A. PPG content. B. Epicuticle. The numbers in the chromatograms refer to the following compounds: 1: (*S*)-2,3-dihydrofarnesoic acid, 2: (*Z*)-9-octadecen-1-ol, 3: 10-nonacosen-2-one, 4: 1-octadecanol, 5: heineicosane, 6: docosane, 7:  $\Delta^{x,y}$ -tricosadiene + (*Z*)-9-tricosene + (*Z*)-7-tricosene, 8: (*Z*)-11-eicosenol, 9: 1-eicosanol, 10: tricosane, 11: unidentified substance 1, 12:  $\Delta^{x,y}$ -tetracosadiene + (*Z*)-9-tetracosene + (*Z*)-7-tetracosene, 13: tetracosane, 14: unidentified substance 2, 15:  $\Delta^{x,y}$ -pentacosadiene + (*Z*)-9-pentacosene + (*Z*)-7-pentacosene, 16: pentacosane, 17: (*Z*)-9-hexacosene, 18: hexacosane, 19:  $\Delta$ -16-pentacosen-8-one, 20:  $\Delta^{x,y}$ -heptacosadiene + (*Z*)-9-heptacosene + (*Z*)-7-heptacosene, 21: heptacosane, 22: octacosane, 23: nonacosane, 24: hentriacontane. Please note that owing to their very low quantities the peaks of the substances 13-methyl pentacosane, 11-methyl pentacosane, 7 methyl-pentacosane, hexacosane and octacosane are not visible in one or both of the chromatograms of one individual male.

lumen of the reservoir. The cells of the epithelium are connected by septate desmosomes. They contain some rough and some smooth endoplasmatic reticulum. Some cells contain a large number of mitochondria but others have only few mitochondria. Multilamellar bodies and Golgi apparatus could rarely be seen. There were only few vesicles in the cells and no microvilli on the inner side of the epithelium. There were, however, basal invaginations on the outer side of the epithelium [Fig. 8.5C].

#### 8.4.2 Chemistry

The gland reservoir samples contained 35 previously described (Schmitt et al., 2003; Kroiss et al., 2006) pheromonal substances [Fig. 8.6A]. Some of the very minor HCs that had been found as components of the gland content in another study (Kroiss et al., 2006) could not be detected, most probably because we analyzed single males with the consequence that the amounts of some substances were below the detection limits of the analytical set-up.

In the cuticle extracts we characterized 19 peaks [Fig. 8.6B]. All these substances also occur in the gland. The concentration of HCs in the cuticle extracts was considerably lower than in the gland extracts and it is likely that some of the HCs that we found in the pheromone but not in the cuticle extracts were below the detection limits of our GC-MS. The amounts of the methyl alkanes were too low to determine the position of the methyl group. Except for  $\Delta$ -16-pentacosen-8-one none of the pheromonal substances with functional groups were present in the cuticle extracts.

For the correlation and regression analysis we included all HC peaks that were present both in the gland and on the cuticle and that could unambiguously be identified. In general, the (Z)-9 and (Z)-7 alkenes as well as the alkadiene of the same chain length could not be entirely separated in the chromatograms and were thus treated as one peak. The (Z)-9-alkene was always the dominant peak among the three. In the chromatograms of the gland extracts, the peaks of  $\Delta^{x,y}$ -tricosadiene, (Z)-9-tricosene, and (Z)-7-tricosene were hidden under the huge peak of (Z)-11-eicosen-1-ol and could not be separated satisfactorily for a quantitative analysis. They were thus excluded from the analyses. The very minor compounds  $\Delta$ -16-pentacosen-8-one and octacosane were only present in traces in most of the gland extracts and not detectable at all in most of the cuticle extracts and were thus also excluded from the analyses. The remaining 12 peaks (Aitchison- and log-transformed) were subjected to the correlation and regression analyses.

The relative amounts of substances in the gland reservoirs showed a strong linear correlation with the corresponding substances on the cuticles [Fig. 8.6]. The slope of the RMA-regression was 1.091 (95% confidence intervals: 0.748 – 1.433). The intercept of the regression line was 0.062 (95% confidence intervals: -0.175 – 0.299). Thus, there was no significant deviation from direct proportionality.

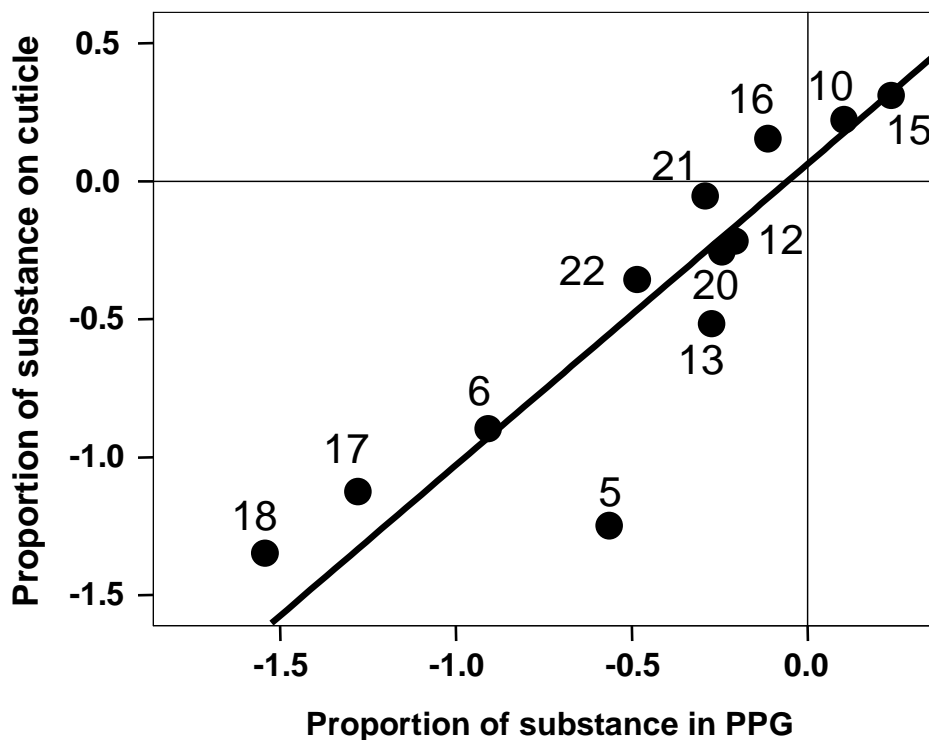


Fig. 8.7. Correlation between the mean relative amount (peak area Aitchison transformed, see text) of a particular hydrocarbon in the PPGs and on the cuticles of beewolf males ( $r^2 = 0.801$ ,  $n = 11$ ,  $p < 0.001$ ). The trend line was generated using reduced major axis regression ( $y = 1.091 * x + 0.062$ ). The data are based on extracts of 14 males and the following 12 peaks (the numbers correspond to the numbers given in figure 6): 5 heneicosane, 6 docosane, 10 tricosane, 12 tetracosene, 13 tetracosane, 15 pentacosenes + pentacosadiene, 16 pentacosane, 17 hexacosene, 18 hexacosane, 20 heptacosenes + heptacosadiene, 21 heptacosane, 23 nonacosane.

## 8.5 Discussion

Our findings strongly support the hypothesis that the large cephalic gland reservoir of male *P. triangulum* is a PPG. Despite the difference in overall appearance, there is considerable similarity between the PPG of male European beewolves and their female conspecifics as well as ants. As in female *P. triangulum* and ants, the presumptive PPG of male beewolves originates from the posterior part of the pharynx and takes up a considerable fraction of the head-capsule volume. Whereas in ants the PPG is made up of only one paired structure (Soroker et al., 1995a; Peregrine et al., 1973), in both sexes of *P. triangulum* the reservoir comprises two parts (the upper and the lower part; Strohm et al., 2007; this study). The overall appearance of these two parts of the reservoir is sexually dimorphic, however. Whereas in males both the upper and the lower part of the reservoir consist of two symmetrical halves, in females only the upper part shows this subdivision, and the lower part consists of a single small sac-like structure (Strohm et al., 2007). Furthermore, in contrast to the overall glove-like structure of the upper part of the PPG in female *P. triangulum* (Strohm et al., 2007) and ants (Lucas et al., 2004; Soroker et al., 1995a; Peregrine et al., 1973), the upper part of the PPG of male *P. triangulum* consists of unbranched simple straight tubes.

The epithelium that forms the wall of the gland reservoir is monolayered in male and female *P. triangulum* (Strohm et al., 2007) and in ants (Peregrine et al., 1973; Soroker et al. 1995a). In both sexes of the beewolf, the epithelium carries long hairs that extend into the lumen of the reservoir and the inner surface is lined with cuticle. With the exception of the hairs, which are not present in ants, these results support the hypothesis that the large cephalic reservoir of male European beewolves is a PPG.

The PPG of ants typically contains long-chain straight and methyl-branched HCs that are sequestered from the hemolymph and/or taken up from the cuticle during self-grooming (Lucas et al., 2004; Hefetz et al., 2001; Soroker and Hefetz, 2000; Soroker et al., 1998, 1995a, b, 1994). In female *P. triangulum* the PPG content also comprises long-chain straight and methyl-branched HCs (plus additionally long-chain unsaturated ketones) and there are indications that these are likewise sequestered from the hemolymph (E. Strohm, G. Herzner, T. Schmitt, unpublished). The glove-like gland shape may facilitate the efficient sequestration of HCs from the hemolymph by enlarging the surface of the gland reservoir. Both in ants (Lucas et al., 2004; Soroker et al., 1995a; Do Nascimento et al., 1993; Bagnères and Morgan, 1991) and female beewolves (E. Strohm, G. Herzner, T. Schmitt, unpublished) the HCs in the PPG match those extractable from the cuticle. In male *P. triangulum* the PPG functions as reservoir of the marking pheromone (Kroiss et al., 2006). As in ants and females it contains (among others) several straight and methyl-branched long-chain HCs. In a previous study on the chemistry of the marking pheromone based mainly on head extracts (Schmitt et al., 2003) we did not include most of the minor HCs. During this previous study we were not aware of the existence of the PPG in male beewolves and we thought that the minor HCs in our head extracts came from the cuticle rather than the cephalic glands. Here and in Kroiss et al. (2006) we show that they are indeed present in the PPG and we thus include them in the description of the marking pheromone. We also found a high congruence between the HCs from the reservoir and the cuticle. This provides further evidence for a homology of the male reservoir with the PPG of ants and female beewolves.

Although in ants the cuticular HC composition generally appears to be congruent with that of the PPG, some deviations from this rule have also been documented (Lucas et al., 2004; Soroker and Hefetz, 2000). However, these differences between PPG and cuticular HCs are mostly quantitative in nature. In male *P. triangulum* we found a high congruency concerning the HC fraction of the PPG and cuticular HCs. In addition to the HCs, the PPG contained several compounds with functional groups that did not occur on the cuticle, i.e. there are large qualitative differences between the gland and the cuticle. This raises the question where this blend of compounds in the PPG of male beewolves comes from.

Chemical analyses of the cuticle as well as preliminary analyses of the hemolymph (M. Kaltenpoth, unpublished data) suggest that the HCs might be either sequestered from the cuticle or from the hemolymph or both. Owing to the large size of the male PPG and the low proportion of HCs in its content (Kroiss et al., 2006; this study) the surface of the gland might be sufficiently large to allow for the sequestration of the HCs, even if the reservoir lacks the surface enlargement of the glove-like female PPG. Sequestration of HCs from the hemolymph by exocrine glands has also been proposed for other species (Dufour's gland of honey bee queens: Katzav-Gozansky et al., 1997; pheromone glands of moths, Jurenka et al., 2003; Schal et al., 1998). The pheromonal compounds with functional groups do not occur in the hemolymph of male *P. triangulum* (M. Kaltenpoth, unpublished data) and must thus come from somewhere else. The lack of typical class 3 gland cells and the thin epithelium with no signs of high glandular activity suggest that these compounds are not synthesized by the PPG itself. Instead, we hypothesize that they are produced in the large mandibular glands (Fig. 8.2; E. Strohm and W. Goettler, unpublished) and transferred to the PPG via the pharynx. Since the substances with functional groups do not occur on the cuticle, it is rather unlikely that male beewolves apply the PPG secretion to their cuticle via self-grooming as demonstrated e.g. for the ant *Cataglyphis niger* (Soroker and Hefetz, 2000). We cannot preclude, however, that the HCs take the opposite route, i.e. that they are incorporated into the PPG during self-grooming, a mechanism proposed e.g. for the ants *Camponotus vagus* (Meskali et al., 1995) as well as *Pachycondyla apicalis* and *P. villosa* (Lucas et al., 2004; Hefetz et al., 2001). The marking pheromone that is stored in the PPG thus seems to be a mixture of substances with different origins.

The finding of a pheromone storing PPG in males of a solitary wasp is surprising. The 3D reconstruction visualizes the impressive size of the pheromone producing and storing glands in male European beewolves. Together, the PPG and the mandibular gland make up approximately one third of the head-capsule volume. The size and content of the male PPG are most probably shaped by strong sexual selection. Whereas the composition of the pheromone might have been influenced by receiver bias processes (Herzner et al., 2005; Endler and Basolo, 1998), the extraordinary size of the glands and consequently the exceptional large amounts of pheromone suggest the involvement of runaway processes (Fisher, 1930) in the exaggeration of this secondary sexual character (see also Herzner, 2004).

It is an intriguing yet common phenomenon that glands that serve particular functions in solitary species (like the PPG in male and female beewolves) have been modified to serve 'social functions' in social species (like the PPG in ants). The Dufour's gland, for example, serves numerous different functions in various solitary and social species. In solitary bees of the families Colletidae, Andrenidae, and Anthophoridae, females use the Dufour's gland secretion to line the walls of underground brood cells with hydrophobic substances to maintain favorable microclimatic conditions for their progeny



(e.g., Vander Wall, 1990). In ants, the Dufour's gland contains (among others) trail, recruitment, and queen pheromones (e.g., Blatrix et al., 2002; Bestmann et al., 1995; Edwards and Chambers, 1984). In honeybee queens, it contains substances that elicit retinue behavior by workers (Katzav-Gozansky et al., 2001). A further example is the poison gland that first served to paralyze hosts or prey (Quicke, 1997), then evolved an additional defensive function in brood caring solitary species (Wilson, 1971) and has finally gained a function in recruitment (Kohl et al., 2001) and trail-establishment (Morgan et al., 1992) in ants.

The occurrence of PPGs in beewolves is remarkable, since it has long been assumed that PPGs are restricted to the family Formicidae (Lenoir et al., 1999; Schoeters and Billen, 1997; Soroker et al., 1995a; Hölldobler and Wilson, 1990), which is phylogenetically not closely related to crabronid wasps (Brothers, 1999). In ants the PPG harbors a heritable blend of cuticular HCs that allows for nestmate recognition and thus contributes to colony integrity, a problem idiosyncratic to social species. Lenoir and coworkers (1999) thus speculated about the evolutionary origin of the PPG: 'Is there a cuticular lipid storing gland in solitary species, or has it evolved specifically in ants' to allow the formation of a colony signature and thus nestmate recognition? Here and in a further study (Strohm et al., 2007) we demonstrate the existence of such a lipid storing gland in a solitary digger wasp. Based on current knowledge it remains unclear whether the PPGs of beewolves and ants are derived from a single evolutionary root or evolved by independent convergent evolution (for a detailed discussion of this issue see Strohm et al., 2007).

In any case, another interesting question that arises is whether the chemical composition of the PPG content in the European beewolf has a genetic basis and thus the potential to enable kin recognition. In *P. triangulum* the PPG is involved in brood care by females (Strohm and Linsenmair, 2001) and in mate attraction by males (Schmitt et al., 2003; Kroiss et al., 2007). It is as yet unknown whether the composition of the PPG secretion of females is heritable. In male *P. triangulum*, however, the overall composition of the PPG content, i.e. the sex pheromone, in fact varies with kinship (Herzner et al., 2006). Taking into account that the ability to recognize nestmates (which usually involves kin recognition to some degree) by virtue of chemical cues is one of the key characteristics of social insects, a genetically based composition of the PPG content of a solitary species, such as *P. triangulum*, could represent a crucial preadaptation for the evolution of nepotism and sociality.

In conclusion, our findings strongly support the existence of a PPG, a gland that was thought to be idiosyncratic to ants, where it guarantees the integrity of the social group, in males of a solitary wasp, the 'lone beewolf' *P. triangulum*.

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## 8.6 References

- Aitchinson, J. (1986). The statistical analysis of compositional data. Chapman & Hall, London.
- Bagnères, A.G., Morgan, E.D. (1991). The postpharyngeal glands and the cuticle of Formicidae contain the same characteristic hydrocarbons. *Experientia* 47: 106-111.
- Bestmann, H.J., Janssen, E., Keru, F., Liepold, B. (1995). All-trans geranylgeranyl acetate and geranyl-geraniol, recruitment pheromone components in the Dufour gland of the Ponerinae ant *Ectatomma ruidum*. *Naturwissenschaften* 82: 334-336.
- Blatrix, R., Schulz, C., Jaisson, P., Francke, W., Hefetz, A. (2002). Trail pheromone of ponerine ant *Gnamptonyx striatula*: 4-Methylgeranyl esters from Dufour's gland. *Journal of Chemical Ecology* 28: 2557-2567.
- Böck, P. (1989). Romeis Mikroskopische Technik. Urban & Schwarzenberg, München, Wien, Baltimore.
- Brothers, D.J. (1999). Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). *Zoologica Scripta* 28 (1-2): 233-249.
- Cabrera, A., Williams, D., Hernández, J.V., Caetano, F.H., Jaffe, K. (2004). Metapleural- and postpharyngeal-gland secretion from workers of the ants *Solenopsis invicta* and *S. geminata*. *Chemistry & Biodiversity* 1: 303-311.
- Crozier, R.H., Dix, M.W. (1979). Analysis of two genetic models for the innate components of colony odour in social Hymenoptera. *Behavioral Ecology and Sociobiology* 4: 217-224.
- Dietemann, V., Peeters, C., Liebig, J., Thivet, V., Hölldobler, B. (2003). Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. *PNAS* 100: 10341-10346.
- Do Nascimento, N.R.R., Billen, J., Morgan, E.D. (1993). The exocrine secretions of the jumping ant *Harpegnathos saltator*. *Comparative Biochemistry and Physiology B* 104: 505-508.
- Edwards, J.P., Chambers, J. (1984). Identification and source of a queen-specific chemical in the pharaohs ant *Monomorium pharaonis*. *Journal of Chemical Ecology* 10: 1731-1748.
- Eelen, D., Borgesen, L., Billen, J. (2006). Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 87: 101-111.
- Endler, J.A., Basolo, A.L. (1998). Sensory ecology, receiver biases and sexual selection. *Trends in Ecology and Evolution* 13: 415-420.
- Evans, H.E., O'Neill, K.M. (1988). The Natural History and Behavior of North American Beewolves. Cornell University Press, Ithaca, New York.
- Fisher, R.A. (1930). The genetical theory of natural selection. Clarendon Press, Oxford.
- Hefetz, A., Errard, C., Cojocar, M. (1992). The occurrence of heterospecific substances in the postpharyngeal gland secretion of ants reared in mixed species colonies (Hymenoptera: Formicidae). *Naturwissenschaften* 79: 417-420.
- Hefetz, A., Soroker, V., Dahbi, A., Malherbe, M.C., Fresneau, D. (2001). The front basitarsal brush in *Pachycondyla apicalis* and its role in hydrocarbon circulation. *Chemoecology* 11: 17-24.
- Herzner, G. (2004). Evolution of the pheromone communication system in the European Beewolf *Philanthus triangulum* F. (Hymenoptera: Crabronidae). PhD thesis, Würzburg University, Germany.
- Herzner, G., Schmitt, T., Linsenmair, K.E., Strohm, E. (2005). Prey recognition by females of the European beewolf and its potential for a sensory trap. *Animal Behaviour* 70: 1411-1418.
- Herzner, G., Schmitt, T., Heckel, F., Schreier, P., Strohm, E. (2006). Brothers smell similar: variation in the sex pheromone of male European beewolves *Philanthus triangulum* F. (Hymenoptera: Crabronidae) and its implication for inbreeding avoidance. *Biological Journal of the Linnean Society* 89: 433-442.
- Hölldobler, B., Wilson, E.O. (1990). The ants. Harvard University Press, Cambridge.
- Jackson, B.D., Morgan, E.D. (1993). Insect chemical communication: pheromones and exocrine glands of ants. *Chemoecology* 4: 125-144.
- Jurenka, R.A., Subchev, M., Abad, J.L., Choi, M.Y., Fabrias, G. (2003). Sex pheromone biosynthetic pathway for disparlure in the gypsy moth, *Lymantria dispar*. *PNAS* 100: 809-814.
- Kaltenpoth, M., Strohm, E. (2006). The scent of senescence: Age-dependent changes in the composition of the marking pheromone of the male European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae). *Journal of Insect Science* 6: 20.
- Katzav-Gozansky, T., Soroker, V., Hefetz, A. (1997). The biosynthesis of Dufour's gland constituents in queens of the honeybee (*Apis mellifera*). *Invertebrate Neuroscience* 3: 239-243.
- Katzav-Gozansky, T., Soroker, V., Ibarra, F., Francke, W., Hefetz, A. (2001). Dufour's gland secretion of the queen honeybee (*Apis mellifera*): an egg discriminator pheromone or a queen signal? *Behavioral Ecology and Sociobiology* 51: 76-86.
- Kohl, E., Hölldobler, B., Bestmann, H.J. (2001). Trail and recruitment pheromones in *Camponotus socius* (Hymenoptera: Formicidae). *Chemoecology* 11: 67-73.

- Kroiss, J., Schmitt, T., Schreier, P., Strohm, E., Herzner, G. (2006). A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 32: 2763-2776.
- Legendre, P., Legendre, L. (1998). Numerical Ecology. Elsevier Science B.V., Amsterdam.
- Lenoir, A., Fresneau, D., Errard, C., Hefetz, A. (1999). Individuality and colonial identity in ants: the emergence of the social representation concept. in Detrain, C., Deneubourg, J.L., Pasteels, J.M., eds. *Information processing in social insects*. Birkhäuser, Basel.
- Lucas, C., Pho, D.B., Fresneau, D., Jallon, J.M. (2004). Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*. *Journal of Insect Physiology* 50: 595-607.
- Meskali, M., Bonavita-Cougourdan, A., Provost, E., Bagnères, A.G., Dusticier, G., Clément, J.L. (1995). Mechanism underlying cuticular hydrocarbon homogeneity in the ant *Camponotus vagus* (Scop.) (Hymenoptera: Formicidae): Role of postpharyngeal glands. *Journal of Chemical Ecology* 21: 1127-1148.
- Morgan, E.D., Hölldobler, B., Vaisar, T., Jackson, B.D. (1992). Contents of poison apparatus and their relation to trail-following in the ant *Daceton armigerum*. *Journal of Chemical Ecology* 18: 2161-2168.
- Noirot, C., Quennedey, A. (1974). Fine-structure of insect epidermal glands. *Annual Review of Entomology* 19: 61-80.
- Peregrine, D.J., Mudd, A., Cherrett, J.M. (1973). Anatomy and preliminary chemical analysis of postpharyngeal glands of leaf-cutting ant, *Acromyrmex octospinosus* (Reich) (Hym, Formicidae). *Insectes Sociaux* 20 (4): 355-363.
- Phillips, S.A., Vinson, B.S. (1980). Comparative morphology of glands associated with the head among castes of the red imported fire ant, *Solenopsis invicta* Buren. *Journal of the Georgia Entomological Society* 15: 215-226.
- Quicke, D. (1997). Parasitic wasps. Chapman & Hall, London.
- Reyment, R.A. (1989). Compositional data analysis. *Terra nova* 1: 29-34.
- Schal, C., Sevala, V., Cardé, R.T. (1998). Novel and highly specific transport of a volatile sex pheromone by hemolymph lipophorin in moths. *Naturwissenschaften* 85: 339-342.
- Schmitt, T., Strohm, E., Herzner, G., Bicchi, C., Krammer, G., Heckel, F., Schreier, P. (2003). (S)-2,3-dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum*. *Journal of Chemical Ecology* 29 (11): 2469-2479.
- Schoeters, E., Billen, J. (1997). The post-pharyngeal gland in *Dinoponera* ants (Hymenoptera: Formicidae): Unusual morphology and changes during the secretory process. *International Journal of Insect Morphology & Embryology* 25 (4): 443-447.
- Simon Thomas, R.T., Poorter, E.P.R. (1972). Notes on the behaviour of males of *Philanthus triangulum* (F.) (Hymenoptera, Sphecidae). *Tijdschrift voor Entomology* 115: 141-151.
- Soroker, V., Vienne, C., Hefetz, A. (1994). The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 81 (11): 510-513.
- Soroker, V., Hefetz, A., Cojocaru, M., Billen, J., Franke, S., Francke, W. (1995a). Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiological Entomology* 20 (4): 323-329.
- Soroker, V., Vienne, C., Hefetz, A. (1995b). Hydrocarbon dynamics within and between nestmates in *Cataglyphis niger* (Hymenoptera: Formicidae). *Journal of Chemical Ecology* 21: 365-378.
- Soroker, V., Fresneau, D., Hefetz, A. (1998). Formation of colony odor in Ponerine ant *Pachycondyla apicalis*. *Journal of Chemical Ecology* 24: 1077-1090.
- Soroker, V., Hefetz, A. (2000). Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. *Journal of Insect Physiology* 46: 1097-1102.
- Strohm, E. (1995). Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). *Wissenschaftliche Schriftenreihe Biologie* 6.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (1997). Low resource availability causes extremely male-biased investment ratios in the European beewolf, *Philanthus triangulum* F. (Hymenoptera, Sphecidae). *Proceedings of the Royal Society of London Series B* 264 (1380): 423-429.
- Strohm, E., Linsenmair, K.E. (1999). Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behavioral Ecology and Sociobiology* 47: 76-88.
- Strohm, E. (2000). Factors affecting body size and fat content in a digger wasp. *Oecologia* 123: 184-191.
- Strohm, E., Lechner, K. (2000). Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 59: 183-191.

- Strohm, E., Linsenmair, K.E. (2000). Allocation of parental investment among individual offspring in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Biological Journal of the Linnean Society* 69 (2): 173-192.
- Strohm, E., Linsenmair, K.E. (2001). Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 26 (2): 198-203.
- Strohm, E., Herzner, G., Goettler, W. (2007). A "social" gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36 (2): 113-122.
- Vander Meer, R.K., Glancey, B.M., Lofgren, C.S. (1982). Biochemical changes in the crop, oesophagus and postpharyngeal gland of colony-founding red imported fire ant queens (*Solenopsis invicta*). *Insect Biochemistry* 12: 123-127.
- Vander Wall, S. (1990). Food hoarding in animals. The University of Chicago Press, Chicago, Illinois.
- Vargo, E.L., Hulse, C. (2000). Multiple glandular origins of queen pheromones in the fire ant *Solenopsis invicta*. *Journal of Insect Physiology* 46 (1151-1159).
- Vienne, C., Soroker, V., Hefetz, A. (1995). Congruency of hydrocarbon patterns in heterospecific groups of ants - transfer and/or biosynthesis. *Insectes Sociaux* 42 (3): 267-277.
- Wilson, E.O. (1971). *Insect societies*. Harvard University Press, Cambridge, MA.

## CHAPTER 9

**MANDIBULAR GLANDS OF MALE EUROPEAN BEEWOLVES,  
*PHILANTHUS TRIANGULUM* (HYMENOPTERA, CRABRONIDAE)**

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**9.1 Summary**

Males of a solitary digger wasp, the European beewolf, *Philanthus triangulum*, possess large mandibular glands that have been reported to produce a scent marking pheromone. We analysed the morphology and ultrastructure of these glands using light microscopy as well as scanning and transmission electron microscopy. The paired glands are located laterally in the head and each side consists of a larger and a smaller part. Both parts possess a collecting duct each with distinct openings at the mandible base. However, the collecting duct of the larger part is additionally connected to the pharynx through a lateral extension. The collecting ducts are bordered by a monolayered epithelium lined with cuticle that exhibits conspicuous ramified protuberances. About 1400 acini consisting of class 3 gland cells surround the ducts and are connected to them through conducting canals. The main components in the cytoplasm of these gland cells are mitochondria, well developed smooth endoplasmatic reticulum, and electron lucent vesicles suggesting a high secretory activity. The connection between the large gland parts and the pharynx suggests that the secretion of the mandibular glands might not only be delivered directly onto the mandibles but might also be transported to and stored in the postpharyngeal gland.

## 9.2 Introduction

Exocrine glands play an important role for all aspects of interactions between insects and their environment. Such interactions have been extensively studied in the Hymenoptera that show a huge variety of glands. In bees, wasps, and ants the secretions of exocrine glands serve diverse functions, e.g. defence against predators and pathogens, digestion, reproduction or inter- and intraspecific communication (Hölldobler and Wilson, 1990; Billen, 1991; Vander Meer, 1998; Ayasse, 2001). In many species males produce and secrete volatile pheromones from exocrine glands to attract potential mates often over long distances. Besides this attraction such male pheromones may contain information about the signaller that could be used by females to choose the most suitable mate (Jones et al., 2000; Herzner et al., 2006; Kaltenpoth and Strohm, 2006).

Males of the European beewolf (*Philanthus triangulum*; Hymenoptera, Crabronidae), a solitary digger wasp, scent mark plants and other structures in territories with cephalic gland secretions (e.g. Evans and O'Neill, 1988; Schmitt et al. 2003; Herzner et al. 2006; Kroiss et al., 2006). The territories are small (about 0.25 m<sup>2</sup>), contain no resources essential to females, and are defended against intruding conspecific males in prolonged flight interactions (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988; Strohm and Linsenmair, 1995, Strohm and Lechner, 2000). To spread the secretions over the surface, males run on plant stems with lowered heads and opened mandibles and drag their clypeal brush over the surface (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988; Strohm, 1995). Since the abdomen is lowered, the body forms an inverted V. This behaviour was initially called abdomen dragging, but there is no evidence that the abdomen has any function in the scent marking (Strohm, unpubl. data). Receptive females enter the territories and copulations take place within or in the vicinity of these territories (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988). Since females approach the territories from the downwind side in a zig-zagging flight they probably find the territories by olfactory sensing of the male pheromone (e.g. Evans and O'Neill, 1988; Schmitt et al. 2003; Herzner et al. 2006; Kroiss et al., 2006).

Hitherto most authors assumed, that the marking secretion is produced and stored in the mandibular glands (MG in the following) (reviewed in Evans and O'Neill, 1988) and a number of publications dealt with the chemical analysis of the putative MG secretions in the subfamily Philanthinae (*Philanthus triangulum*: Kaltenpoth and Strohm, 2006; Kroiss et al., 2006; Schmitt et al., 2003; Schmidt et al., 1990; Borg-Karlsön and Tengö, 1980; *P. basilaris* / *bicinctus*: McDaniel et al., 1987; Schmidt et al., 1985; *P. crabroniformis* / *barbatus* / *pulcher*: McDaniel et al., 1992; *Eucerceris conata* / *montana* / *rubripes* / *tricolor*: Clarke et al., 2001). However, the mandibular glands are not the only possible source of cephalic secretion since Kroiss et al. (2006) and Herzner et al. (2007) described a large postpharyngeal gland (PPG in the following) in the head of males of *P. triangulum*. This PPG shows a tube-like structure and, when filled, comprises about one third of the head capsule volume

(Herzner et al., 2007). Combined gas-chromatography and mass-spectrometry revealed that the PPG content resembles the marking pheromone found in scent marked territories (Kroiss et al., 2006; Herzner et al., 2007). The absence of gland cells as well as the occurrence of substances in the PPG, that could not be found in the hemolymph suggest, that at least parts of the PPG contents originate from the mandibular glands (Kroiss et al., 2006; Herzner et al., 2007).

Unfortunately, morphological data on mandibular glands in the Philanthinae are fragmentary: The only detailed investigation of male mandibular glands of a species of the subfamily Philanthinae, *Cerceris rybyensis*, was published by Ågren (1977). Furthermore basic descriptions of the overall morphology are available for *P. bicinctus* (Gwynne, 1978) and *P. albopilosus* (Evans and O'Neill, 1988).

In this study we investigated the morphology, fine- and ultrastructure of the mandibular glands of male beewolves. Moreover, we wanted to test the hypothesis that at least the major components of the pheromone are synthesised in the MG and then transported into the PPG via the pharynx. Therefore, one particular goal was to analyse where exactly the mandibular glands discharge and whether this might allow for an uptake of the products of the MG by the PPG. We produced 3D-reconstructions based on continuous series of semithin sections and analysed the glands' structure using light microscopy, scanning and transmission electron microscopy.

### 9.3 Materials and Methods

#### 9.3.1 Specimens

Male European beewolves were obtained from a laboratory population at the University of Regensburg, Germany. For detailed information about the rearing conditions see e.g. Strohm and Linsenmair (1997).

#### 9.3.2 Semithin sections and 3D-reconstruction

Male beewolves were anaesthetised with CO<sub>2</sub>, killed with diethyl ether and decapitated. The compound eyes were abscised sagittally for the most part with a razor blade to facilitate penetration of the fixation/embedding media. The heads were fixed in alcoholic Bouin for 3 hours at 4°C, dehydrated in a graded ethanol series and embedded in Poly/Bed<sup>®</sup> 812 (Polysciences, Eppelheim, Germany). Sections of 4 µm thickness were made with a diamond knife on a Reichert 2040 Autocut and stained with 1% toluidine blue buffered with 1% Di-sodium-tetraborate in distilled water.

To reveal the three-dimensional structure of the glands we used continuous series of sagittal semithin sections of whole heads. Digital photos of the sections were obtained with a Nikon DS-2Mv camera



attached to a Zeiss Axioplan microscope. The slices were stacked and aligned to each other using the 3D-visualization software Amira<sup>®</sup> (Mercury Computer Systems, Berlin). The collecting ducts, acini and other structures of interest were manually marked with different colours in all slices. Finally, the program computed the 3D-structure of the marked structures.

### 9.3.3 Electron microscopy

After removing the front cuticle of the head capsule of freshly killed males using razor blades on a blade holder, the mandibular glands were grasped with tweezers near the mandible base and gently pulled out. For scanning electron microscopy (SEM) the glands were 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 collecting ducts, the glands were intersected with a razor blade before sputtering. For transmission electron microscopy (TEM), the mandibular glands were fixed overnight at 4 °C in a solution of 2.5 % glutardialdehyd / 2 % formaldehyde in 0.1 M sodium cacodylate buffer at pH 7.2, followed by postfixation with 2% osmium tetroxide. After dehydration in a graded ethanol series and propylene oxide the specimens were embedded in Poly/Bed<sup>®</sup> 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.

## 9.4 Results

### 9.4.1 Overall morphology

Mandibular glands of males of the European beewolf (*Philanthus triangulum*) consist of two pairs of gland structures located laterally in the head capsule. From each mandible base a slightly contorted, large collecting duct extends anterior to the brain upwards to about 4/5th of the head capsule height [Figs. 9.1, 9.2A,B]. A second considerably smaller collecting duct extends posterior from each mandible base [Figs. 9.1, 9.2B]. It consists of a single tube which divides into two branches that surround the ventral part of the optical lobe [Fig. 9.2B]. The anterior and the posterior collecting ducts each have distinct openings into the intersegmental gap at the mandible base [Fig. 9.3]. The opening of the posterior collecting duct is located ventrally to the mandible base [Figs. 9.2B, 9.3]. The anterior part, by contrast, opens dorsally to the mandible base [Figs. 9.2B, 9.3]. Most notably, the anterior part is additionally connected to the pharynx through an oval shaped duct that runs along the ventral rim of the clypeus [Figs. 9.2A,B, 9.3]. This lateral duct is not completely closed and therefore extends the opening of the anterior collecting duct from the mandible base to the pharynx [Figs. 9.2B, 9.3].

The two pairs of collecting ducts are surrounded by a total of about 1400 mostly spherical acini with diameters ranging from 20 to 80 µm [Figs. 9.1, 9.2A,B]. The acini consist of up to 20 class 3 gland

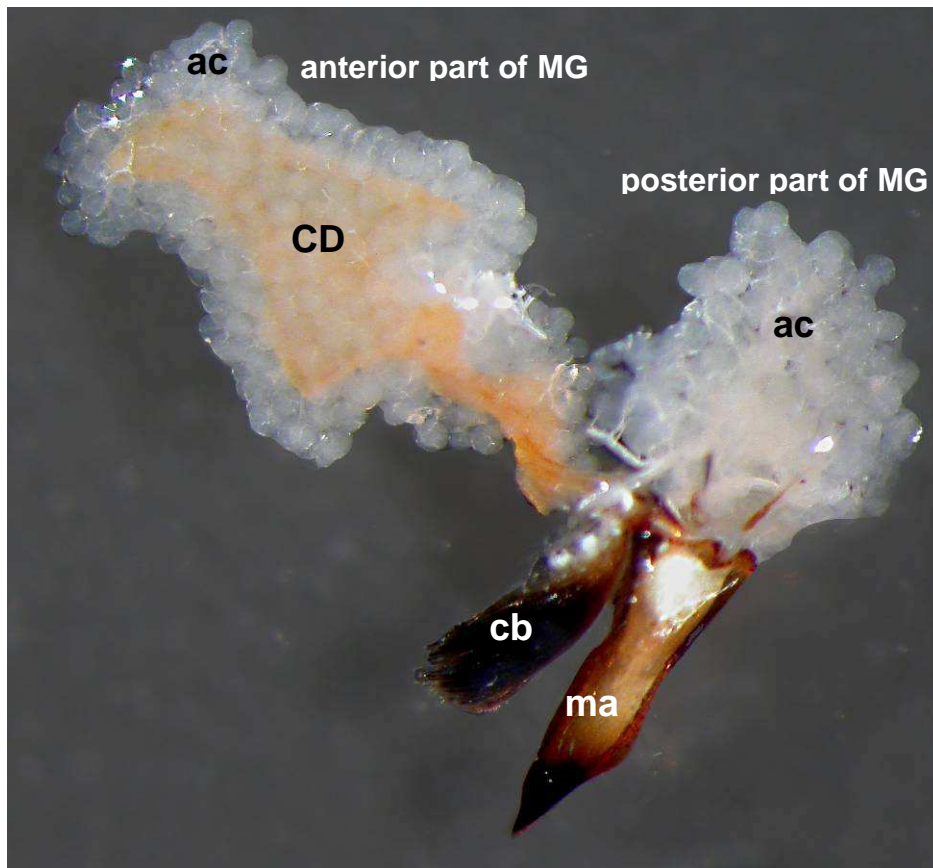


Fig. 9.1. Dissected left mandibular glands of male *P. triangulum* with mandible (ma) and clypeal brush (cb). Note the large collecting duct (CD) of the anterior part of the MG shining through the surrounding acini (ac) in light brown.

cells (according to Noirot and Quennedey, 1974) and are connected to the collecting ducts through bundles of conducting canals [Fig. 9.4]. From air sacs that are located between brain and the mandibular glands a number of tracheoles branch off and run to the acini [Fig. 9.4]. Some tracheoles even extend in between the gland cells that form the acini [Fig. 9.4]

#### 9.4.2 Fine-/Ultrastructure

The class 3 gland cells composing the acini are pervaded by convoluted receiving canals [Figs. 9.4, 9.5A,C]. Such a canal is bordered by an open porous cuticle and enclosed by tightly packed microvilli [Fig. 9.5C]. The most prominent cell organelles in the class 3 gland cells are the well developed smooth endoplasmatic reticulum, spherical mitochondria with diameters about 1  $\mu\text{m}$ , and electron lucent vesicles with diameters up to 3  $\mu\text{m}$  [Fig. 9.5A,B,C]. Most of the vesicles bear membranous inclusions [Fig. 9.5A,C]. The cell membrane is frequently invaginated, at the hemolymph side as well as at the contact zone to the neighbouring class 3 cells [Fig. 9.5A, B].

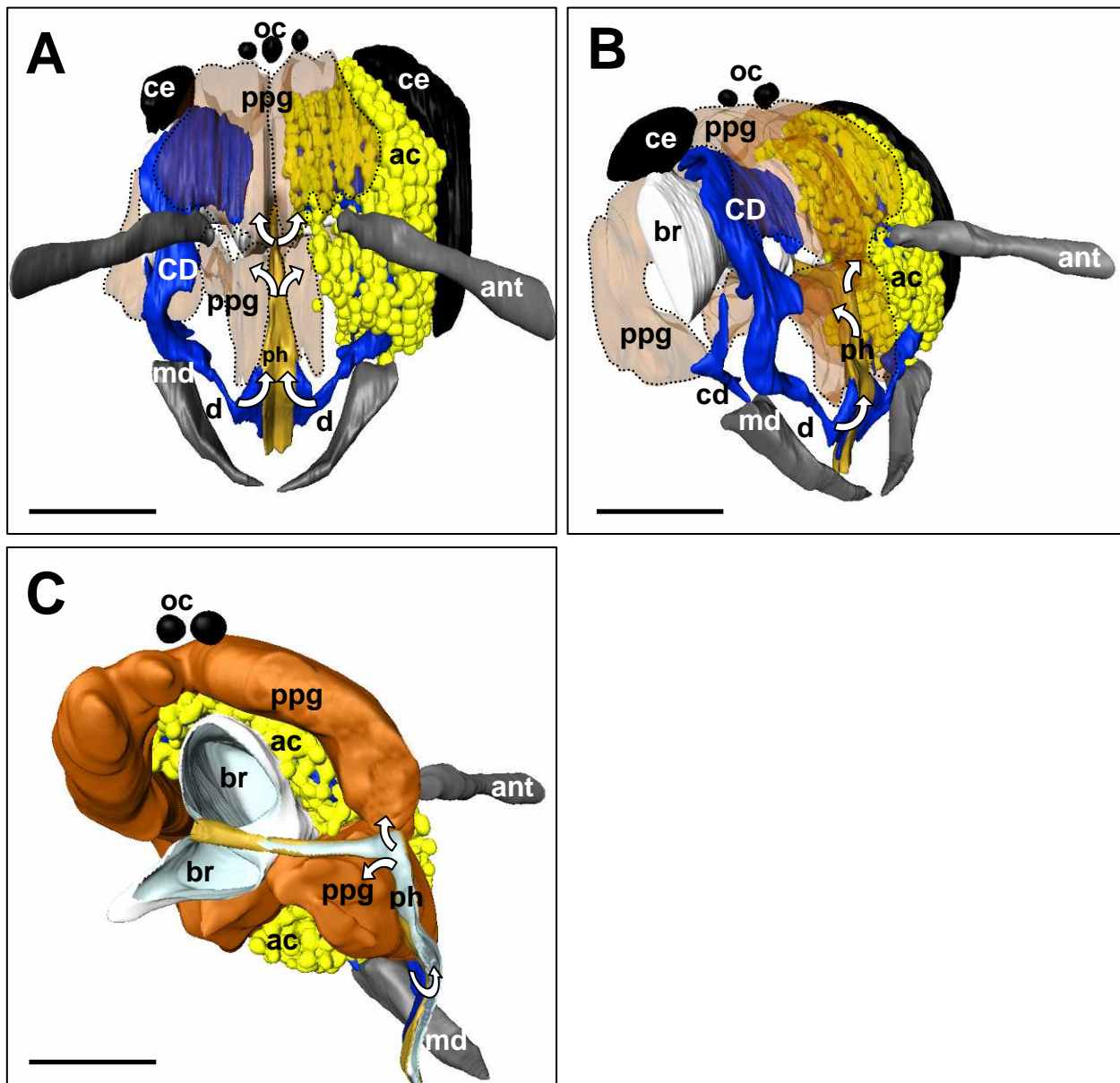


Fig. 9.2. 3D-reconstruction of male *P. triangulum* head. Arrows show the putative way of MG secretion from the anterior collecting duct (CD) through the pharynx (ph) into the postpharyngeal gland (ppg). **A** Frontal view. Acini (ac, yellow) surrounding the collecting ducts (CD, blue) of the mandibular glands are only shown at the left body side. The postpharyngeal gland (ppg) is displayed half transparent (outline pointed). Note the lateral ducts (d) connecting the anterior collecting duct (CD) with the pharynx (ph). The compound eyes (ce) were cut off during preparation. **B** View from lateral/frontal. The small posterior collecting duct (cd) of the mandibular gland is visible. **C** Lateral view. The right half of the head is not shown, scale bars A-C = 1mm. (ac) acini, (ant) antennae, (br) brain, (CD/cd) Anterior/posterior collecting ducts of MG, (ce) compound eyes, (d) lateral ducts, (md) mandibles, (oc) ocelli, (ph) pharynx, (ppg) postpharyngeal gland, (arrows) putative way of secretion from MG into PPG

The conducting canals leading from the acini to the collecting duct are 2  $\mu\text{m}$  thick (outer diameter) and bordered by massive cuticle [Fig. 9.6]. The conducting canals of each acinus form a bundle and open out in the collecting duct in groups [Fig. 9.4]. The monolayered epithelium bordering the lumen of the collecting ducts is about 10  $\mu\text{m}$  thick [Fig. 9.6]. The epithelial cells bear large nuclei and are connected to each other by septate desmosomes [Fig. 9.6]. In the cytoplasm mitochondria and coiled up rough endoplasmic reticulum are the most conspicuous structures [Fig. 9.6].

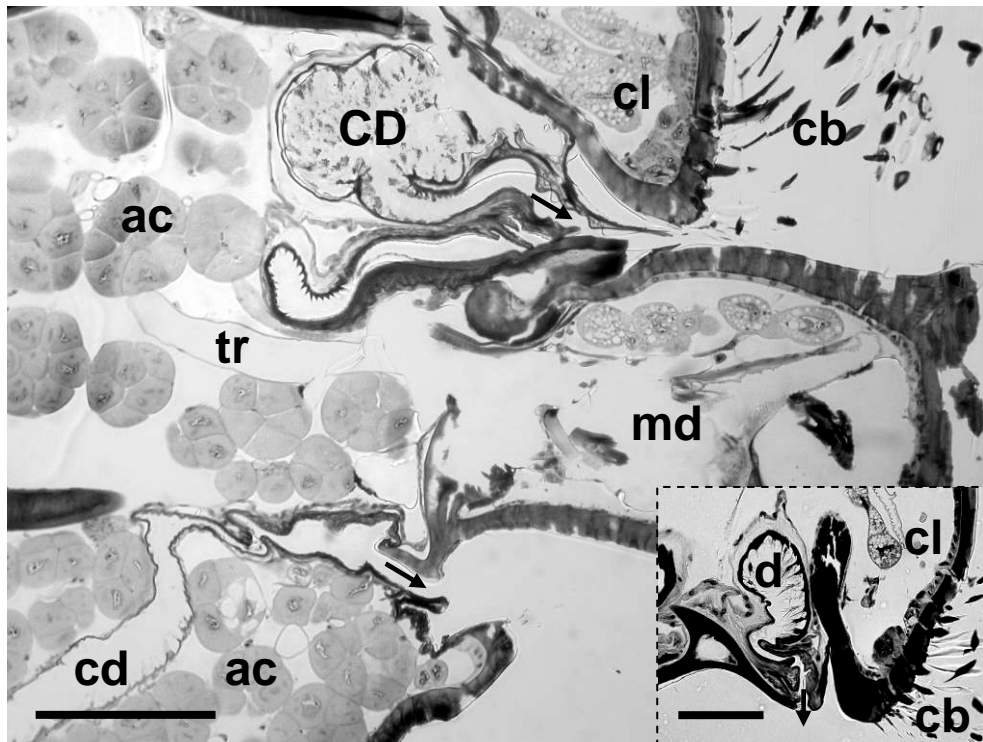


Fig. 9.3. Sagittal semithin section through head of male *P. triangulum* with distinct openings (arrows) of the anterior (CD) and posterior (cd) collecting duct at the mandible base. Inset shows a more central section with the lateral duct (d) that connects the anterior collecting duct to the pharynx, scale bars 100  $\mu\text{m}$ . (ac) acini, (cb) clypeal brush, (cl) clypeus, (md) mandible, (tr) tracheole, (arrows) openings of collecting ducts

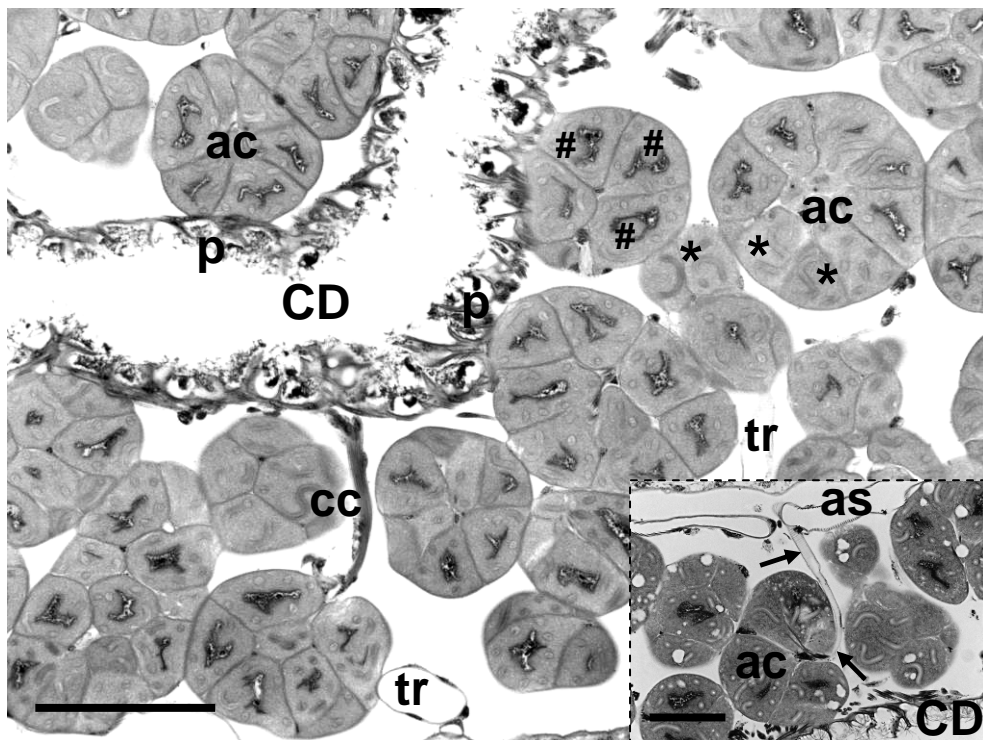


Fig. 9.4. Semithin section through the large anterior part of the mandibular gland with the collecting duct (CD) and surrounding acini (ac). Cuticular protuberances (p) with adherent secretion (dark granules) reach into the duct lumen. The class 3 gland cells show convoluted end apparatuses (\*) as well as nuclei (#) and are connected to the reservoir through bundles of conducting canals (cc). Inset shows tracheole (tr) leading from air sac (as) into acinus, scale bars 50  $\mu\text{m}$ . (tr) tracheole



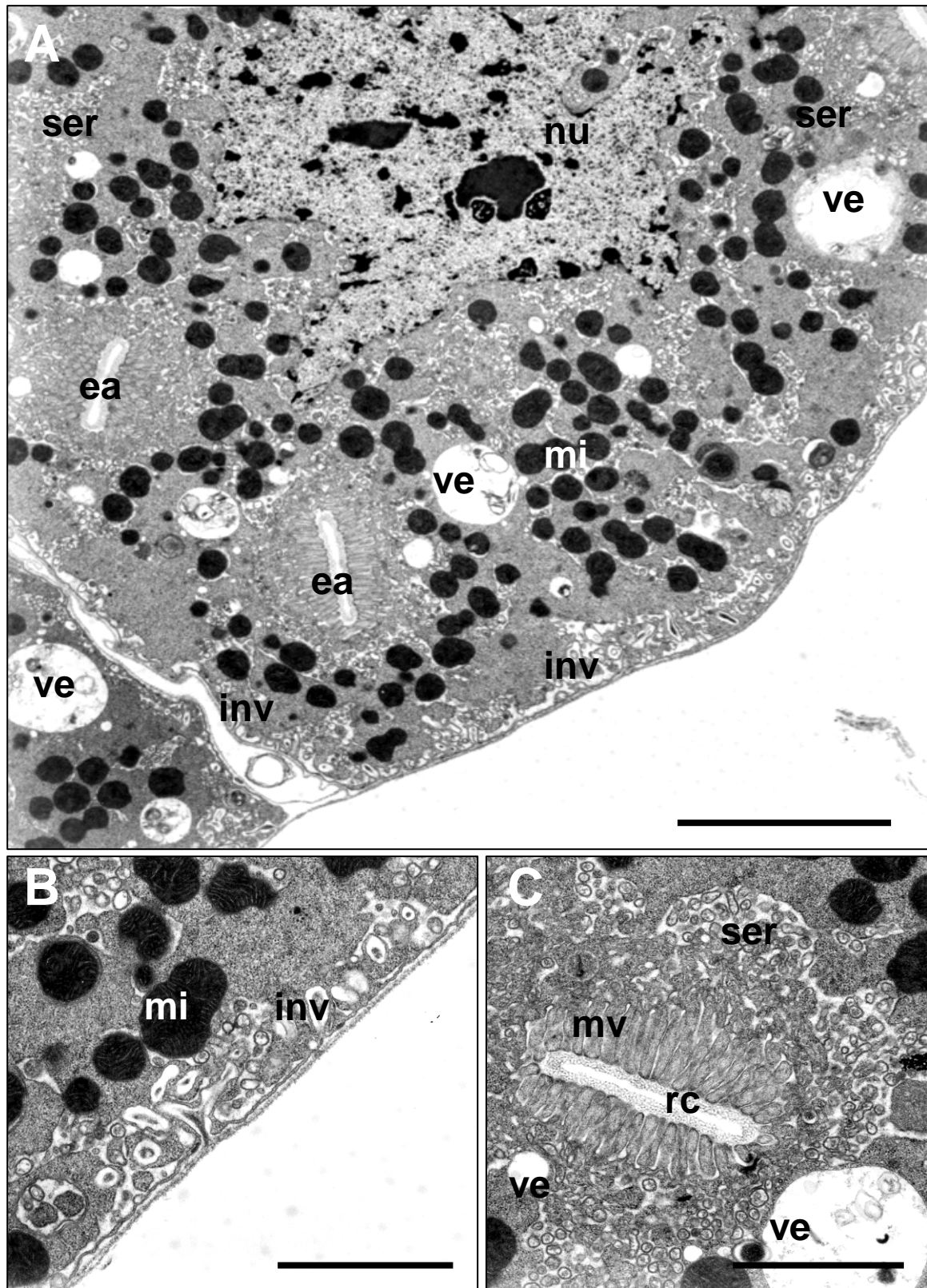


Fig. 9.5. TEM micrographs of class 3 gland cells of the acini. **A** Class 3 gland cell with spherical mitochondria (mi), smooth endoplasmatic reticulum (ser), electron lucent vesicles (ve) with membranous inclusions and end apparatuses (ea). **B** Invaginations (inv) of the plasma membrane. **C** End apparatus consisting of receiving canal (rc) with open porous cuticle, surrounded by microvilli (mv). Next to the end apparatus smooth endoplasmatic reticulum (ser) and vesicles (ve) are visible, scale bars A = 5  $\mu\text{m}$ , B,C = 2  $\mu\text{m}$ .

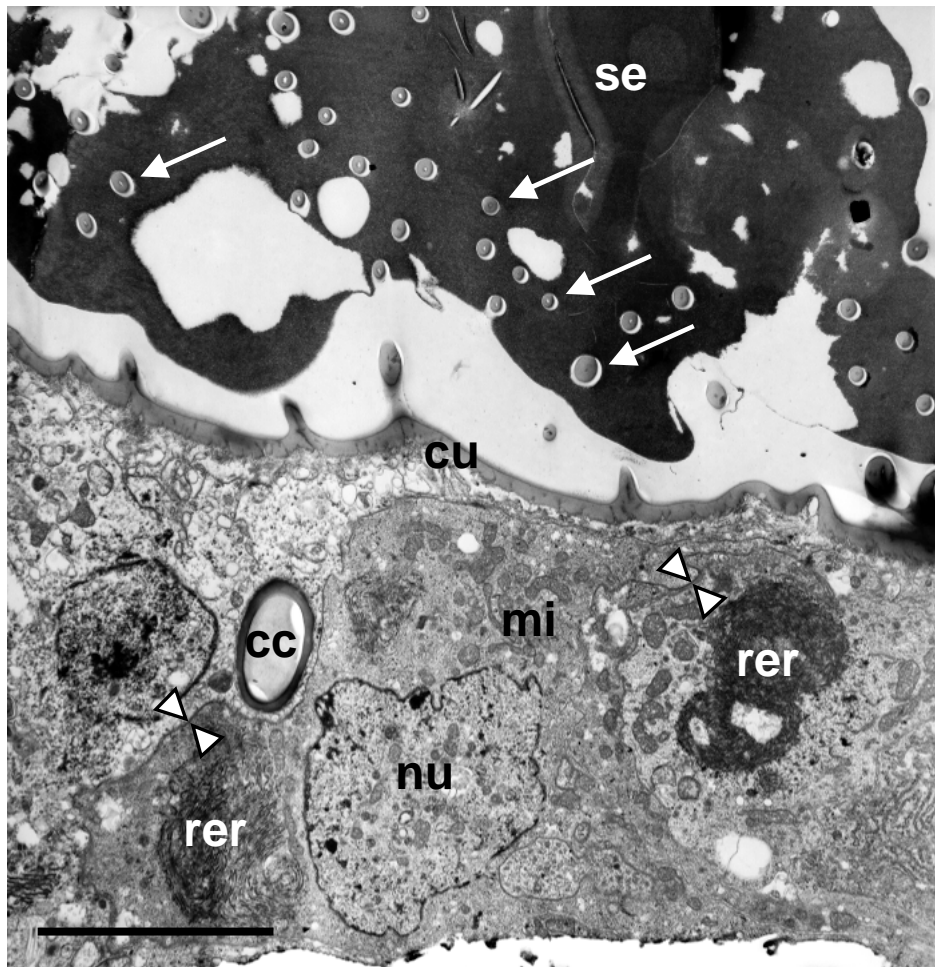


Fig. 9.6. TEM micrograph of monolayered epithelium bordering the collecting ducts. At the lumen side, the epithelium is bordered by cuticle (cu). The cross-sectioned tips of the scale-like cuticle protuberances (arrows) are embedded in electron dense secretion (se). The cells are connected by septate desmosomes (double arrows). A conducting canal (cc) leads through the epithelium. Coiled rough endoplasmatic reticulum (rer) and mitochondria (mi) can be seen in the cytoplasm, scale bar 5  $\mu\text{m}$ . (nu) nucleus

No invaginations of the basal lamina or microvilli at the apical side could be found. The epithelium of the collecting ducts is lined with a 0.5  $\mu\text{m}$  thick cuticle that shows two types of conspicuous protuberances reaching into the lumen. In most parts the cuticle forms tipped scales that are filled with cytoplasm [Figs. 9.4, 9.6, 9.7A,B]. The 15  $\mu\text{m}$  wide scales are arranged in parallel rows and bear 10-15 finger-like tips (up to 20  $\mu\text{m}$  long) at their distal ends [Fig. 9.7A,B]. In the parts of the collecting ducts that are closer to the opening the cuticle forms ramified, tree-like structures consisting of trunks (40  $\mu\text{m}$  long/ 2  $\mu\text{m}$  thick) with approx. 50 branches (20  $\mu\text{m}$  long/ 0.5  $\mu\text{m}$  thick) [Fig. 9.7C,D]. The openings of the conducting canals leading from the acini to the collecting ducts are located at the bases of the scales and trees.

### 9.4.3 Gland contents

In semithin sections intensely stained secretory material between the protuberances of the collecting duct cuticle can be seen [Fig. 9.4]. TEM micrographs show electron dense material between the cuticle structures [Fig. 9.6]. In SEM micrographs irregular granules appear between the rows of the cuticular scales and the branches of the tree-like structures [Fig. 9.7A,B,C,D]. SEM investigations also revealed less secretion in the collecting duct of young male beewolves compared to older males. One day after eclosion the collecting ducts bear only some secretion flakes [Fig. 9.7D] whereas in 5 days old animals large clusters of secretion can be found [Fig. 9.7A,B,C].

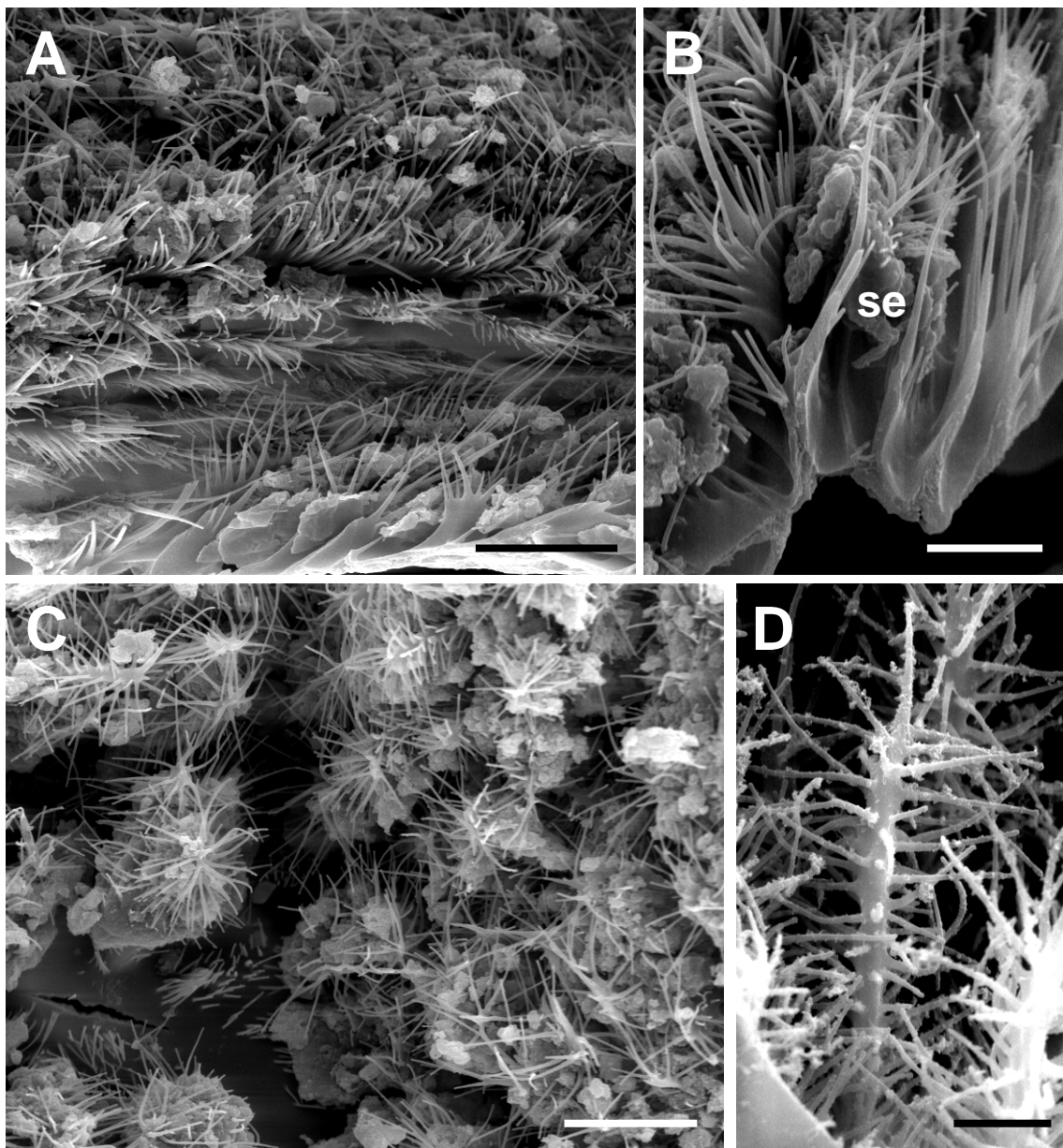


Fig. 9.7. SEM micrographs of cuticular protuberances inside the collecting ducts. **A/B** Scale-like structures. (se) secretion. **C/D** Tree-like structures, each consisting of a trunk with radial branches. The amount of secretion in 5 day old males (A, B, C) is considerably higher than in 1 day old males (D), scale bars A,C = 20 µm, B = 10 µm, D = 5 µm.

## 9.5 Discussion

Male *P. triangulum* possess large and complex paired mandibular glands. On each side of the head capsule there are two parts of different sizes. Each part consists of a collecting duct with a large number of surrounding acini that are connected to it by conducting canals. The walls of the collecting duct bear conspicuous cuticular scales and tree like structures that are partly covered by granules. Both collecting ducts discharge near the base of the mandibles. However, there is also a connection between the anterior collecting duct and the pharynx. Invaginations of the plasma membranes of the class 3 gland cells suggest a high rate of uptake of substances from the hemolymph. The abundance of vesicles and mitochondria and the well developed smooth endoplasmatic reticulum within the cytoplasm of the class 3 cells forming the acini suggest a high secretory activity of the gland cells of the MG. Similarly, the remarkable supply of the acini of the MG with tracheoles suggests very high aerobic metabolism rate.

The glands in male *P. triangulum* resemble the mandibular glands in male *P. bicinctus* (Gwynne, 1978), one of the two other *Philanthus* species for which some information is available. However, mandibular glands of *P. bicinctus* seemingly lack the smaller posterior parts. The MGs are probably the source for the marking pheromone that males apply to their territories (Evans and O'Neill, 1988). This hypothesis is supported by the fact that males of *P. albopilosus*, a species which does not establish and scent mark territories, possess considerably smaller mandibular glands than *P. triangulum* and *P. bicinctus* and also lack a clypeal brush (Evans and O'Neill, 1988).

There are only few other philanthine wasps whose mandibular glands have been investigated. In males of *C. rybyensis* (Philanthinae), mandibular glands consist of two clusters of class 3 gland cells that are connected through canals to "common ducts" at the mandible bases (Ågren, 1977). Furthermore in *C. rybyensis* a cuticular "tube" was described that resembles the collecting duct of the anterior part of the mandibular gland of *P. triangulum* in size and shape, but no associated gland cells have been found (Ågren, 1977).

The function of the unique cuticular protuberances that reach into the lumen of the collecting ducts is not clear. In postpharyngeal gland reservoirs of male and female *P. triangulum* (Herzner et al, 2007; Strohm et al., 2007) there are also digitated scales and hairs (but no tree-like structures) whose function is unknown as well. In *C. Rybyensis*, cuticular pegs with unknown function appear in the common duct of the mandibular glands (Ågren, 1977). Taking into account that the amount of granules on the scales and trees increases with male age in *P. triangulum*, the cuticular protuberances might function as a filter to prevent the blockage of the openings. However, at the moment this is pure speculation. Alternatively, the cuticular scales and trees could also be spacers that prevent the



reservoir from collapsing when the neighbouring PPG (for PPG morphology see: Herzner et al., 2007) expands with increasing filling status and squeezes the mandibular glands.

Kroiss et al. (2006) and Herzner et al. (2007) have shown that most of the secretion that males apply onto the territory is stored in their hugely enlarged PPGs. Since the PPGs do not have any glandular structures that could produce these large amounts of chemicals, these authors hypothesised that most of the secretion is synthesised in the MG and then transported and stored in the PPG. The existence of an internal connection between the anterior part of the mandibular gland and the pharynx in *P. triangulum* supports the hypothesis, that mandibular gland secretion can be transported into the pharynx and taken up by the PPG by a not yet known mechanism. There is no internal connection between the posterior part of the MG and the pharynx. Thus, an uptake of the products of the posterior part of the MG by the PPG might require that the secretion is swallowed.

During marking the large amounts of pheromone stored in the PPG could be delivered through the pharynx tract and the lateral duct onto the clypeal brush. This increase in size and complexity of the cephalic glands in beewolves probably is the result of strong sexual selection for high intensity of scent marking. Whereas the MG seems to be the production site of most of the pheromone components the PPG has undergone a change in function and serves as a huge reservoir. This division of functions is supported by preliminary data obtained by careful dissection and separation of the PPGs and MGs and subsequent GC-MS analysis: In males that are actively scent marking territories, the MG shows only very small amounts whereas the PPGs contains huge amounts of the marking secretion that seem to deplete during the 2-6 h of daily territory activity (G. Herzner, E. Strohm, pers. comm.). This suggests that the mandibular glands are not active during the daily scent marking activity but that the stores in the PPG are replenished during the night. This would make sense if the rate of pheromone production of the MG is too low to warrant high scent marking activity and if the costs of pheromone production during the day would interfere with the territorial behaviour that includes extended aerial combats with intruders.

The mandibular glands of male European beewolves represent complex organs that are probably subject to strong sexual selection. Therefore they are very large and possess abundant gland cells that allow a high rate of pheromone production. Most probably, huge amounts of pheromone are stored in the accordingly enlarged PPG. Thus, there has probably been a change in function of the cephalic glands of male European beewolves to enable males to scent mark at high rates in order to attract receptive females. The lack of detailed information on other species of the genus *Philanthus* and the subfamily Philanthinae currently does not allow a comparative analysis. However, we expect that the glands in species with male scent marking behaviour have also undergone some enlargement as a solution to the problem of how to produce and store large amounts of pheromone.

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## 9.6 References

- Ågren, L. (1977). Mandibular gland morphology of *Cerceris rybyensis* (L.), (Hymenoptera: Philanthidae). *Zoon* 5: 91-95.
- Ayasse, M., Paxton, R.J., Tengö, J. (2001). Mating behaviour and chemical communication in the order Hymenoptera. *Annual Review of Entomology* 46: 31-78.
- Billen, J. (1991). Ultrastructural organization of the exocrine glands in ants. *Ethology Ecology & Evolution* (1): 67-73.
- Borg-Karlson, A.K., Tengö, J. (1980). Pyrazines as marking volatiles in philanthine and nyssonine wasps (Hymenoptera: Sphecidae). *Journal of Chemical Ecology* 6 (4): 827-835.
- Clarke, S.R., Dani, F.R., Jones, G.R., Morgan, E.D., Schmidt, J.O. (2001). (Z)-3-Hexenyl(R)-3-hydroxybutanoate: A male specific compound in three North American decorator wasps *Eucerceris rubripes*, *E. conata* and *E. tricolor*. *Journal of Chemical Ecology* 27: 1437-1447.
- Evans, H.E., O'Neill, K.M. (1988). The Natural History and Behavior of North American Beewolves. Cornell University Press, Ithaca, New York.
- Gwynne, D. (1978). Male territoriality in the bumblebee wolf, *Philanthus bicinctus* (Mickel)(Hymenoptera, Sphecidae): observations on the behaviour of individual males. *Zeitschrift für Tierpsychologie* 47: 89-103.
- Herzner, G., Schmitt, T., Heckel, F., Schreier, P., Strohm, E. (2006). Brothers smell similar: variation in the sex pheromone of male European beewolves *Philanthus triangulum* F. (Hymenoptera: Crabronidae) and its implication for inbreeding avoidance. *Biological Journal of the Linnean Society* 89: 433-442.
- Herzner, G., Goettler, W., Kroiss, J., Pura, A., Webb, A.G., Jakob, P.M., Rössler, W., Strohm, E. (2007). Males of a solitary wasp possess a postpharyngeal gland. *Arthropod Structure & Development* 36 (2): 123-133.
- Hölldobler, B., Wilson, E.O. (1990). The ants. Harvard University Press, Cambridge.
- Jones, T.M., Balmford, A., Quinnell, R.J. (2000). Adaptive female choice for middle-aged mates in a lekking sandfly. *Proceedings of the Royal Society of London Series B* 267: 681-686.
- Kaltenpoth, M., Strohm, E. (2006). The scent of senescence: Age-dependent changes in the composition of the marking pheromone of the male European beewolf (*Philanthus triangulum*, Hymenoptera, Crabronidae). *Journal of Insect Science* 6: 20.
- Kroiss, J., Schmitt, T., Schreier, P., Strohm, E., Herzner, G. (2006). A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 32: 2763-2776.
- McDaniel, C.A., Howard, R.W., O'Neill, K.M., Schmidt, J.O. (1987). Chemistry of male mandibular gland secretions of *Philanthus basilaris* (Cresson) and *Philanthus bicinctus* (Mickel)(Hymenoptera: Sphecidae). *Journal of Chemical Ecology* 13: 227-235.
- McDaniel, C.A., Schmidt, J.O., Howard, R.W. (1992). Mandibular gland secretions of the male beewolves *Philanthus crabroniformis*, *P. barbatus*, and *P. pulcher* (Hymenoptera: Sphecidae). *Journal of Chemical Ecology* 18 (1): 27-37.
- Noirot, C., Quennedey, A. (1974). Fine-structure of insect epidermal glands. *Annual Review of Entomology* 19: 61-80.
- Schmidt, J.O., O'Neill, K.M., Fales, H.M., McDaniel, C.A., Howard, R.W. (1985). Volatiles from mandibular glands of male beewolves (Hymenoptera: Sphecidae, *Philanthus*) and their possible roles. *Journal of Chemical Ecology* 11 (7): 895-901.
- Schmidt, J.O., McDaniel, C.A., Simon Thomas, R.T. (1990). Chemistry of male mandibular gland secretions of *Philanthus triangulum*. *Journal of Chemical Ecology* 16 (7): 2135-2143.
- Schmitt, T., Strohm, E., Herzner, G., Bicchì, C., Krammer, G., Heckel, F., Schreier, P. (2003). (S)-2,3-dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum*. *Journal of Chemical Ecology* 29 (11): 2469-2479.
- Simon Thomas, R.T., Poorter, E.P.R. (1972). Notes on the behaviour of males of *Philanthus triangulum* ((F.) (Hymenoptera, Sphecidae). *Tijdschrift voor Entomology* 115: 141-151.
- Strohm, E. (1995). Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). *Wissenschaftliche Schriftenreihe Biologie* 6.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (1997). Female size affects provisioning and sex allocation in a digger wasp. *Animal Behaviour* 54: 23-34.
- Strohm, E., Lechner, K. (2000). Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 59: 183-191.

- Strohm, E., Herzner, G., Goettler, W. (2007). A "social" gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthropod Structure & Development* 36 (2): 113-122.
- Vander Meer, R.K., Breed, M.D., Espelie, K.E., Winston, M.L. (1998). Pheromone communication in social insects. Westview Press, Boulder, CO.



## CHAPTER 10

**DOES REARING TEMPERATURE AFFECT SYNAPTIC ORGANIZATION  
IN THE BRAIN OF THE SOLITARY RED MASON BEE,  
*OSMIA BICORNIS* (HYMENOPTERA, MEGACHILIDAE)?**

*Brain, Behaviour and Evolution, submitted*

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### 10.1 Summary

Honey bees keep the temperature in their hives remarkably constant. Even small deviations from optimal temperature in the magnitude of 2°C have adverse effects on brain organization and learning of the honey bee progeny. Larvae of solitary bees, by contrast, experience highly variable nest temperatures during their development. In order not to suffer high fitness losses, solitary species should be selected to be more tolerant against changing temperatures during their development. To test this hypothesis, we raised both sexes of the Red Mason bee, *Osmia bicornis* (Hymenoptera, Megachilidae), at three different temperatures (20°C, 25°C, 30°C) and investigated the synaptic organization of the brain in the adults. We focused on the mushroom bodies as these are known as centers of higher integration in the brain of Hymenoptera. Distinct synaptic complexes, so-called microglomeruli, within the mushroom body calyces were visualized by fluorophore-conjugated phalloidin. Although body size and survival rate was reduced in the 30°C group, no significant differences between the three groups according to the abundance of microglomeruli in the mushroom bodies were found. Our results suggest that the development of the brain of *O. bicornis* is buffered against different temperatures during postembryonic development.

## 10.2 Introduction

For holometabolous insects temperature is a crucial abiotic factor that influences postembryonic development and therefore the anatomy, physiology and behaviour of adults (Bosch and Blas, 1994; Blanckenhorn, 1997; Bosch and Kemp, 2004, Jones et al., 2005). Social insects like termites, ants and honey bees actively control climatic factors, like temperature, humidity and carbon-dioxide level in their nests to obtain constant rearing conditions for the brood (e.g. Seeley and Heinrich, 1981; Kronenberg and Heller, 1982; Korb and Linsenmair, 1998, 1999).

Honey bees, *Apis mellifera*, maintain a remarkably constant temperature in their brood combs by active temperature control (Seeley and Heinrich, 1981; Fahrenholz, 1989; Heinrich and Esch, 1994). It has been shown that even small deviations from the optimal temperature affect behavioral performance (Tautz et al., 2003; Jones et al., 2005), short- and long-term-memory (Jones et al., 2005) as well as synaptic organization of the honey bee brain (Groh et al., 2004, 2006). According to Groh et al. (2004, 2006) slight variations in the temperature during pupal development of queens and workers of *A. mellifera* cause significant differences in the amounts of synaptic complexes in the mushroom bodies (MB).

MB are recognized as centers of memory, learning, and orientation in the brain of Hymenoptera (Hammer and Menzel, 1995; Heisenberg, 1998; Gronenberg, 2001; Heisenberg and Gerber, 2002; Strausfeld, 2002; Fahrbach, 2006). Due to its complex geometry the calyx neuropil within the MB is a favourable candidate for structural investigations (Heisenberg, 1998; Strausfeld, 2002; Frambach et al., 2004; Fahrbach, 2006; Kirschner et al., 2006). In honey bees the three subdivisions of the MB calyx, as there are lip, collar and basal ring (Mobbs, 1982), receive mainly olfactory and visual input (Abel et al., 2001; Gronenberg, 2001; Ehmer and Gronenberg, 2002). Olfactory afferents from the antennal lobe innervate the lip, the collar receives visual input from medulla/lobula and the basal ring receives mixed input (Gronenberg and Hölldobler, 1999; Gronenberg, 2001). In holometabolous insects the metamorphosis includes a complete remodelling of the nervous system (Weeks and Levine, 1990; Farris et al., 2004; Ganeshina et al., 2006). The intricate physiological processes of this neuronal reorganization, including apoptosis and synaptogenesis, might be adversely affected by non optimal environmental conditions, like temperature and humidity.

In contrast to honey bees that keep the brood combs at an almost constant temperature (Seeley and Heinrich, 1981; Fahrenholz, 1989; Heinrich and Esch, 1994), the nests of solitary Hymenoptera are subject to environmental temperature fluctuations that are only buffered in brood that is situated deep in the substrate (e.g. Strohm and Linsenmair 1995). The solitary Red Mason bee, *Osmia bicornis* (Hymenoptera, Megachilidae) is one of the most abundant Megachilids in Central Europe (Westrich, 1989). It is polylectic and accepts a large scope of nesting sites (Raw, 1972; Westrich, 1989). Females

build their nests from early April till mid of June in small cavities like holes in walls, hollow stems and crevices in all kinds of material. Each nest consists of several brood cells separated by partitions made of loam (Westrich, 1989; Strohm et al., 2002). The female provisions each brood cell mainly with pollen (>96%) and nectar (Maddocks and Paulus, 1987; Strohm et al., 2002), lays a single egg and closes the cell. After feeding on the pollen the larva spins into a cocoon pupates and passes through metamorphosis during the summer. The completely developed adults hibernate in the cocoon and leave the nest in early spring. Nests of *O. bicornis* are not well insulated since they usually are located near the surface of the substrate. Thus, the temperature within the nests largely depends on outside air temperature and solar irradiation. Consequently, the offspring of *O. bicornis* has to deal with much more variable temperatures during development compared to honey bees. This environmental variability would probably cause adverse effects on the development of complex structures. Thus, *O. bicornis* should have evolved a developmental program that compensates such temperature variation and canalizes the development of crucial organs such as the MB.

In this study we investigated the effects of different rearing temperatures on the synaptic organization in the brain of the Red Mason bee, *O. bicornis*. The temperature regimes we used were more variable than those that produced clear effects in honey bees (Groh et al. 2004, 2006). We predicted that the Red Mason bee is able to compensate for these environmental differences during development and should, thus, show no or much less variation in neuronal structures than observed in honey bees. Since male bees are haploid they might be less able to buffer the environmental differences than females (Clarke and Oldroyd 1996, Clarke 1997). Therefore, males might show more variation in neuronal structures than females. Thus, by examining both males and females of *O. bicornis* the probability of detecting differences in this study was increased. In order to further increase the probability of detecting significant temperature effects, we manipulated the temperature during the whole period from the larval to the adult stage (with one exception, see below) whereas only the pupal period was manipulated in the studies on honey bees. Distinct synaptic complexes within the MB were visualized with fluorophore-conjugated phalloidin which binds specifically to dendritic f-actin (Groh et al., 2004, 2006). We analyzed, whether the size and the number of synaptic complexes in the mushroom bodies are affected by temperature during development.

### 10.3 Materials and Methods

#### 10.3.1 Animals and temperature treatment

Specimens were obtained between May 8<sup>th</sup> and June 14<sup>th</sup> 2006 from a population of *Osmia bicornis cornigera* at the Biocenter, University of Würzburg/Germany nesting in trap nests made of Styrofoam (for details see Strohm et al., 2002). The ceiling of the trap nests consisted of transparent



polycarbonate, so the stage of nest provisioning and development of the brood could be observed continuously.

Completed brood cells with eggs or one-day old larvae were separated by cross cutting the whole block of Styrofoam near the cell partitions. The open sides were sealed with paper that was fixed with adhesive tape. The separated brood cells were randomly split into 3 groups that were subjected to different rearing temperatures. The brood cells were incubated in climate chambers (Ehret ATS 1373; Emmendingen, Germany) at constant 20°C, 25°C and 30°C. To this end the blocks with brood cells were transferred to plastic boxes (20x20x6 cm) that were covered with gauze and additionally put into cardboard boxes to prevent desiccation. To ensure that both sexes were represented in the three groups we made use of the fact that female offspring are mostly reared in the more basal brood cells of a trap nest whereas males are produced in cells closer to the entrance (Raw, 1972). Definitive determination of the sex of an individual was done after metamorphosis (see below). A total of 205 brood cells (36 females, 147 males, 22 dead larvae) from 22 different nests were incubated (68 at 20°C, 72 at 25°C, 65 at 30°C).

The brood cells kept at 20°C and 25°C remained at this temperature for about 14 weeks. Brood cells in which the development to the imago was completed were transferred to a climate chamber with 10°C for diapause. However, in the group initially kept at 30°C, most larvae did not consume all their provisions, thus remained rather small and showed a high mortality of 20% until the end of the feeding period of about four weeks. To avoid the loss of the whole treatment group we transferred these brood cells from 30°C to 25°C immediately after the larvae spun their cocoons. After metamorphosis these brood cells were also transferred to 10°C for diapause. In the following we refer to this group as 30°C group. Between March 27<sup>th</sup> and April 5<sup>th</sup> 2007 all 183 cocoons were opened with razor blades to determine mortality during the pupal phase and the sex of the individual. The maximum head capsule width was determined to the nearest 0.1 mm under a stereo microscope using an eyepiece micrometer.

### *10.3.2 Fluorescent labelling with fluorophore-conjugated phalloidin*

For brain preparations we used 4-5 animals of each sex for each temperature treatment (20°C/25°C/30°C, female 5/5/4, male 4/4/5). To avoid pseudoreplication due to relatedness, all specimens of the same sex *and* temperature treatment were from different nests. Brain preparations were made between March 27<sup>th</sup> and April 5<sup>th</sup> 2007 mainly according to a protocol used for honey bees (Groh et al., 2004). Imagos that were still in diapause at 10°C were taken out of their cocoons, anaesthetised with CO<sub>2</sub>, and decapitated. The heads were immersed in physiological saline solution (130mM NaCl, 5mM KCl, 4mM MgCl<sub>2</sub>, 5mM CaCl<sub>2</sub>, 15mM Hepes, 25mM glucose, 160mM sucrose, pH 7.2). Brains were carefully dissected and fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M PBS, pH 7.2. After washing (3xPBS) brains were embedded in a mixture (1:6.25) of gelatine (Sigma,

G2500) and ovalbumin (Sigma, A5253) and postfixed in 4% paraformaldehyde in 0.1 M PBS, pH 7.2. Brain sections of 60  $\mu\text{m}$  were cut with a vibratome (Leica VT 1000S, Nussloch, Germany) in a frontal plane, so the four calyces were cut transversely and pedunculi and the central body were visible [Fig. 10.1A,B]. Free floating sections were washed (3x PBS) and preincubated in 2% normal goat serum (Sigma, G9023) in PBS with 0.2 % Triton X-100 for 1 h at room temperature. To label neuronal F-actin, the sections were incubated in 0.5% Alexa Fluor 488 phalloidin (Invitrogen, Karlsruhe, Germany) in 1% normal goat serum and 0.2 % Triton in PBS for 2 days at 4°C. After washing (5x PBS) brains were mounted on glass slides in vectashield mounting medium (Vector Labs., H-1000).

### *10.3.3 Confocal Laser Scanning Microscopy, Imaging, Statistics*

Sections were viewed with a confocal laser scanning microscope (Zeiss LSM 510) equipped with an Ar-Laser (488nm). Pseudocolor images were taken with a Zeiss HRc Axiocam at a resolution of 2048x2048 pixels. Tif-images were generated using Zeiss LSM Image Browser (Carl Zeiss, Jena). Measurements of MG size and areas of the lip/collar were performed with Inkscape (freeware, [www.inkscape.org](http://www.inkscape.org)) and Adobe Photoshop Elements 2.0.

Statistical tests were conducted using SPSS 15.0 (Chicago) and BiAS 8.2 (Darmstadt). Effects of head capsule width on other parameters were tested using correlation analyses. Differences among groups were tested using Mann-Whitney U-test ( $N_{\text{females}}=14$ ;  $N_{\text{males}}=13$ ) or exact Kruskal-Wallis test ( $N_{\text{females}20/25/30^\circ\text{C}}=5/5/4$ ;  $N_{\text{males}20/25/30^\circ\text{C}}=5/4/4$ ) followed by Dunn's multiple comparison tests. In case of a non significant result there is the question whether it would have been possible to yield a significant result for a meaningful effect size with the available data, i.e., whether the type II error " $\beta$ " is small enough to warrant the conclusion that there is no effect of a given size. Such a meaningful effect size is given by the studies of Groh et al. (2006) on honey bees. Thus, using power analysis (G-Power 3.0) we estimated the probability ( $1 - \beta$ ) for detecting the difference in the number of MG that would be expected if the Red Mason bee had similar temperature dependence as the honey bee.

### *10.3.4 Counting of MG, Estimating neuropil sizes*

MG countings and area measurements were performed on the inner half of the lateral calyx and the outer half of the medial calyx on both sides [Fig. 10.1A,B]. Only clearly distinguishable MG were counted in three circles with 1000  $\mu\text{m}^2$  each [Fig. 10.1D]. The mean value was estimated from the two circles in the collar. All counts were done independently by two persons; individual differences between these countings were average 8.3 %.

Areas of lip and collar were estimated in Adobe Photoshop by marking the contours with the magnetic lasso [Fig. 10.1D]. Unlike to previous studies on honey bees (Groh et al., 2004, 2006) we did not distinguish between a dense and a loose area in the collar. The extrapolation of MG densities per 1000

$\mu\text{m}^2$  to the whole lip/collar areas resulted in MG abundance per lip/collar. To reveal a possible relation between temperature and MG diameters we measured 3 MG in lip and collar of six randomly chosen calyces for each temperature/sex.

## 10.4 Results

### 10.4.1 Mortality, Body size

Mortality during the larval phase was 6% at 20°C and 7% at 25°C. In the 30°C group most larvae consumed their provisions only partly and, thus, remained considerably smaller when they spun into cocoons and the mortality until this stage was 20%. In order not to lose too many specimens of this group they were transferred to 25°C directly after cocoon spinning. Mortality during the subsequent pupal stage was 9% at 20°C, 10.5% at 25°C and 47% in the 30°C group although these had been transferred to 25°C. None of the surviving animals showed any morphological defects.

As expected due to the sexual dimorphism of *O. bicornis*, females had a larger head capsule width than males (head capsule width, Mann-Whitney U: N=27, U=38, p=0.009) [Fig. 10.2]. In females head capsule width differed significantly between groups (female head capsule width, exact Kruskal-Wallis:  $\chi^2=8.431$ , df=2, p=0.014). The 20°C and 30°C groups differed in pairwise comparisons (Dunn's multiple comparison: 20 vs. 25°C, p=0.175; 25 vs. 30°C, p=0.208; 20 vs. 30°C, p=0.013;). In males both the 20°C and 25°C group showed a significant larger head capsule width than the 30°C group (male head capsule width, exact Kruskal-Wallis:  $\chi^2=8.055$ , df=2, p=0.018; Dunn's multiple comparison: 20 vs. 25°C, p=0.665; 25 vs. 30°C, p=0.028; 20 vs. 30°C, p=0.042;).

### 10.4.2 Phalloidin labelling of synaptic complexes

Similar to honey bees, fluorophore-conjugated phalloidin labelled all neuropils in the brain of *O. bicornis* due to its affinity to dendritic F-actin [Fig. 10.1A,B,C] (Wieland, 1987; Rössler et al., 2002; Frambach et al., 2004; Groh et al., 2004, 2006). We found no conspicuous differences between sexes or groups with regard to gross morphology of the MB and intensity of labelling [Fig. 10.1A,B]. The overall shape of the neuropils of *O. bicornis* resembles that of honey bee queens and workers (Groh, 2004, 2006).

In the calyces of the MB circular structures with outer diameters of 3-3.8 $\mu\text{m}$  were clearly visible [Fig. 10.1D]. The phalloidin-labelled circular structures represent the outer sphere of spherical microglomeruli (MG) which consist of a central, non-labelled, bouton formed by axons from projection neurons that is surrounded by dendritic spines mostly from Kenyon cells (KC) (Frambach et al., 2004; Gronenberg, 2001).

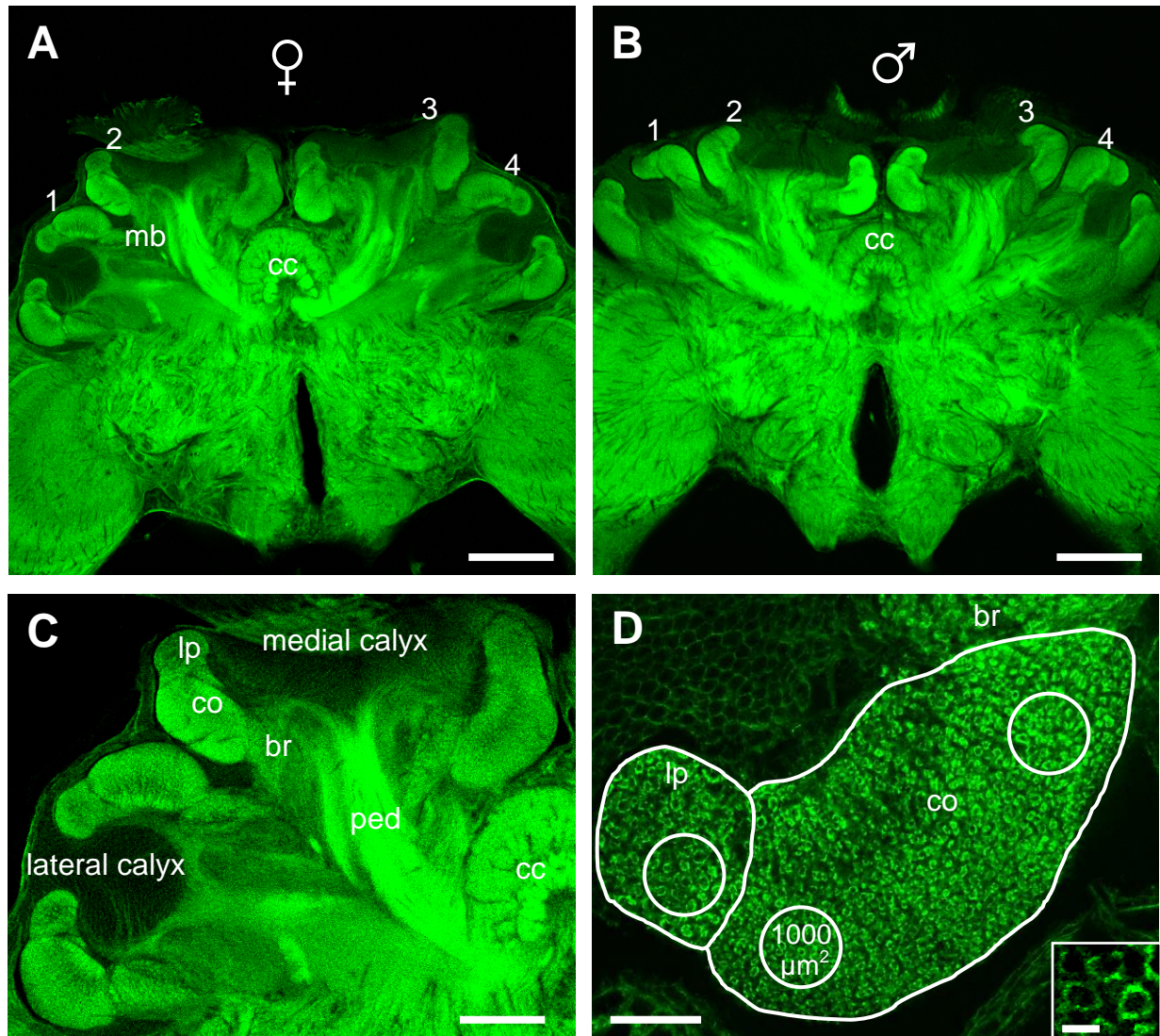


Fig. 10.1. **A/B** Frontal view of brains of female (A) and male (B) *O. bicornis* labelled with fluorophore-conjugated phalloidin. The central complex (cc) and mushroom bodies (mb) are visible. Calyx branches used for MG counting and area measurement are marked by numbers (1-4). scale bars = 200  $\mu\text{m}$ . **C** Mushroom bodies of female *O. bicornis* with their subdivisions lip (lp), collar (co), basal ring (br) and peduncle (ped). scale bar = 100  $\mu\text{m}$ . **D** MB calyx branch with visible MG; the measured lip and collar areas are indicated; MG were counted in three circles of 1000  $\mu\text{m}^2$ . scale bar = 20  $\mu\text{m}$ . **inset** MG at higher magnification. scale bar = 3  $\mu\text{m}$ .

#### 10.4.3 Temperature effects on MB size and MG abundance

Correlation analyses showed that head capsule width is not significantly correlated with MB size, MG density or total number of MG. Thus it was not necessary to correct for body size.

As expected due to different body sizes the measured areas of both lip and collar were larger in females than in males (Mann-Whitney U, N=27: lip area, U=14,  $p < 0.001$ ; collar area, U=31,  $p = 0.003$ ) [Fig. 10.3A]. In females the areas were average 3522  $\mu\text{m}^2$  (lip) and 9685  $\mu\text{m}^2$  (collar), in males 2860  $\mu\text{m}^2$  (lip) and 8215  $\mu\text{m}^2$  (collar). Significant effects of temperature on

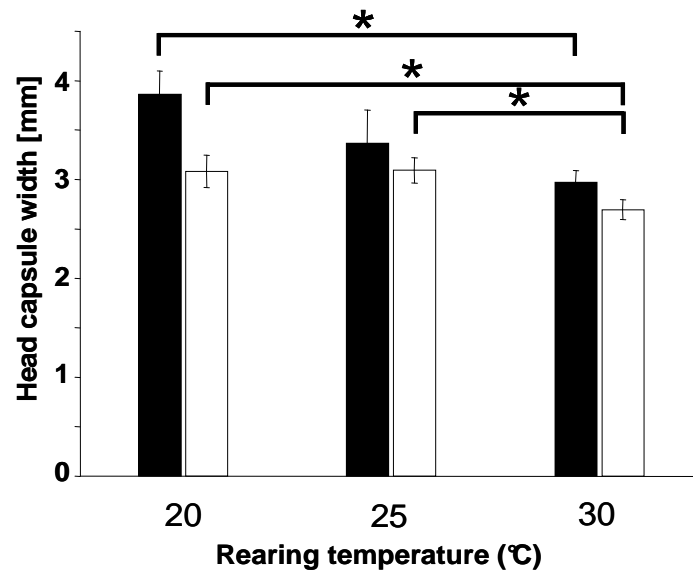


Fig. 10.2. Head capsule width of female (black bars) and male (white bars) *O. bicornis* in the three temperature groups (mean  $\pm$  SD). Asterisks and bars indicate significance.

the areas of lip and collar were found in females between the 25°C and 30°C group (lip area, exact Kruskal-Wallis:  $\chi^2=8.691$ ,  $df=2$ ,  $p=0.004$ ; Dunn's multiple comparison: 20 vs. 25°C,  $p=0.406$ ; 25 vs. 30°C,  $p=0.012$ ; 20 vs. 30°C,  $p=0.072$ ; collar area, exact Kruskal-Wallis:  $\chi^2=8.683$ ,  $df=2$ ,  $p=0.004$ ; Dunn's multiple comparison: 20 vs. 25°C,  $p=0.226$ ; 25 vs. 30°C,  $p=0.010$ ; 20 vs. 30°C,  $p=0.144$ ). In males temperature had no significant effect on neuropil areas (exact Kruskal-Wallis: lip area,  $\chi^2=2.733$ ,  $df=2$ ,  $p=0.271$ ; collar area,  $\chi^2=1.273$ ,  $df=2$ ,  $p=0.556$ ).

The MG densities, meaning MG per 1000  $\mu\text{m}^2$ , were not different between sexes (Mann-Whitney U,  $N=27$ : density lip,  $U=70$ ,  $p=0.325$ ; density collar,  $U=74.5$ ,  $p=0.430$ ) [Fig. 10.3B]. In females densities were 16.6 (lip) and 26.6 (collar), in males 15.1 (lip) and 27.5 (collar). Temperature dependent effects on MG density differed between sexes and again were only significant in females, where MG density was significantly increased in the 30°C group (MG per 1000 $\mu\text{m}^2$  lip, exact Kruskal-Wallis:  $\chi^2=7.243$ ,  $df=2$ ,  $p=0.017$ ; Dunn's multiple comparison: 20 vs. 30°C,  $p=0.044$ ; 20 vs. 25°C,  $p=0.880$ ; 25 vs. 30°C,  $p=0.044$ ; MG per 1000 $\mu\text{m}^2$  collar, exact Kruskal-Wallis:  $\chi^2=8.131$ ,  $df=2$ ,  $p=0.008$ ; Dunn's multiple comparison: 20 vs. 30°C,  $p=0.013$ ; 20 vs. 25°C,  $p=0.199$ ; 25 vs. 30°C,  $p=0.202$ ). In males temperature had no significant effect on the MG density (MG per 1000 $\mu\text{m}^2$  lip, exact Kruskal-Wallis:  $\chi^2=4.579$ ,  $df=2$ ,  $p=0.098$ ; MG per 1000 $\mu\text{m}^2$  collar,  $\chi^2=3.613$ ,  $df=2$ ,  $p=0.170$ ).

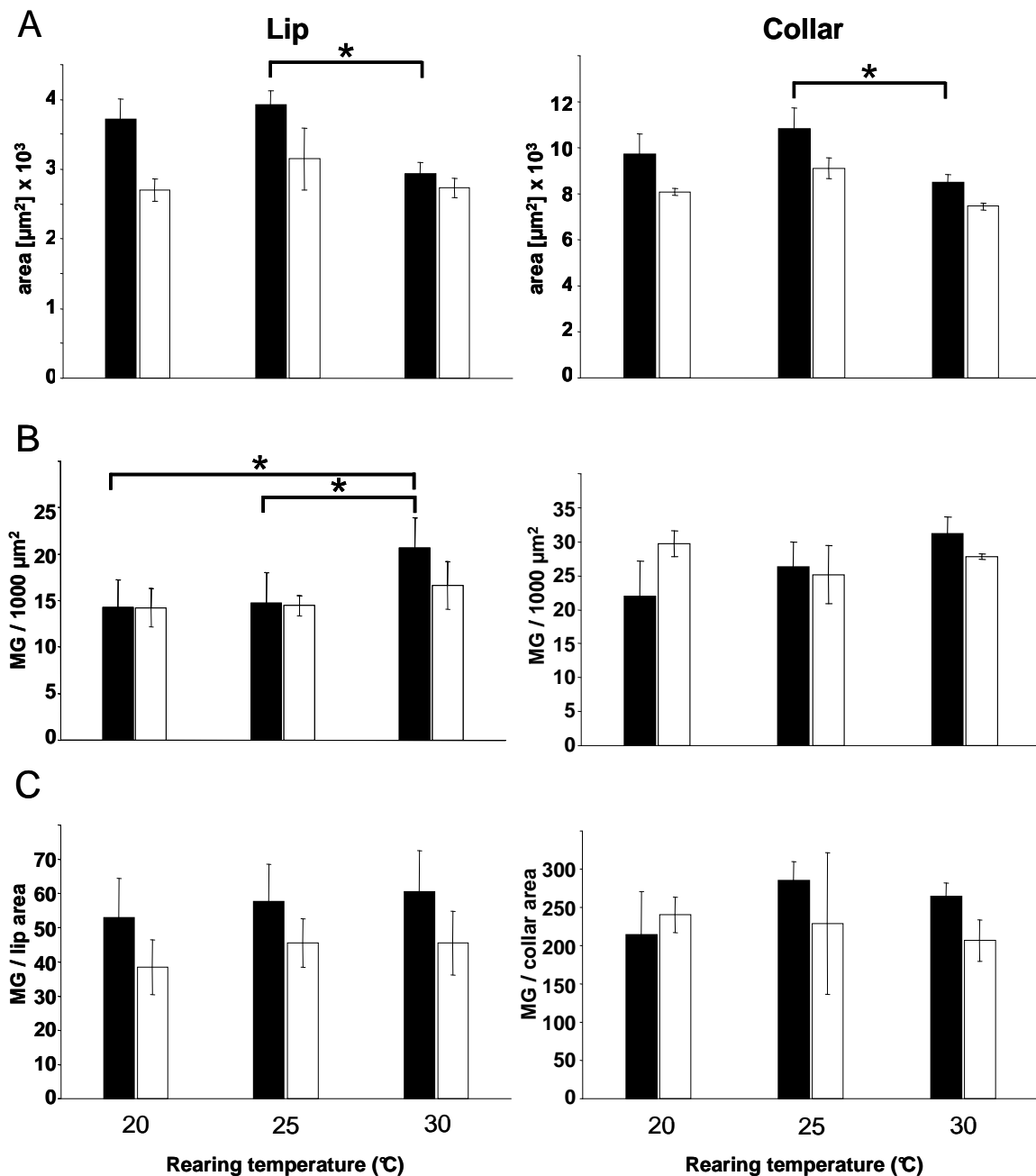


Fig. 10.3. **A** Area of lip and collar region of MB **B** MG density in lip and collar and **C** estimated total number of MG in the lip and collar of female (black bars) and male (white bars) *O. bicornis* reared in the three temperature regimes. Values for the lip region on the left, for the collar on the right. Asterisks and bars indicate significance.

We calculated the total number of MG per lip and collar by extrapolating the MG densities (MG per  $1000\mu\text{m}^2$ ) to the respective areas. Total number in lip and collar were higher in females than in males (Mann-Whitney U, N=27: MG per lip, U=24.5, p=0.001; MG per collar, U=46.5, p=0.029)[Fig.10.3C]. In females the average number of MG were 57 (lip) and 254 (collar). Males MG numbers were 43 (lip) and 225 (collar). The total number of MG per lip and collar was not significantly influenced by temperature in both sexes (exact Kruskal-Wallis: female lip,  $\chi^2=0.672$ ,

df=2, p=0.732; female collar,  $\chi^2=4.657$ , df=2 p=0.092; male lip,  $\chi^2=4.141$ , df=2, p=0.126; male collar,  $\chi^2=2.601$ , df=2, p=0.288).

MG sizes did not differ between sexes (Mann-Whitney U, N=27: MG size lip, U=134, p=0.389; MG size collar, U=138, p=0.462) or between temperatures (exact Kruskal-Wallis: female lip,  $\chi^2=1.180$ , df=2, p=0.574; female collar,  $\chi^2=2.376$ , df=2, p=0.321; male lip,  $\chi^2=2.608$ , df=2, p=0.271; male collar,  $\chi^2=0.143$ , df=2, p=0.939). Average MG sizes were larger in the lip ( $3.63 \pm 0.32 \mu\text{m}$ ) than in the collar ( $3.12 \pm 0.19 \mu\text{m}$ ) (Mann-Whitney U, N=96: U=268, p<0.0001)

To test for the possibility that the non significant effect of temperature on the number of MG in the brain of *O. bicornis* results from inadequate data, we conducted a power analysis. The effect sizes obtained for the honey bee were derived from the values for the difference between 29°C and 34.5°C documented in Figure 2 of Groh et al. (2004). The estimated probability (1- $\beta$ ) of detecting such effects with our data (given the actual sample size and variance,  $\alpha \leq 0.05$ ) would have been 1 both for the lip and for the collar. Thus, it can be excluded that *O. bicornis* might show temperature dependence similar to honey bees but that this effect was not detected due to high variance of small sample size.

### 10.5 Discussion

In this study we tested the hypothesis that different temperatures during development should affect synaptic organization in the brain of the solitary Red Mason bee, *Osmia bicornis*, less severely than has been shown for honey bees (Groh et al. 2004, 2006). Our results support the hypothesis. Although the size of the individuals was significantly influenced by temperature treatment, the total number of MG was not affected by temperature during development.

In order to increase the probability to detect temperature effects we also included males in our study. Male Hymenoptera are (mostly) haploid and might therefore show decreased developmental stability (Clarke and Oldroyd, 1996; Clarke, 1997). Contrary to this prediction, males showed even smaller effects of temperature with regard to both body size and synaptic organization in the MB.

This result differs from investigations on honey bees that exhibit a high degree of temperature control in the hive. Thus, synaptic organization of the brain of honey bees is considerably affected by even small deviations from the optimum (Jones et al., 2005; Groh et al., 2004, 2006). Probably as an adaptation to the inevitable temperature variation in their nests *O. bicornis* shows a much higher degree of developmental stability of brain structures than honey bees.

In the study on honey bees (Groh et al., 2004, 2006), only the temperature during metamorphosis was manipulated. In this study on *O. bicornis*, the temperature during the whole larval and pupal period was experimentally varied, with the exception of the 30°C group that had to be transferred to 25°C after the larval phase to avoid disproportionately high mortality rates. Thus, for the 20°C and 25°C groups the impact of temperature on development should have been stronger than in honey bees but no effect on synaptic organization in the MB was observed. At 30°C larvae of both sexes of *O. bicornis* consumed less pollen than larvae kept at 20°C or 25°C and showed a three times higher mortality during the larval phase. Although we transferred the specimens of the 30°C group to 25 °C after cocoon spinning about half of these bees did not survive the following pupal stage and the survivors were significant smaller than in the other groups. Despite the fact that the 30°C treatment during the larval phase had severe and enduring effects with regard to body size and survival no significant effects on the number of MG in the MB were detected.

In honey bees, bumble bees and in the megachilid *Osmia lignaria* a clear correlation between body size and brain size was found in animals from the field (Mares et al., 2005; Withers et al., 2007). However, in this study the sizes of the MB subdivisions of the temperature treated *O. bicornis* were not correlated to body size. That suggests the correlation between body size and neuropil size is masked by temperature effects on the brain volume.

For honey bees a constant temperature around 35°C is crucial for an optimal brood development (Himmer, 1927; Koeniger, 1978; Tautz et al., 2003; Groh et al., 2004, 2006; Jones et al., 2005). In solitary bees passive temperature control due to insulation of the nest is not given at least in species whose nests are located near the surface of a substrate like in *O. bicornis*. During the development from egg to the adult (April-August) temperatures are rather variable. During cold periods, temperatures could drop near 0°C, whereas during the day the nest temperature could exceed the outside temperature if the nest is exposed to direct sunlight. Therefore we did not expect to find a decrease of larval fitness already at constant 30°C, which is probably not an extreme value in natural nests. This detrimental effect of 30°C is even more surprising taking into account that the temperature optimum for honey bee development, 35°C, is considerably higher.

In honey bees deviations from the optimal rearing temperature not only influence neuronal structures but also affect performance in behavioural assays (Tautz et al., 2003). Since there are no strong temperature effects on brain structure in *O. bicornis*, such detrimental effects of temperature on behavioural performance are not expected. However, temperature might affect behavioural performance through other effects, e.g. body size, or the development of other complex structures. Possibly the proboscis extension reflex could be used in upcoming learning experiments with *O. bicornis* that were reared at different temperatures.



This study exemplifies the different evolutionary solutions to the problem of attaining developmental stability by the eusocial honey bee and the solitary Red Mason bee: In the honey bee, environmental variation is buffered due to the costly homeostasis inside the hive but the individuals have lost the ability to compensate environmental variation. In the Red Mason bee by contrast, environmental conditions are not (or only slightly) buffered in the nest but the individuals show a remarkable ability to canalize the development of important neuronal structures.

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## 10.6 References

- Abel, R., Rybak, J., Menzel, R. (2001). Structure and response patterns of olfactory interneurons in the honeybee, *Apis mellifera*. *Journal of Comparative Neurology* 437: 363-383.
- Blanckenhorn, W.U. (1997). Effects of temperature on growth, development and diapause in the yellow dung fly - against all the rules? *Oecologia* 111: 318-324.
- Bosch, J., Blas, M. (1994). Effect of over-wintering and incubation temperatures on adult emergence in *Osmia cornuta* Latr (Hymenoptera, Megachilidae). *Apidologie* 25: 265-277.
- Bosch, J., Kemp, W.P. (2004). Effect of pre-wintering and wintering temperature regimes on weight loss, survival, and emergence time in the mason bee *Osmia cornuta* (Hymenoptera: Megachilidae). *Apidologie* 35: 469-479.
- Clarke, G.M., Oldroyd, B.P. (1996). The genetic basis of developmental stability in *Apis mellifera*. II. Relationships between character size, asymmetry and single-locus heterozygosity. *Genetica* 97: 211-224.
- Clarke, G.M. (1997). The genetic basis of developmental stability. III. Haplodiploidy: are males more unstable than females? *Evolution* 51: 2019-2026.
- Ehmer, B., Reeve, H.K., Hoy, R.R. (2001). Comparison of brain volumes between single and multiple foundresses in the paper wasp *Polistes dominulus*. *Brain Behavior & Evolution* 57: 161-168.
- Fahrbach, S.E. (2006). Structure of the mushroom bodies of the insect brain. *Annual Review of Entomology* 51: 209-232.
- Fahrenholz, L., Lamprecht, I., Schrickler, B. (1989). Thermal investigations of a honey bee colony: thermoregulation of the hive during summer and winter and heat production of members of different bee castes. *Journal of Comparative Physiology B* 159: 551-560.
- Farris, S.M., Abrams, A.I., Strausfeld, N.J. (2004). Development and morphology of class II kenyon cells in the mushroom bodies of the honey bee, *Apis mellifera*. *Journal of Comparative Neurology* 474: 325-339.
- Frambach, I., Rössler, W., Winkler, M., Schürmann, F.W. (2004). F-Actin at identified synapses in the mushroom body neuropil of the insect brain. *Journal of Comparative Neurology* 475: 303-314.
- Ganeshina, O., Vorobyev, M., Menzel, R. (2006). Synaptogenesis in the mushroom body calyx during metamorphosis in the honeybee *Apis mellifera*: an electron microscopic study. *Journal of Comparative Neurology* 497: 876-897.
- Groh, C., Tautz, J., Rössler, W. (2004). Synaptic organization in the adult honey bee brain is influenced by brood-temperature control during pupal development. *PNAS* 101 (12): 4268-4273.
- Groh, C., Ahrens, D., Rössler, W. (2006). Environment- and age-dependent plasticity of synaptic complexes in the mushroom bodies of honeybee queens. *Brain Behavior & Evolution* 68: 1-14.
- Gronenberg, W., Hölldobler, B. (1999). Morphologic representation of visual and antennal information in the ant brain. *Journal of Comparative Neurology* 412: 229-240.
- Gronenberg, W. (2001). Subdivisions of hymenopteran mushroom body calyces by their afferent supply. *Journal of Comparative Neurology* 436: 474-489.
- Hammer, M., Menzel, R. (1995). Learning and memory in the honeybee. *Journal of Neuroscience* 15 (3): 1617-1630.
- Heinrich, B., Esch, H. (1994). Thermoregulation in bees. *American Scientist* 82 (2): 164-170.
- Heisenberg, M. (1998). What do the mushroom bodies do for the insect brain? An introduction. *Learning & Memory* 5: 1-10.
- Heisenberg, M., Gerber, B. (2002). Common design of mushroom bodies in bees and flies? *Journal of Comparative Neurology* 450: 1-3.
- Himmer, A. (1927). Ein Beitrag zur Kenntnis des Wärmehaushalts im Nestbau sozialer Hautflügler. *Zeitschrift für vergleichende Physiologie* 5: 375-389.
- Jones, J.C., Helliwell, P., Beekmann, M., Maleszka, R., Oldroyd, B.P. (2005). The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*. *Journal of Comparative Physiology A* 191: 1121-1129.
- Kirschner, S., Kleineidam, C.J., Zube, C., Rybak, J., Grünewald, B., Rössler, W. (2006). Dual olfactory pathway in the honeybee, *Apis mellifera*. *Journal of Comparative Neurology* 499: 933-952.
- Koeniger, N. (1978). Das Wärmen der Brut bei der Honigbiene (*Apis mellifera* L.). *Apidologie* 9 (4): 305-320.
- Korb, J., Linsenmair, K.E. (1998). The effects of temperature on the architecture and distribution of *Macrotermes bellicosus* (Isoptera, Macrotermitinae) mounds in different habitats of a West African Guinea savanna. *Insectes Sociaux* 45: 51-65.
- Korb, J., Linsenmair, K.E. (1999). The architecture of termite mounds: a result of a trade-off between thermoregulation and gas exchange? *Behavioral Ecology* 10 (3): 312-316.
- Kronenberg, F., Heller, H.C. (1982). Colonial Thermoregulation in Honey Bees (*Apis mellifera*). *Journal of Comparative Physiology* 148: 65-76.

- Maddocks, R., Paulus, H. (1987). Quantitative Aspekte der Brutbiologie von *Osmia rufa* L. und *Osmia cornuta* Latr. (Hymenoptera: Megachilidae): Eine vergleichende Untersuchung zu Mechanismen der Konkurrenzreduktion zweier nahe verwandter Bienenarten. *Zoologisches Jahrbuch Systematik* 114: 15-44.
- Mares, S., Ash, L., Gronenberg, W. (2005). Brain allometry in bumblebee and honey bee workers. *Brain Behavior & Evolution* 66: 50-61.
- Mobbs, P.G. (1982). The brain of the honey bee, *Apis mellifera*. The connections and spatial organization of the mushroom bodies. *Philosophical Transactions of the Royal Society of London* 298: 309-354.
- Raw, A. (1972). The biology of the solitary bee *Osmia rufa* (L.) (Megachilidae). *Transactions of the Royal Entomological Society of London* 124 (3): 213-229.
- Seeley, T., Heinrich, B. (1981). Insect thermoregulation. Wiley, New York.
- Strausfeld, N.J. (2002). Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes. *Journal of Comparative Neurology* 450: 4-33.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beeswolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Marliani, A. (2002). The cost of parental care: prey hunting in a digger wasp. *Behavioral Ecology* 13 (1): 52-58.
- Tautz, J., Maier, S., Groh, C., Rössler, W., Brockmann, A. (2003). Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. *PNAS* 100 (12): 7343-7347.
- Weeks, J.C., Levine, R.B. (1998). Postembryonic Neuronal Plasticity and its Hormonal Control During Insect Metamorphosis. *Ann. Rev. Neurosci.* 13: 183-194.
- Westrich, P. (1989). Die Wildbienen Baden-Württembergs. Spezieller Teil. Die Gattungen und Arten. Eugen Ulmer GmbH & Co., Stuttgart.
- Withers, G.S., Day, N.F., Talbot, E.F., Dobson, H.E.M., Wallace, C.S. (2007). Experience-dependent plasticity in the mushroom bodies of the solitary bee *Osmia lignaria* (Megachilidae). *Developmental Neurobiology* 68 (1): 73-82.

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## CHAPTER 11

### GENERAL DISCUSSION

#### 11.1 Antennal glands and symbiotic bacteria

In chapters 2 to 6 of this thesis we describe the mutualism between *Philanthus* digger wasps (Hymenoptera, Crabronidae) and symbiotic *Streptomyces* bacteria. The streptomycetes are cultivated in specialized glands in the female digger wasps' antennae and protect the beewolf offspring from pathogenic fungi.

The *Philanthus-Streptomyces* symbiosis is unique with regard to the location of the bacteria-cultivation organs within the beewolves antennae where space is limited and circulation of hemolymph is probably not optimal compared to antennae without the additional obstacle of glands. To proliferate, the bacterial symbionts need nutrients which have to be transported inside the antennal segments via the hemolymph. In insects the dorsal vessel is not able to pump hemolymph in body appendages like antennae and therefore accessory pulsatile organs at the base of extremities function as auxiliary hearts (reviewed in Pass, 2000). Due to increased nutrition requirements inside antennae that result from the bacteria cultivation process we expect enlarged antennal pulsatile organs compared to other Hymenoptera (Matus and Pass, 1999; Pass, 2000). Such organs could also enhance the secreting of the reservoir content into the brood cells by increasing the hemolymph pressure in the antennae. However, this hypothesis has to be tested with detailed morphological investigations on antennal circulatory organs in the genus *Philanthus*.

Our studies on beewolves represent, to our knowledge, the first descriptions of insect antennal glands that are used for bacteria cultivation. Future studies should reveal, whether closely related taxa like the sister genus *Trachypus* (for phylogeny of Philanthinae see Alexander, 1992) also possess antennal glands. Particularly the discovery of less complex glands without bacteria could provide insights about the possible morphology of predecessors of *Philanthus* antennal glands and how they changed during evolution.

*Streptomyces* bacteria are commonly found in the soil (Kutzner, 1981; Dari et al., 1995) and insect antennae with their intersegmental gaps and overlapping segments have numerous cavities which microorganisms could invade and proliferate in – if a nutritional basis is present. Therefore it seems plausible that bacteria infected the antennal glands of beewolf ancestors as commensales or even parasites, possibly using the gland secretions as substrate. Future investigations should reveal the

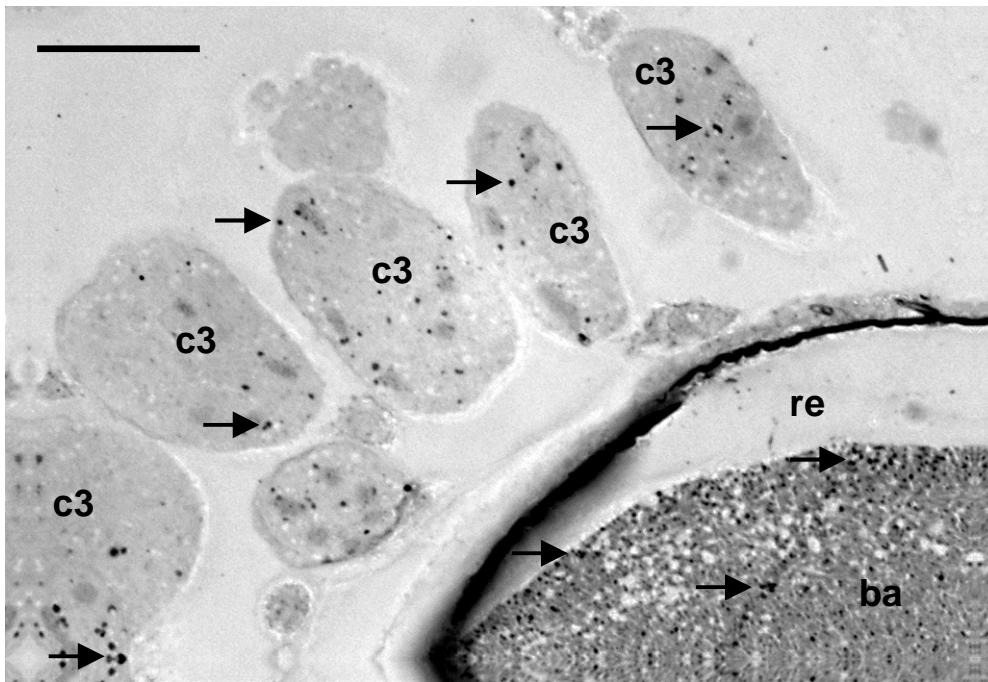


Fig. 11.1 Semithin section of *P. triangulum* antennal glands. Histological staining with Coomassie brilliant blue revealed numerous proteinaceous droplets (some indicated by arrows) within gland cells (c3) and between bacteria (ba) inside the gland reservoir (re). scale bar = 20  $\mu$ m.

chemical nature of the beewolves' antennal gland cell secretions and their role for the bacteria as nutrition basis. Preliminary histological tests for lipids were negative (Goettler, unpubl. data). However, we could identify proteinaceous vesicles within the antennal gland cells and also between the bacteria in the reservoirs (Fig. 11.1; Goettler, unpubl. data). The production of proteins is costly for beewolves, but it seems that the selective advantage of rearing symbiotic bacteria compensates this investment sufficiently.

During the vertical transmission from the beewolf mother to its daughters and at the bottlenecks during brood cell building the streptomycetes probably engage increased genetic drift and an accumulation of deleterious mutations, a process that is known as Muller's ratchet (Lynch and Gabriel, 1990; Gabriel et al., 1993; Lynch et al., 1993; Andersson and Kurland, 1998). However, selection on the beewolf host probably limits the accumulation of detrimental mutations in obligate symbionts and thus counteracts this bias (Andersson and Kurland, 1998; Pettersson and Berg, 2007). Actually there are still many open questions about the beewolf symbionts. Future projects should e.g. reveal whether the mode of the bacteria transmission is strictly vertical or exhibits also horizontal transfer between different *Philanthus* species (Kaltenpoth, in prep.). Another topic is the search for antibiotics which are probably involved in the protection of the beewolf cocoon by the *Streptomyces* bacteria (Kaltenpoth, in prep.).

Cocoon alignment like in *P. triangulum* (Strohm and Linsenmair, 1995) has also been reported for the solitary sphecid wasp *Ammophila pubescens* (Hymenoptera, Sphecidae) (Honomichl, 1998). Females of *A. pubescens* dig a relative short burrow with a single brood cell at the distal side in sandy soil and successively provision the larva with paralyzed caterpillars (Evans, 1965; Field, 2007). The last larval stage spins the cocoon in a way that the head of the pupa points towards the nest entrance (Honomichl, 1998). However, it is not known how the larva of this species obtains the directional cues for cocoon spinning. The larva could for example orientate itself on temperature differences within the substrate, gravitational cues or, like beewolves, on information provided by its mother. If the latter is true *A. pubescens* could possess hitherto undescribed exocrine glands, which secretions act as information carriers. However, this hypothesis is pure speculation and has to be tested by behavioural and anatomical investigations in the future.

Comparable to the *Philanthus-Streptomyces* mutualism attine ants (Formicidae) evolved glands and cuticle crypts on their body surface where symbiotic *Pseudonocardia* bacteria proliferate (Currie et al., 2006). Both, *Pseudonocardia* and *Streptomyces* bacteria belong to the actinomycetes. This group is well known for its antimicrobial secondary metabolites which are frequently used as antibiotics in human medicine (e.g. Kutzner, 1981; Behal, 2000). So it appears that fungus-growing ants, beewolves and – only very recently on the evolutionary timescale – humans use actinomycetes as partners against microbial opponents.

Social insects like bees, wasps, ants and termites are known to produce various antibiotics in exocrine glands. The secretions of mandibular, metapleural, salivary, venom and Dufour's glands have been frequently demonstrated to possess fungistatic and bacteriostatic properties (Hefetz, 1987; Veal et al., 1992; Schmid-Hempel, 1995; Rosengaus et al., 2000, 2004; Ayasse and Paxton, 2002). From a number of solitary insects parental care is known, e.g. earwigs (Dermaptera), burying beetles (Silphidae) or scarab beetles (Scarabaeidae) protect their offspring effectively against pathogenic microorganisms e.g. by eliminating fungal spores through licking and digestion (Tallamy, 1984; Clutton-Brock, 1991; Rankin et al., 1995; Halffter et al., 1996, Eggert et al., 1998). Mass-provisioning of the progeny with perishable food occurs in various insect taxa without parental care, e.g. digger wasps (Crabronidae; Sphecidae) or dung beetles (Geotrupidae). It is unknown how these species deal with the risk of fungal infestation, but maybe symbiosis between insects and protective bacteria is more widespread than we hitherto recognized. The discovery of new effective antibiotics produced by bacteria involved in such symbioses would be an important step in the race of human medicine against multi-resistant pathogens.

## 11.2 Female postpharyngeal glands

In chapter 7 we describe a cephalic gland in the head of female European beewolves which resembles the postpharyngeal gland (PPG) of ants (Formicidae) with regard to location and basic aspects of morphology and ultrastructure. Based on our results we propose the female beewolf gland to be also a PPG. Hitherto it was thought that a PPG is idiosyncratic to ants (Billen, 1990; Hölldobler and Wilson, 1990; Schoeters and Billen, 1997; Lenoir et al., 1999; Eelen et al., 2006) where it plays a crucial role for the organization and maintenance of eusociality by establishing the nest-specific ‘Gestalt’ odour (e.g. Crozier and Dix, 1979; Hefetz et al., 1992; Soroker et al., 1994; Dahbi et al., 1998; Lahav et al., 1999; Oldham et al., 1999). Our description of a PPG in a female crabronid wasp raises the question of the evolutionary origin of the gland and their original function.

Within the aculeate Hymenoptera the genus *Philanthus* (Crabronidae) and the Formicidae are not closely related (see Brothers, 1999; Fig. 11.2). In case that all other aculeate taxa in the phylogenetic tree lack a PPG a homologous origin of this organ in ants and beewolves would be rather unlikely. However, it must be considered that the PPG in female and male European beewolves was found only now, despite the fact that *P. triangulum* is one of the best studied solitary aculeate species (e.g. Tinbergen, 1932, 1935; Rathmayer, 1962; Strohm, 2000; Strohm und Lechner, 2000; Strohm and Linsenmair, 1995, 1997, 1999, 2000, 2001). Therefore other Hymenoptera could possess undiscovered PPG and there is in fact strong evidence for such glands in two other aculeate families, the Ampulicidae and the more basal Chrysididae (Herzner, Strohm, Goettler, unpubl. data; Fig. 11.2) suggesting that this organ is more widespread than hitherto assumed.

According to GC-MS analysis a high congruency between the cuticular HC profiles of females of the cuckoo wasp *Hedychrum rutilans* (Hymenoptera, Chrysididae), and their host species *P. triangulum* was observed (Kroiss, 2008). Additionally behavioural observations showed that the parasitoid *H. rutilans* is rarely attacked by female *P. triangulum* inside the beewolves’ nests. These data suggest that *H. rutilans* uses a kind of chemical mimicry to stay unoffended by the female beewolves (Kroiss, 2008; Strohm et al., 2008). A recently discovered gland in the head of female *H. rutilans* resembles in a number of aspects the PPG in ants/beewolves (Strohm, Goettler, unpubl. data) and could be involved in this kind of chemical camouflage [Fig. 11.3]. Possibly this Chrysidid gland represents another hitherto unknown example of morphological and functional adaptation of a PPG in aculeate Hymenoptera.

As a conclusion we can say, that our results on the location, morphology and ultrastructure of the female PPG in beewolves provide evidence for a homology with the PPG in ants. This view is further supported by other studies about the function and the chemical composition of the PPG secretion (Strohm and Linsenmair, 2001; Herzner and Strohm, 2007; Herzner et al., 2007; Herzner and Strohm,

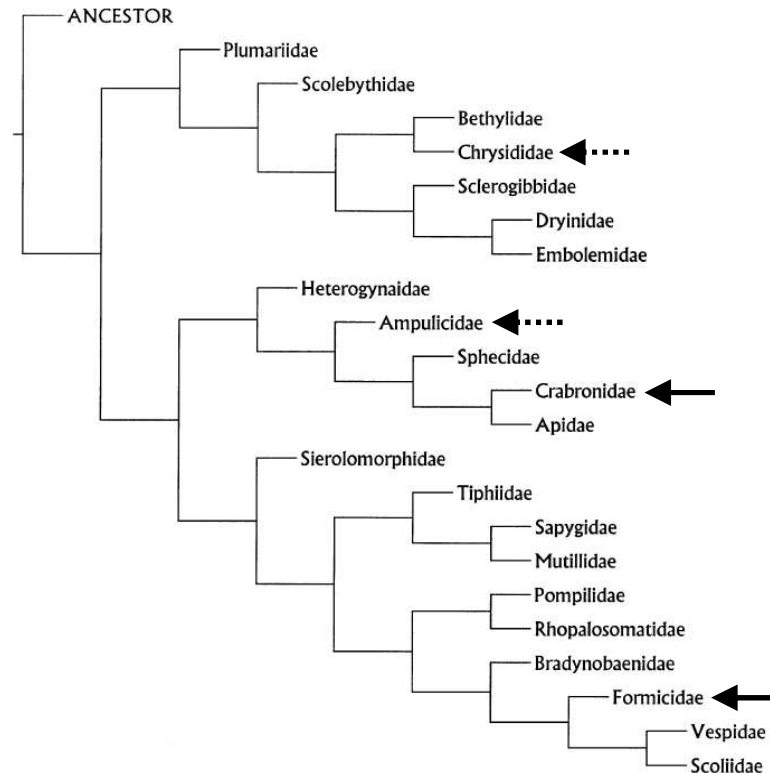


Fig. 11.2. Phylogenetic tree of aculeate Hymenoptera on the family level (Brothers, 1999). Hitherto a PPG has been described in Crabronidae and Formicidae (solid arrows). Preliminary investigations (Herzner, Strohm, Goettler) suggest presence of a PPG also in Chrysididae and Ampulicidae (dotted arrows).

2008). Our hypothesis is also supported by preliminary investigations which revealed that two other aculeate taxa probably possess a PPG (Herzner, Strohm, Goettler, unpubl. data). Therefore we propose that the PPG is not an apomorphism of ants but evolved at a more basal position in the phylogenetic tree of aculeate Hymenoptera, thus representing a plesiomorphic character of the aculeates and has undergone alterations in form and function in different taxa.

It appears that the PPG and antennal glands of female beewolves complement one another in the protection of the offspring against pathogenic fungi. The PPG secretion reduces fungal growth on the honey bee prey during the larval phase (Strohm and Linsenmair, 2001; Herzner et al., 2007; Herzner and Strohm, 2007), whereas the streptomycetes from the antennal glands protect the cocoon from fungal infestation. It seems that only the egg is unprotected against microbial attack, but maybe beewolves evolved – hitherto unknown – mechanisms to protect this vulnerable embryonic stage, too.



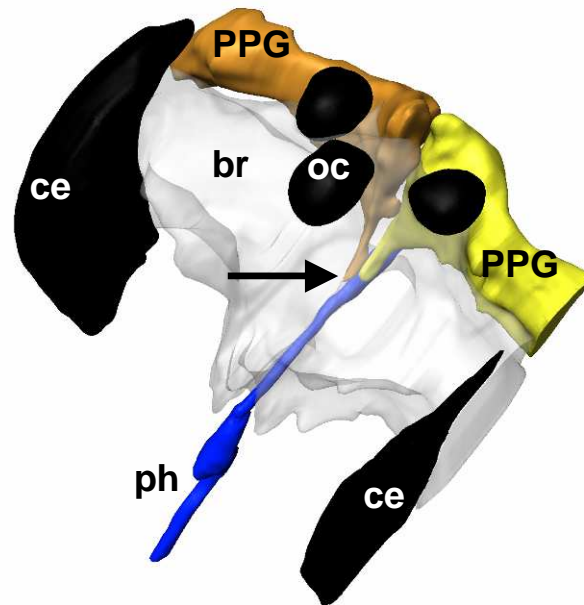


Fig. 11.3. 3D-reconstruction of head of female *Hedychrus rutilans* with putative PPG. View from oblique frontal/dorsal. Note the connections of the two gland parts with the pharynx (arrow) behind the brain (br, half transparent). ce – compound eyes; oc – ocelli; ph – pharynx; PPG – putative postpharyngeal gland.

### 11.3 Male cephalic glands

In chapters 8 and 9 we describe two cephalic glands in male European beewolves, the postpharyngeal gland (PPG) and the mandibular gland (MG). Our morphological studies provide evidence for the hypothesis that both glands are involved in pheromone production and storage. Earlier studies revealed that the PPG contains substances which are also present in the MG (Kroiss et al., 2006) but not in the hemolymph (Strohm, pers. comm.). These facts were the first hints that at least parts of the MG secretions are transported to and stored in the PPG from where they are delivered during the scent-marking process.

Chapter 8 shows that the male PPG shows no enlarged surface or a glandular epithelium as it is the case in female PPG. However the filling status of male PPG varies greatly within short periods of time. One day after eclosion from the cocoon the PPG of the young imagos is empty and collapsed (Goettler, unpubl. data; Fig. 11.4A), whereas it is completely filled with secretion in three day old males which had no opportunity to scent mark territories [Fig. 11.4B]. It seems hardly possible that this remarkable increase of PPG content during 2 days could be achieved by secretion from the PPG epithelium since we did not find any elaborate glandular structures directly associated with this gland. We suppose that only the numerous and well developed acini of the MG have the potential to produce the huge amounts of pheromones that males apply to their territories.

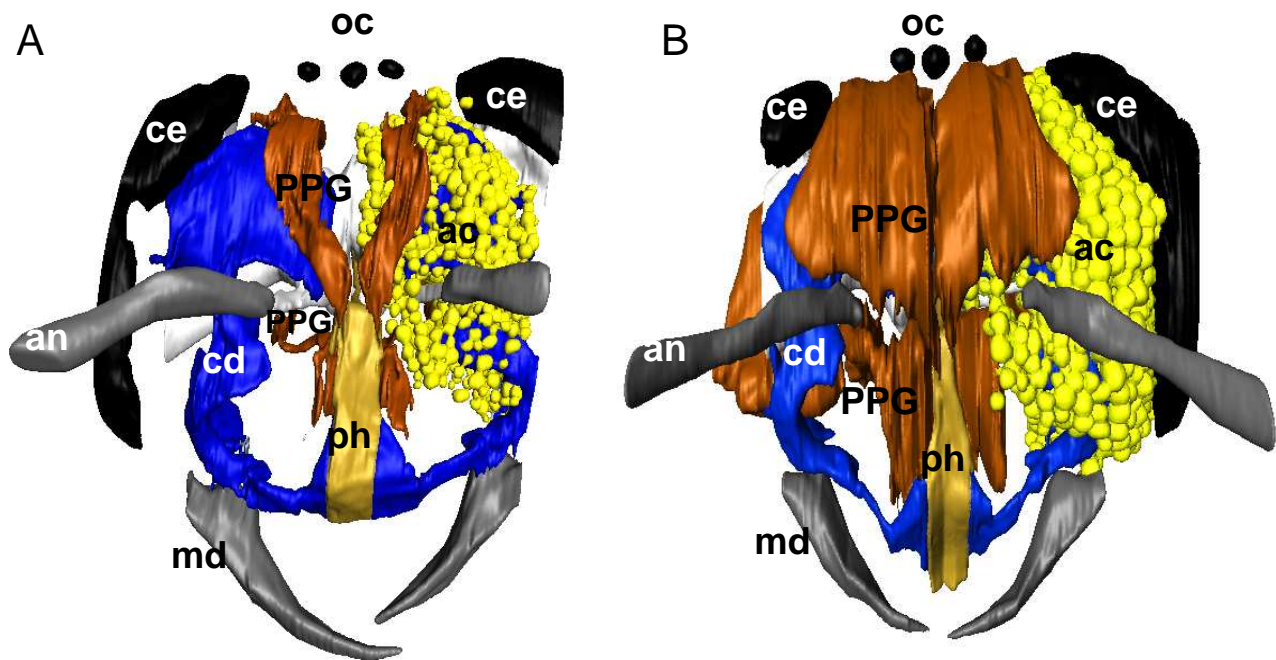


Fig. 11.4. 3D-reconstructions of heads of different aged males of *P. triangulum*. **A** The postpharyngeal gland (PPG) is empty and collapsed in imago directly after eclosion from the cocoon. **B** PPG is filled with secretion in imago 3 days after eclosure. Acini (ac) surrounding MG are only shown on the left body side. an – antenna; cd – collecting duct of MG; ce – compound eyes; md – mandible; ph – pharynx; oc – ocelli

In chapter 9 we describe a lateral duct between the MG and the pharynx that probably enables male European beewolves to transport MG content into the PPG via the pharynx. These morphological characteristics and the varying filling status further support our assumption that the male PPG is in fact a reservoir for the male scent-pheromone.

Male European beewolves extensively scent mark their territories during the day with pheromone and additionally engage conspecific intruders in aerial combat flights (Simon Thomas and Poorter, 1972; Evans and O'Neill, 1988; Strohm and Linsenmair, 1995; Strohm and Lechner, 2000). Both behaviours are energy consuming and probably interfere with a simultaneous production of large pheromone amounts. A solution to this problem could be that pheromone production is done during the night, then stored in the huge PPG and delivered during scent-marking during the day. The observation that male beewolves stay in burrows near the warm surface at night (Strohm, pers. comm.) might be an adaptation to increase the metabolic rate during the night. The PPG could be the evolutionary solution for the need to store the pheromone between production and application in male beewolves.

It is known that males of *P. albopilosus*, which do not scent-mark territories, possess only very small MG and lack a clypeal brush (Evans and O'Neill, 1988). Preliminary investigations of other species in the genus *Philanthus* revealed that the morphology of the male MG and PPG differ greatly even between closely related *Philanthus* species (Goettler, Strohm, unpubl. data). Differences are found in

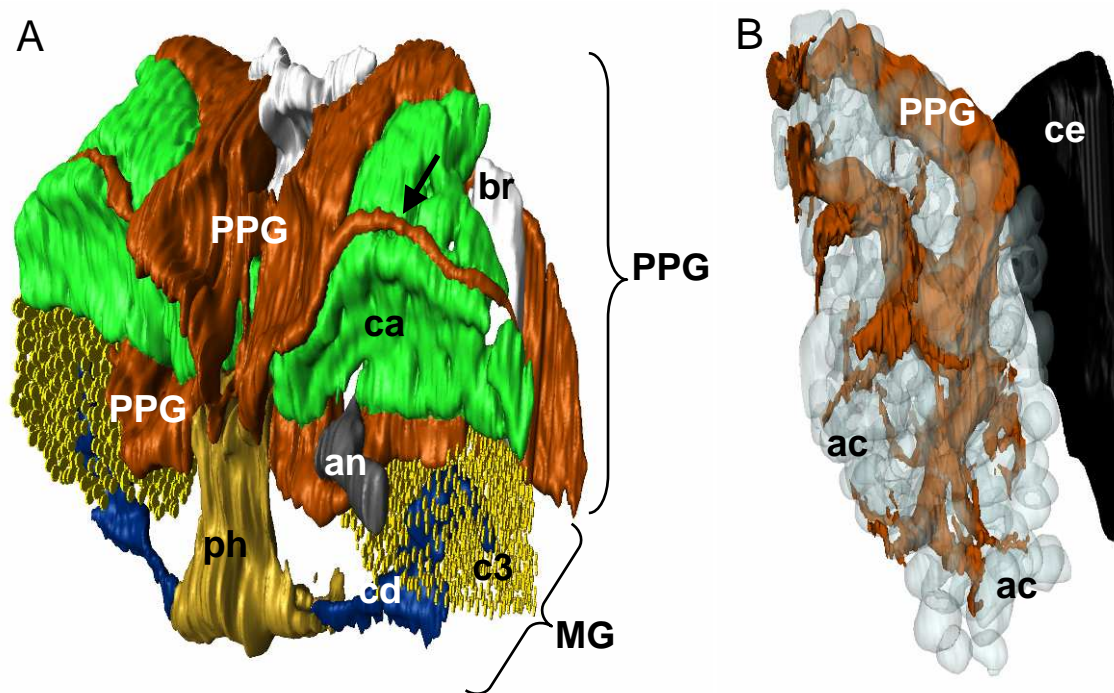


Fig. 11.5. Preliminary 3D-reconstructions of two male heads of the genus *Philanthus*. **A** *P. rugosus* Frontal/oblique view. The collecting ducts (cd) of the small mandibular glands (MG) are surrounded by single class 3 gland cells (c3). The postpharyngeal gland (PPG) shows a conspicuous extension (arrow) surrounded by cell aggregations (ca). **B** PPG of *P. gloriosus*. The ramified PPG is surrounded by numerous acini (ac). ce – compound eye; an – antenna; br – brain; ph – pharynx; reconstructions by Julius Ossowski (A) and Thomas Heimerl (B).

all aspects of the glands' morphology like size, shape, gland cell numbers and types. In males of *P. rugosus* for example the MG collecting ducts are smaller than in *P. triangulum* and surrounded by single class 3 cells instead of acini. Furthermore, in *P. rugosus* the upper part of the PPG shows a conspicuous tube that is surrounded by acini-like cell aggregations [Fig. 11.5A]. However, in the semithin sections we found no canals or end apparatuses connected to this cells, so their function is unclear. Male *P. gloriosus* in contrast exhibit an extensively ramified PPG with surrounding gland cell clusters and only a much reduced MG [Fig. 11.5B]. Further morphological and chemical analyses as well as behavioural observations on territorial males have to reveal the relations between anatomy and function of male cephalic glands in the genus *Philanthus*. This could possibly bring to light that some published data on male MG contents in the subfamily Philanthinae are in fact descriptions of PPG contents. Finally it appears that natural selection led to more or less similar antennal glands in females throughout the genus *Philanthus*, whereas sexual selection heavily changed the morphology of male MG and PPG of even closely related species.

#### 11.4 Temperature and brain development

Chapter 10 of this thesis is a study about temperature-dependent effects on the postembryonic brain-development of the Red Mason bee, *Osmia bicornis*. We used this Megachilid bee for our investigations because it is abundant, undemanding according to its food sources, and has a large scope of nesting sites and therefore easily accepts artificial trap nests (Westrich, 1989; Strohm et al., 2002). We focused our investigations on the mushroom bodies (MB) in the brain since this structure is recognized as centres of higher integration with regard to orientation, memory and learning (Hammer and Menzel, 1995; Heisenberg, 1998; Gronenberg, 2001; Heisenberg and Gerber, 2002; Strausfeld, 2002; Fahrbach, 2006). Temperature-dependent changes in the neuronal patterns of the MB would therefore most likely lead to detectable changes in brain performance like it has been shown for honey bees before (Tautz et al., 2003; Jones et al., 2005).

The temperature regimes we used in our study had no significant influence on the number of synaptic complexes in the MB of *O. bicornis*. This supports our hypothesis that in this species the postembryonic brain development is canalized and shows only small phenotypic variation due to different ambient temperatures. Nevertheless other relevant effects on the brain that we were not able to detect could occur, e.g. changes in ‘quality’ of synaptic organization or physiological defects. Biotests with adult *O. bicornis* which were reared under different temperatures could reveal such effects. Learning and memory skills could be tested by using the proboscis extension reflex, orientational abilities by changing nest positions and the overall fitness of females could be measured as the performance in nest-building, provisioning and number of offspring.

In our study we present data of *O. bicornis* which were reared under different constant temperatures whereas in the field temperatures fluctuate within a broad range. Throughout all insect taxa investigations revealed that temperature cycles (thermoperiods) could influence growth rates in another way than constant temperatures (reviewed in Beck, 1983). In fact investigations on *O. bicornis* from a control group of the field (Goettler, unpubl. data) with natural temperature variations show that these animals obtain a larger body size and also have more synaptic complexes within their MB than the groups with constant temperatures. This suggests that in fact *O. bicornis* not only can stand, but crucially needs thermoperiods during its postembryonic phase for optimal development. Maybe different physiological processes like e.g. ingestion and synaptogenesis need particular ranges of temperature and therefore only alternating temperatures could optimize all of these processes. Upcoming experiments have to determine the range as well as the absolute values of these fluctuating temperatures that would result in an optimal development for *O. bicornis*.

### 11.5 Final Conclusions

The studies presented in this thesis demonstrate that the genus *Philanthus* is a suitable model system for investigating the influence of natural and sexual selection on morphological and physiological traits in aculeate Hymenoptera. The described exocrine glands in the genus *Philanthus* serve unusual functions like bacteria cultivation and food-wrapping or combine the production and storage of pheromones. The antennal glands of *Philanthus* females and the mutualism with *Streptomyces* bacteria provide an amazing example of an obligate insect-bacteria symbiosis. It also impressively demonstrates how coevolutionary processes can change the anatomy and behaviour of insect hosts. The descriptions of postpharyngeal glands in *P. triangulum* show that the knowledge about morphology and the respective functions of organs in the order Hymenoptera is still fragmentary.

Our study on the Red Mason bee, *Osmia bicornis*, confirmed our hypothesis that the postembryonic brain development of this solitary species is buffered against different temperatures during the larval and pupal phase. Further investigations on *O. bicornis* have to reveal where the limits of this temperature tolerance are and whether there is a trade-off between brain buffering and other physiological and morphological traits.

## 11.6 References

- Alexander, B.A. (1992). A cladistic analysis of the subfamily Philanthinae (Hymenoptera: Sphecidae). *Systematic Entomology* 17: 91-108.
- Andersson, S.G., Kurland, C.G. (1998). Reductive evolution of resident genomes. *Trends in Microbiology* 6 (7): 263-268.
- Ayasse, M., Paxton, R.J. (2002). Brood protection in social insects. in Hilker, M., Meiners, T., eds. *Chemoecology of insect eggs and egg deposition*. Blackwell Science, Oxford, UK.
- Beck, S.D. (1983). Insect thermoperiodism. *Annual Review of Entomology* 28: 91-108.
- Behal, V. (2000). Bioactive products from *Streptomyces*. Pages 113-156 in Neidleman, S., Laskin, A., Bennett, J., Gadd, G., eds. *Advances in applied microbiology. Volume 47*. Academic Press, New York.
- Billen, J. (1990). Phylogenetic aspects of exocrine gland development in the Formicidae. Pages 317-318 in Veeresh, G.K., Mallik, B., Viraktamath, C.A., eds. *Social insects and environment*. Oxford and IBH Publishing, New Delhi, Bombay, Calcutta.
- Brothers, D.J. (1999). Phylogeny and evolution of wasps, ants and bees (Hymenoptera, Chrysidoidea, Vespoidea and Apoidea). *Zoologica Scripta* 28 (1-2): 233-249.
- Clutton-Brock, T.H. (1991). The evolution of parental care. Princeton University Press, Princeton, New Jersey.
- Crozier, R.H., Dix, M.W. (1979). Analysis of two genetic models for the innate components of colony odour in social Hymenoptera. *Behavioral Ecology and Sociobiology* 4: 217-224.
- Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J., Billen, J. (2006). Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311 (28): 81-83.
- Dahbi, A., Cerda, X., Lenoir, A. (1998). Ontogeny of colonial hydrocarbon label in callow workers of the ant *Cataglyphis iberica*. *Comptes Rendus de L'Academie des Sciences - Series III - Sciences de la Vie* 321: 395-402.
- Dari, K., Bechet, M., Blondeau, R. (1995). Isolation of soil *Streptomyces* strains capable of degrading humic acids and analysis of their peroxidase activity. *FEMS Microbiology Ecology* 16 (2): 115-122.
- Eelen, D., Borgesen, L., Billen, J. (2006). Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zoologica* 87: 101-111.
- Eggert, A.K., Reinking, M., Mueller, J. (1998). Parental care improves offspring survival and growth in burying beetles. *Animal Behaviour* 55 (1): 97-101.
- Evans, H.E. (1965). Observations on the nesting behavior of digger wasps of the genus *Ammophila*. *American Midland Naturalist* 62 (2): 449-473.
- Evans, H.E., O'Neill, K.M. (1988). The Natural History and Behavior of North American Beewolves. Cornell University Press, Ithaca, New York.
- Fahrbach, S.E. (2006). Structure of the mushroom bodies of the insect brain. *Annual Review of Entomology* 51: 209-232.
- Field, J., Turner, E., Fayle, T., Foster, W.A. (2007). Costs of egg-laying and offspring provisioning: multifaceted parental investment in a digger wasp. *Proceedings of the Royal Society of London Series B* 274: 445-451.
- Gabriel, W., Lynch, M., Burger, R. (1993). Muller's ratchet and mutational meltdowns. *Evolution* 47 (6): 1744-1757.
- Gronenberg, W. (2001). Subdivisions of hymenopteran mushroom body calyces by their afferent supply. *Journal of Comparative Neurology* 436: 474-489.
- Halfpeter, G., Huerta, C., Lopez-Portillo, J. (1996). Parental care and offspring survival in *Copris incertus* Say, a sub-social beetle. *Animal Behaviour* 52: 133-139.
- Hammer, M., Menzel, R. (1995). Learning and memory in the honeybee. *Journal of Neuroscience* 15 (3): 1617-1630.
- Hefetz, A. (1987). The role of Dufour's gland secretions in bees. *Physiological Entomology* 12: 243-253.
- Hefetz, A., Errard, C., Cojocaru, M. (1992). The occurrence of heterospecific substances in the postpharyngeal gland secretion of ants reared in mixed species colonies (Hymenoptera: Formicidae). *Naturwissenschaften* 79: 417-420.
- Heisenberg, M. (1998). What do the mushroom bodies do for the insect brain? An introduction. *Learning & Memory* 5: 1-10.
- Heisenberg, M., Gerber, B. (2002). Common design of mushroom bodies in bees and flies? *Journal of Comparative Neurology* 450: 1-3.
- Herzner, G., Schmitt, T., Peschke, K., Hilpert, A., Strohm, E. (2007). Food wrapping with the post pharyngeal gland secretion by females of the european beewolf *Philanthus triangulum*. *Journal of Chemical Ecology* 33 (4): 849-859.
- Herzner, G., Strohm, E. (2007). Fighting fungi with physics: food wrapping by a solitary wasp prevents water condensation. *Current Biology* 17: R46-R47.

- Herzner, G., Strohm, E. (2008). Food wrapping by females of the European beewolf *Philanthus triangulum* retards water loss of larval provisions. *Physiological Entomology* in press.
- Hölldobler, B., Wilson, E.O. (1990). The ants. Harvard University Press, Cambridge.
- Honomichl, K. (1998). Biologie und Ökologie der Insekten. Fischer, Stuttgart, Jena, Lübeck.
- Jones, J.C., Helliwell, P., Beekmann, M., Maleszka, R., Oldroyd, B.P. (2005). The effects of rearing temperature on developmental stability and learning and memory in the honey bee, *Apis mellifera*. *Journal of Comparative Physiology A* 191: 1121-1129.
- Kroiss, J., Schmitt, T., Schreier, P., Strohm, E., Herzner, G. (2006). A selfish function of a "social" gland? A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *Journal of Chemical Ecology* 32: 2763-2776.
- Kroiss, J. (2008). Chemische Tarnkappe bei einem spezialisierten Brutparasiten des Bienenwolfs. *Naturwissenschaftliche Rundschau* 61 (4): 191-193.
- Kutzner, H.J. (1981). The family Streptomycetaceae. Pages 2028-2090 in Starr, M.P., Stolp, H., Trüper, H.G., Balows, A., Schlegel, H.G., eds. *The Prokaryotes*. Springer, Berlin.
- Lahav, S., Soroker, V., Hefetz, A. (1999). Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften* 86: 246-249.
- Lenoir, A., Fresneau, D., Errard, C., Hefetz, A. (1999). Individuality and colonial identity in ants: the emergence of the social representation concept. in Detrain, C., Deneubourg, J.L., Pasteels, J.M., eds. *Information processing in social insects*. Birkhäuser, Basel.
- Lynch, M., Gabriel, W. (1990). Mutation load and the survival of small populations. *Evolution* 44 (7): 1725-1737.
- Lynch, M., Burger, R., Butcher, D., Gabriel, W. (1993). The mutational meltdown in asexual populations. *Journal of Applied Microbiology* 84 (5): 339-344.
- Matus, S., Pass, G. (1999). Antennal circulatory organ of *Apis mellifera* L. (Hymenoptera: Apidae) and other Hymenoptera: functional morphology and phylogenetic aspects. *International Journal of Insect Morphology* 28: 97-106.
- Oldham, N.J., Morgan, E.D., Agosti, D., Wehner, R. (1999). Species recognition from postpharyngeal gland contents of ants of the *Cataglyphis bicolor* group. *Journal of Chemical Ecology* 25 (6): 1383-1393.
- Pass, G. (2000). Accessory pulsatile organs: Evolutionary innovations in insects. *Annual Review of Entomology* 45: 495-518.
- Petersson, M.E., Berg, O.G. (2007). Muller's ratchet in symbiont populations. *Genetica* 130 (2): 199-211.
- Rankin, S.M., Storm, S.K., Pioto, D.L., Risser, A.L. (1995). Maternal behavior and clutch manipulation in the ring-legged earwig (Dermaptera: Carcinophoridae). *Journal of Insect Behavior* 9 (1): 85-103.
- Rathmayer, W. (1962). Paralysis caused by the digger wasp *Philanthus*. *Nature* 196: 1149-1151.
- Rosengaus, R.B., Lefebvre, M.L., Traniello, J.F.A. (2000). Inhibition of fungal spore germination by nasutitermes: evidence for a possible antiseptic role of soldier defensive secretions. *Journal of Chemical Ecology* 26 (1): 21-39.
- Rosengaus, R.B., Traniello, J.F.A., Lefebvre, M.L., Maxmen, A.B. (2004). Fungistatic activity of the sternal gland secretion of the dampwood termite *Zootermopsis angusticollis*. *Insectes Sociaux* 51 (3): 259-264.
- Schmid-Hempel, P. (1995). Parasites in social insects. *Apidologie* 26: 255-271.
- Schoeters, E., Billen, J. (1997). The post-pharyngeal gland in *Dinoponera* ants (Hymenoptera: Formicidae): Unusual morphology and changes during the secretory process. *International Journal of Insect Morphology & Embryology* 25 (4): 443-447.
- Simon Thomas, R.T., Poorter, E.P.R. (1972). Notes on the behaviour of males of *Philanthus triangulum* (F.) (Hymenoptera, Sphecidae). *Tijdschrift voor Entomology* 115: 141-151.
- Soroker, V., Vienne, C., Hefetz, A. (1994). The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 81 (11): 510-513.
- Strausfeld, N.J. (2002). Organization of the honey bee mushroom body: representation of the calyx within the vertical and gamma lobes. *Journal of Comparative Neurology* 450: 4-33.
- Strohm, E., Linsenmair, K.E. (1995). Leaving the cradle: How beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology Jena* 98 (3): 137-146.
- Strohm, E., Linsenmair, K.E. (1997). Low resource availability causes extremely male-biased investment ratios in the European beewolf, *Philanthus triangulum* F. (Hymenoptera, Sphecidae). *Proceedings of the Royal Society of London Series B* 264 (1380): 423-429.
- Strohm, E., Linsenmair, K.E. (1999). Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behavioral Ecology and Sociobiology* 47: 76-88.
- Strohm, E. (2000). Factors affecting body size and fat content in a digger wasp. *Oecologia* 123: 184-191.
- Strohm, E., Lechner, K. (2000). Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Animal Behaviour* 59: 183-191.



- Strohm, E., Linsenmair, K.E. (2000). Allocation of parental investment among individual offspring in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Biological Journal of the Linnean Society* 69 (2): 173-192.
- Strohm, E., Linsenmair, K.E. (2001). Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecological Entomology* 26 (2): 198-203.
- Strohm, E., Daniels, H., Warmers, C., Stoll, C. (2002). Nest provisioning and a possible cost of reproduction in the megachilid bee *Osmia rufa* studied by a new observation method. *Ethology Ecology & Evolution* 14: 255-268.
- Strohm, E., Kroiss, J., Herzner, G., Laurien-Kehnen, C., Boland, W., Schreier, P., Schmitt, T. (2008). A cuckoo in wolves' clothing? Chemical mimicry in a specialized cuckoo wasp of the European beewolf (Hymenoptera, Chrysididae and Crabronidae). *Frontiers in Zoology* 5:2.
- Tallamy, D.W. (1984). Insect parental care. *BioScience* 34 (1): 20-24.
- Tautz, J., Maier, S., Groh, C., Rössler, W., Brockmann, A. (2003). Behavioral performance in adult honey bees is influenced by the temperature experienced during their pupal development. *PNAS* 100 (12): 7343-7347.
- Tinbergen, N. (1932). Über die Orientierung des Bienenwolfes (*Philanthus triangulum* Fabr.). *Zeitschrift für vergleichende Physiologie* 16: 305-335.
- Tinbergen, N. (1935). Über die Orientierung des Bienenwolfes II. Die Bienenjagd. *Zeitschrift für vergleichende Physiologie* 16: 305-355.
- Veal, D.A., Trimble, J.E., Beattie, A.J. (1992). Antimicrobial properties of secretions from the metapleural glands of *Myrmecia gulosa* (the Australian bull ant). *Journal of Applied Microbiology* 72 (3): 188-194.
- Westrich, P. (1989). Die Wildbienen Baden-Württembergs. Spezieller Teil. Die Gattungen und Arten. Eugen Ulmer GmbH & Co., Stuttgart.





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## CHAPTER 12

### SUMMARY

#### 12.1 Antennal glands and symbiotic bacteria in beewolves

The development and survival of insects frequently depend on mutualistic associations with endosymbiotic bacteria. Insects profit by their bacterial partners in terms of digestion, pheromone production or pathogen-defence and in return provide the bacteria with a free ecological niche and a reliable transmission to the next generation. During coevolution insect hosts frequently developed specialized cells and organs which symbiotic bacteria are reared in and evolved behavioural patterns that ensure the vertical transfer of symbionts to the offspring.

In this thesis we describe an unusual symbiosis between solitary digger wasps of the genus *Philanthus* (Hymenoptera, Crabronidae) and *Streptomyces* bacteria. The antennae of the female digger wasps bear unique glands which consist of large reservoirs and numerous surrounding gland cells. Within the gland reservoirs *Streptomyces* bacteria proliferate and probably use the primary gland cell secretions as nutrition basis. Observations in our model species, the European beewolf (*Philanthus triangulum*) revealed that the streptomycetes are secreted in large amounts by the female digger wasps into the subterranean brood cells. The beewolf larva actively takes up the bacteria with its mouthparts and applies them to the silk of its cocoon. Biotests showed that the presence of the *Streptomyces* bacteria drastically reduces the risk of fungal infestation of the cocoon and thus enhance the survival rate of the offspring. Streptomycetes belong to the actinomycetes-group, which members are well known for their production of antibiotics. Therefore we assume that also the fungicide properties of the beewolf symbionts are based on – maybe hitherto unknown – antibiotics.

*Streptomyces* bacteria were found with genetic methods in antennae of all of the 31 so far investigated species of the genus *Philanthus* but were absent in closely related taxa. As a whole the streptomycetes associated with *Philanthus* digger wasps represent a monophylum inside the *Streptomyces* group. Based also on morphological, ecological and genetic data we therefore propose a new monophyletic taxon “*Candidatus Streptomyces philanthi*”. The transmission route of the symbiotic streptomycetes is not yet clear, but there is some evidence that young females take up small amounts of bacteria into their antennae during the eclosure from the cocoon. Quantitative investigations of the amount of bacteria within the antennal glands at different age support this hypothesis. Our investigations also revealed that there are severe bottlenecks during the transmission between mother and daughter as well as during the secretion process.

The comparative investigation of the antennal gland morphology in 15 *Philanthus* species from Africa, Europe and North America revealed limited interspecific differences in gland shape, size and the number of gland cells. Our results suggest that already the common ancestor of the genus *Philanthus* possessed complex antennal glands and bacteria. Probably the successful symbiosis between the *Philanthus* ancestor and streptomycetes enhanced the radiation and world-wide dispersal of the genus *Philanthus*.

## 12.2 Cephalic glands in European beewolves

Natural and sexual selection resulted in an enormous variety of morphological and functional adaptations of insects' exocrine glands. Whereas in social hymenoptera most glands are well studied, the knowledge about the biggest part of these important organs in solitary species is still fragmentary or non-existent.

This thesis provides evidence that both sexes of the European beewolf exhibit a postpharyngeal gland (PPG) which was hitherto thought to be idiosyncratic to ants. We could show that morphological traits are very similar between the PPG of ants and beewolves, albeit the respective functions are different. Our results together with preliminary investigations in other families of Hymenoptera provided strong evidence that a PPG is in fact a homologous characteristic in aculeate Hymenoptera, which function was changed in different taxa by natural selection.

In male beewolves the PPG most likely functions as a reservoir for the male sex pheromone which is produced in the neighbouring mandibular glands (MG). Male MG show a simple tube-like structure without any associated gland cells. Morphological investigations of PPG and MG in beewolf males revealed the existence of a connecting duct between both glands and therefore support the hypothesis of distinct pheromone production and storage. As the male sex pheromone plays an important role in the mating behaviour of beewolves the involved glands are probably subject to strong sexual selection. This hypothesis is supported by preliminary investigations on males of other *Philanthus* species that show a wide range of different morphological traits of PPG and MG.

## 12.3 Temperature-effects on brain-development of Red Mason bees

Insects are poikilothermic and therefore face the problem to attain developmental stability in an environment with varying temperatures. In particular the complex anatomical reorganization of holometabolous insects during metamorphosis is most likely affected by temperature. Social insects avoid the problem of environmental-effects on postembryonic development by controlling temperature actively inside their nests. Honey bees (*Apis mellifera*) for example keep the temperature of their

brood combs constant at about 35°C and it could be shown in former studies that even small deviations from this temperature during postembryonic development result amongst other things in deficits of the synaptic organization of distinct brain areas.

The question is whether/how solitary species which do not develop in homeothermic nests are influenced by different temperatures. We therefore manipulated temperatures during the postembryonic development of the Red Mason bee, *Osmia bicornis* (Hymenoptera, Megachilidae) and investigated, whether this species shows comparable temperature-effects on brain-development like honey bees. We focussed our investigations on distinct synaptic complexes (microglomeruli) within the mushroom bodies, as the latter are recognized as centers of higher integration in the insect brain. We used fluorophore-conjugated phalloidin to visualize microglomeruli and therefore could estimate their total number and density. Our results on *O. bicornis* suggest that brain-development in this species is buffered against different temperatures and thus adapted to the naturally occurring temperature-conditions in its non-insulated nests.



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## CHAPTER 13

### ZUSAMMENFASSUNG

#### 13.1 Antennendrüsen und symbiotische Bakterien bei Bienenwölfen

Die Entwicklung und das Überleben von Insekten beruhen häufig auf mutualistischen Beziehungen mit endosymbiotischen Bakterien. Insekten profitieren von ihren bakteriellen Partnern im Bezug auf Ernährung, Pheromonproduktion oder Pathogenabwehr und bieten den Bakterien im Gegenzug eine freie ökologische Nische und eine zuverlässige Weitergabe in die nächste Generation. Im Laufe der Koevolution bildeten die Insekten-Wirte oft spezialisierte Zellen und Organe aus, in denen sie die symbiotischen Bakterien züchteten und entwickelten Verhaltensweisen, die die vertikale Weitergabe der Symbionten in die nächste Generation sicher stellten.

In dieser Arbeit beschreiben wir eine ungewöhnliche Symbiose zwischen solitären Grabwespen der Gattung *Philanthus* (Hymenoptera, Crabronidae) and *Streptomyces* Bakterien. In den Antennen der weiblichen Grabwespen sitzen einzigartige Drüsen, die aus großen Reservoirs und vielen umgebenden Drüsenzellen bestehen. Innerhalb den Reservoirs vermehren sich *Streptomyces* Bakterien und nutzen höchst wahrscheinlich die primären Zellsekrete als Nahrungsgrundlage. Beobachtungen an unserem Model, dem Europäischen Bienenwolf (*Philanthus triangulum*) zeigten, dass die Streptomyceten von den weiblichen Grabwespen in großen Mengen in die unterirdischen Brutzellen abgegeben werden. Die Bienenwolf-Larve nimmt die Bakterien aktiv mit ihren Mundwerkzeugen auf und appliziert sie auf die Seide ihres Kokons. Biotests zeigten, dass die Anwesenheit der *Streptomyces* Bakterien das Risiko, dass der Kokon von Schimmelpilzen befallen wird, drastisch reduziert und dadurch die Überlebensrate des Nachwuchses steigert. Streptomyceten gehören zur Gruppe der Actinomyceten, deren Mitglieder bekannt für die Herstellung von Antibiotika sind. Wir nehmen deshalb an, dass die fungizide Wirkung der Bienenwolf-Symbionten auf – möglicherweise noch unbekanntes – Antibiotika beruht.

*Streptomyces* Bakterien konnten mit genetischen Methoden in den Antennen aller bisher untersuchten 31 Arten der Gattung *Philanthus* nachgewiesen werden, nicht jedoch in nahe verwandten Taxa. Zusammen bilden die mit *Philanthus*-Grabwespen assoziierten Streptomyceten ein Monophylum innerhalb der Gruppe *Streptomyces*. Basierend auf morphologischen, ökologischen und genetischen Daten schlagen wir deshalb ein neues monophyletisches Taxon mit dem Namen „*Candidatus* *Streptomyces philanthi*“ vor. Der Übertragungsweg der symbiotischen Bakterien ist noch unklar, es gibt jedoch einige Hinweise darauf, dass junge Weibchen beim Schlupf aus dem Kokon kleine Mengen von Bakterien in ihre Antennen aufnehmen. Quantitative Untersuchungen der Menge an Bakterien in den Antennendrüsen bei verschiedenem Alter unterstützen diese Hypothese. Unsere

Untersuchungen zeigten außerdem, dass es starke Flaschenhals-Effekte sowohl während der Übertragung zwischen Mutter und Tochter, als auch während des Sekretionsprozesses gibt.

Vergleichende Untersuchungen zur Morphologie der Antennendrüsen in 15 *Philanthus*-Arten aus Afrika, Europa und Nordamerika zeigten kleinere Unterschiede zwischen den Arten in Bezug auf Drüsenform, -größe und die Zahl der Drüsenzellen. Unsere Ergebnisse weisen darauf hin, dass bereits der gemeinsame Vorfahre der Gattung *Philanthus* komplexe Antennendrüsen und Bakterien besessen hat. Wahrscheinlich hat die erfolgreiche Symbiose zwischen *Philanthus*-Vorfahren und Streptomyceten die Aufspaltung und die Verbreitung der Gattung *Philanthus* beschleunigt.

### 13.2 Kopfdrüsen des Europäischen Bienenwolfes

Natürliche und sexuelle Selektion führten zu einer enormen Fülle von morphologischen und funktionellen Anpassungen bei exokrinen Drüsen von Insekten. Während in sozialen Hymenopteren die meisten Drüsen gut untersucht sind, ist das Wissen über diese wichtigen Organe bei solitären Arten größtenteils noch bruchstückhaft oder gar nicht vorhanden.

Diese Arbeit zeigt, dass beide Geschlechter des Europäischen Bienenwolfes eine Postpharyngealdrüse (PPG) besitzen, die bisher als idiosynkratisch für Ameisen galt. Wir konnten zeigen, dass sich die morphologischen Merkmale der PPG in Ameisen und Bienenwölfen sehr ähnlich sind, wobei sich die jeweilige Funktion unterscheidet. Zusammen mit vorläufigen Untersuchungen in anderen Hymenopterenfamilien deuten unsere Ergebnisse stark darauf hin, dass eine PPG in Wirklichkeit ein homologes Merkmal aculeater Hymenopteren ist, deren Funktion sich in den unterschiedlichen Taxa unter dem Einfluss natürlicher Selektion änderte.

In männlichen Bienenwölfen fungiert die PPG höchst wahrscheinlich als Reservoir für das männliche Sexualpheromon, das in den benachbarten Mandibeldrüsen (MG) gebildet wird. Die männlichen Mandibeldrüsen haben eine einfache, schlauchförmige Struktur ohne zugehörige Drüsenzellen. Morphologische Untersuchungen von Postpharyngeal- und Mandibeldrüse zeigten einen Verbindungsgang zwischen beiden Drüsen der die Hypothese von getrennter Pheromonproduktion und -speicherung unterstützt. Das männliche Sexualpheromon spielt eine wichtige Rolle im Paarungsverhalten von Bienenwölfen und die beteiligten Drüsen werden deshalb sicherlich stark durch sexuelle Selektion beeinflusst. Diese Hypothese wird auch durch vorläufige Untersuchungen an Männchen anderer *Philanthus*-Arten gestützt, die eine Vielzahl unterschiedlicher morphologischer Ausprägungen von Postpharyngeal- und Mandibeldrüse zeigen.

### 13.3 Effekte der Temperatur auf die Gehirnentwicklung der Roten Mauerbiene

Insekten sind poikilotherm und stehen deshalb vor dem Problem, ihre Entwicklung in einer Umwelt mit wechselnden Temperaturen stabil zu halten. Besonders der komplexe anatomische Umbau holometaboler Insekten während der Metamorphose wird höchst wahrscheinlich durch die Temperatur beeinflusst. Soziale Insekten umgehen das Problem umweltbedingter Effekte auf ihre postembryonale Entwicklung, indem sie die Temperatur in ihren Nestern aktiv kontrollieren. Honigbienen (*Apis mellifera*) zum Beispiel halten die Temperatur in ihren Brutwaben konstant bei ca. 35°C und es konnte in früheren Untersuchungen gezeigt werden, dass bereits kleine Abweichungen von dieser Temperatur während der postembryonalen Entwicklung unter anderem zu Defiziten im synaptischen Aufbau bestimmter Hirnbereiche führen.

Die Frage ist nun, ob bzw. wie solitäre Arten, die sich nicht in Nestern mit konstanter Temperatur entwickeln, von unterschiedlichen Temperaturen beeinflusst werden. Wir manipulierten deshalb die Temperatur während der postembryonalen Entwicklung der Roten Mauerbiene, *Osmia bicornis* (Hymenoptera, Megachilidae) und untersuchten, ob diese Art vergleichbare Temperatureffekte auf die Gehirnentwicklung zeigt, wie sie bei Honigbienen gefunden wurden. Wir konzentrierten unsere Untersuchungen auf bestimmte synaptische Komplexe (Microglomeruli) innerhalb der Pilzkörper, da letztere als Zentren für übergeordnete Prozesse im Insektenhirn gelten. Wir verwendeten Fluorophor-gebundenes Phalloidin um Microglomeruli sichtbar zu machen und konnten so deren Anzahl und Dichte bestimmen. Unsere Ergebnisse bei *O. bicornis* deuten darauf hin, dass die Gehirnentwicklung dieser Art gegen verschiedene Temperaturen gepuffert ist, sie sich also an natürlich auftretende Temperaturbedingungen in ihren unisolierten Nestern angepasst hat.





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**ERKLÄRUNG**

nach § 6, Abs. 1, Nr. 3 der Ordnung zum Erwerb des akademischen Grades eines  
Doktors der Naturwissenschaften (Dr. rer. nat.) an der Universität Regensburg

Ich erkläre hiermit, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

Weitere Personen waren an der inhaltlich-materiellen Herstellung der vorliegenden Arbeit nicht beteiligt. Insbesondere habe ich hierfür nicht die entgeltliche Hilfe eines Promotionsberaters oder anderer Personen in Anspruch genommen. Niemand hat von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen.

Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

Regensburg, den \_\_\_\_\_

\_\_\_\_\_  
Wolfgang Göttler