Protective *Streptomyces* in beewolves

- Ecology, evolutionary history and specificity of symbiont-mediated defense in Philanthini wasps (Hymenoptera, Crabronidae) -

Dissertation zur Erlangung des akademischen Grades „doctor rerum naturalium“ (Dr. rer. nat.)

vorgelegt dem Rat der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena

von

Dipl.-Biol. Sabrina Koehler
geboren am 07.11.1982 in Zwickau

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"There is nothing like looking, if you want to find something. You certainly usually find something, if you look, but it is not always quite the something you were after."

The Hobbit, J.R.R. Tolkien

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

Symbiotic Planet, Lynn Margulis

CONTENTS

LIST OF PUBLICATIONS

The thesis is based on following manuscripts

Koehler S, Doubský J, Kaltenpoth M (2013): Dynamics of symbiont-mediated antibiotic production reveal efficient long-term protection for beewolf offspring. *Frontiers in Zoology, 10(3),* (Chapter 2)

Koehler S and Kaltenpoth M (2013): Maternal and environmental effects on symbiont-mediated antimicrobial defense. *Journal of Chemical Ecology, 39(7), 978-988,* (Chapter 3)

Koehler S and Kaltenpoth M (in preparation): Gut excretions of European beewolf larvae stimulate symbiont-produced antimicrobial protection. (Chapter 4)

Kaltenpoth M, Goettler W, Koehler S, Strohm E (2010): Life cycle and population dynamics of a protective insect symbiont reveal severe bottlenecks during vertical transmission. *Evolutionary Ecology, 24(2), 463-477,* (Chapter 5)

Kaltenpoth M, Roeser-Mueller K, Koehler S, Peterson A, Nechitaylo T, Stubblefield JW, Herzner G, Seger J and Strohm E: Partner choice and fidelity stabilize co-evolution in a cretaceous-age defensive symbiosis. *PNAS, under revision,* (Chapter 6)

Koehler S and Kaltenpoth M (in preparation): Experimental symbiont exchange provides evidence for partner specificity in the defensive beewolf-*Streptomyces* symbiosis. (Chapter 7)

CHAPTER 1

GENERAL INRODUCTION

1.1 THE BIOLOGY OF *STREPTOMYCES*

Before the early 20th century, medical treatments for microbial infections were primarily based on herbalism and naturopathic medicine paired with medicinal folklore. However, mixtures with antimicrobial properties against various diseases have already been described over 2000 years ago from several ancient cultures, using molds and plant materials as well as crude extracts of the latter to treat infections (Forrest 1982; Wainwright 1989; Nelson et al. 2010). The discovery of penicillin by Alexander Fleming in 1828 finally ushered a whole era of modern antibiotic research.
The word "antibiotic" was first used by Selman Waksman in 1941 to describe any small molecule produced by microbes which antagonizes the growth of competing microorganisms (Waksman 1947). Since then many antibiotic substances from various bacterial strains have been utilized in pharmaceuticals and medicine as chemotherapeutic agents to cure microbial infections (Demain and Fang 1995; Demain and Sanchez 2009), and the ability to synthetize and mass produce these compounds still remains to be the greatest success in microbial biosynthesis.
The history of antibiotics which are derived from the actinobacterial genus *Streptomyces* was initiated by the discovery of streptothricin in 1942. However, only the subsequent discovery of streptomycin shortly after triggered the systematic screening of antibiotics from this genus (Waksman 1963; Berdy 1974). In the following decades, efforts towards the discovery of novel antimicrobial compounds from *Streptomyces* increased tremendously. Between 1955 and 1962, about 80% of the antibiotic substances discovered originated from actinomycete bacteria, with the genus *Streptomyces* claiming the lion's share (Berdy 1974).

1.1.1 *Streptomyces* life cycle

Binomial nomenclature often reflects the specific biological characteristics of the organism at hand. The name *Streptomyces* describes a discriminatory morphology, albeit imprecisely: "*Streptomyces*" literally means "chain fungus", although streptomycetes have long been assigned to prokaryotes rather than to eukaryotic fungi, where they have been grouped before (Glauert and Hopwood 1961). With about 900 described species, *Streptomyces* forms the largest genus of Actinobacteria. Belonging to the group of gram-positive, filamentous bacteria, they are characterized by a high G+C content in the genome, and are predominantly found in soil and decaying vegetation. *Streptomyces* produce vegetative, branched hyphae resulting in the formation of a complex substrate mycelium (Figure 1.1) that penetrates and solubilizes organic debris in soil, by the action of extracellular hydrolytic enzymes, and thereby aids in scavenging nutrients from their surrounding substrate (Chater 1984; Chater et al. 2010). The immobility of the mycelium and aerial hyphae constrains the bacteria´s dispersal, a

problem solved by the formation and, subsequently, distribution of metabolically inactive exospores (Chater 1984). Long chains of spores, which can contain up to 50 of these specialized cells, are normally formed from aerial hyphae (Figure 1.1), while simultaneously consuming the vegetative mycelium (Chater 1984). When a typical *Streptomyces* spore encounters favorable conditions, it germinates and one or two germ tubes emerge from the spore (Figure 1.1) Finally, the germ tubes grow by tip extension and branching to form a vegetative mycelium again (Chater 1984).

The formation of spores, however, results in a very sensitive phase of development where the *Streptomyces* bacteria can easily be attacked by competing microorganisms. Thus, it is not surprising to find chemical defense mechanisms, such as the production of antimicrobial substances, in most *Streptomyces* species. To date, *Streptomyces* is the largest antibiotic-producing genus, with various antibacterial, antifungal, and antiparasitic drugs, but also a wide variety of other bioactive compounds, such as immune-suppressants, being used in human medicine (Watve et al. 2001). The production of these substances usually coincides with aerial mycelium formation, suggesting that the expression of genes related to antibiotic production is tightly linked to the regulatory mechanisms involved in initiating aerial mycelium formation (Chater 1993; Champness and Chater 1994; Flärdh and Buttner 2009; Chater et al. 2010).

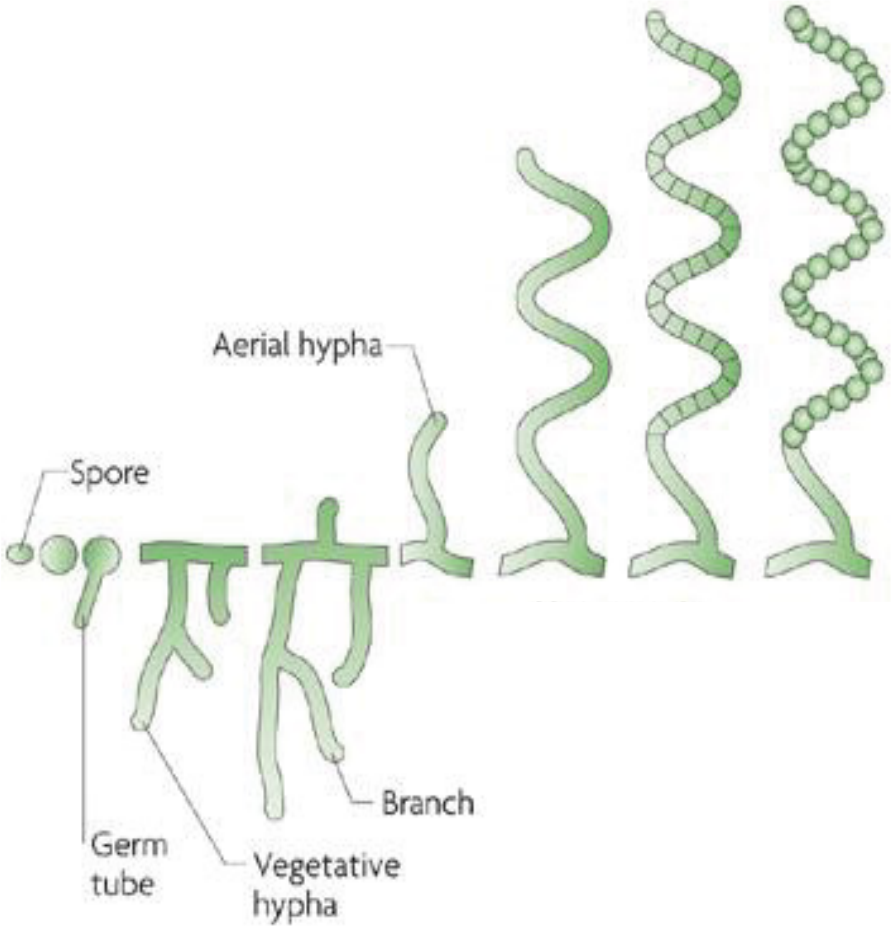

Fig. 1.1 Life cycle of *Streptomyces*. Modified from Flärdh and Buttner (2009), *Nature Reviews Microbiology* 7, 36-49

1.1.2 *Streptomyces* secondary metabolism

The genus *Streptomyces* produces an extraordinary variety of secondary small molecules, usually ranging from 100 to 3000 Da, which are biologically active outside their producer´s cell (Vining 1990; Solecka et al. 2012). Many of those substances are antibiotic compounds restraining the growth of competitors as well as inhibiting enzymes and other cellular processes. To date approximately 7000 different secondary metabolites have been discovered in *Streptomyces* (Berdy 2005), and recent research in the field of *Streptomyces* microbiology reaffirms the potential for natural product discovery. In general, *Streptomyces* genomes can contain up to 30 gene sets for secondary metabolism, with approximately one third of this genes involved in antibiotic production (Bentley et al. 2002; Challis 2008).

The produced secondary metabolites are structurally among the most diverse and complex molecules in nature (Berdy 2005). Among others, many *Streptomyces* species as well as other actinomycetes produce also volatile odors such as geosmin, an abundant substance in nature that contributes to the characteristic earthy smell of moist soils. However, the production of most antibiotic substances in *Streptomyces* appears to be strain-specific and therefore provides different strains with a unique capability to out-compete other microorganisms as well as closely related *Streptomyces*, which may rival for the same ecological niche or nutritional resources. Because antibiotic production is generally delayed until most of the growth has been completed, however, antimicrobial small molecules might serve to defend the *Streptomyces´* colony biomass against overgrowth by other organisms during the vulnerable phase of re-allocating resources into spores, rather than helping them with competition for primary biomass accumulation (Chater and Merick 1979; Chater and Hopwood 1993; Wiener 1996).

In addition to gaining a selective advantage against competitors, particularly in nutrient-poor environments, a recent hypothesis has been driven forward stating that microbe-produced antibiotics can also act as inter- and intracellular signaling molecules (Goh et al. 2002; Tsui et al. 2004; Linares et al. 2006; Dufour and Rao 2011). As such they can affect gene expression (Goh et al. 2002), orchestrate microbial growth (Hoffman et al. 2005; Linares et al. 2006; Lopez et al. 2009), and initiate morphological differentiation (Miall 1971; Vanek and Mikulik 1978; Gräfe 1983; Chater 1989), especially when present in low concentrations (Yim et al. 2007). Thus, the frequent detection of sub-inhibitory antibiotic concentrations in the environment suggests that bacterial communities are not only highly competitive but might as well be highly communicative.

In the context of inter-specific interactions, it has been shown that the protective benefits derived from the ability to produce antibiotic substances can also be exploited by macroorganisms, contributing to the formation of mutualistic interactions. In animals, there are several examples of antibiotic-producing *Streptomyces* involved in defensive symbiotic relationships by providing protection against pathogens for the eukaryotic host or it´s nutritional resources (Currie et al. 1999a; Currie et al. 2003; Kaltenpoth et al. 2005; Kaltenpoth et al. 2006; Mueller et al. 2008; Scott et al. 2008; Haeder et al. 2009; Selvin 2009). Additionally, there are examples of antibiotic compounds mediating symbiotic interactions between microorganisms and plants (Tokala et al. 2002; Schrey and Tarkka 2008; Sadeghi et al. 2012).

1.1.3 *Streptomyces* as symbionts

In 1877, the british botanist and publisher Alfred William Bennett first used the word "symbiosis" to describe the mutualistic relationship in lichens. Later, the german botanist and mycologist Heinrich Anton de Bary defined "symbiosis" as "the living together of organisms from different species" (de Bary 1879). However, this first definition of symbiosis was interdisciplinary declared controversial because it describes a wide range of interactions between different organisms, including parasitism, commensalism as well as mutualism (Douglas 1994). Traditionally, researchers have especially focused on parasitic interactions because of their importance for human welfare. However, mutualistic associations between different species have recently been shown to play a key role in ecology and evolution (Moran 2006), resulting in increasing research efforts to characterize the diversity and function of eukaryote-associated symbionts.

Symbiotic microorganisms can provide a wide range of benefits to their insect hosts (Margulis and Fester 1991; Douglas 1994; Moran and Baumann 2000; Bourtzis and Miller 2003; Douglas 2003). Well known symbiotic associations comprise interactions for the exchange of essential dietary compounds between host and symbionts (e.g. Baines 1956; Sasaki et al. 1996; Douglas 1998; Dillon and Dillon 2004; Douglas 2006; Moya et al. 2008; Salem et al. 2012) or the supply of constituents that can be used as pheromone components by the host (Dillon et al. 2000; Dillon et al. 2002). Moreover, symbiotic microbes can affect the host´s hydrocarbon profiles that are important for nestmate recognition (Matsuura 2003) or defend the host or the host's nutritional resources against pathogens (Currie et al. 1999a; Kellner 2002; Kaltenpoth et al. 2005; Scott et al. 2008; Brownlie and Johnson 2009; Mattoso et al. 2012) or parasitoids (Oliver et al. 2003).

Actinobacteria are especially predisposed towards engaging in defensive symbiotic interactions for a number of reasons (Kaltenpoth 2009). They are common soil bacteria and, therefore, can easily be encountered by soil-dwelling animals and plant roots. In addition, some taxa can form spores, thereby surviving unfavorable conditions, they can exploit a wide range of nutrient sources, and they exhibit an extraordinary potential to produce secondary metabolites with antibiotic properties (Goodfellow et al. 1983; Goodfellow and Williams 1983). The best studied genus of Actinobacteria so far is the genus *Streptomyces*, which have already been proven to be essential in human medicine, producing novel antimicrobial substances, as well as in agriculture, operating as growth promoters and biological control agents. Thus, considering their ecological and physiological features, it is evident that *Streptomyces* constitute a particularly suitable model system to elucidate evolutionary aspects of symbiotic associations between bacteria and other macroorganisms.

In plants, several *Streptomyces* species have been found to be closely associated with their host (Seipke et al. 2012b). The interactions are diverse, ranging from parasitism to beneficial interactions (Sardi et al. 1992; Doumbou et al. 2001; Loqman et al. 2009; Yandigeri et al. 2012; Seipke et al. 2012b). Beneficial interactions with *Streptomyces* bacteria mainly comprise (i) host plant defense via the production of antimicrobials that inhibit the growth of competing microbes such as fungal phytopathogens (Doumbou et al. 1998; Taechowisan et al. 2003; Bascom-Slack et al. 2009; Qin et al. 2011), (ii) the interaction with the host plant´s immune system (Conn et al. 2008; Kinkel et al. 2012; Lin et al. 2012; Schrey et al. 2012), and (iii) the promotion of host plant growth via the production of plant hormones such as auxin (Sadeghi et al. 2012). Auxins play a cardinal role in the coordination of many growth related processes in the plant's life cycle and are essential for plant development. On a cellular level, auxin is essential for cell growth, affecting both cell division and cellular expansion. The auxin concentration, together with other local factors inside the tissue, contributes to cell differentiation and specification, resulting in growth of specific tissues. In endophytic *Streptomyces*

species, the ability to produce such plant hormones could be utilized by the host plant to promote growth and plant development (Overvoorde et al. 2010; Sadeghi et al. 2012), gaining advantages over other plants that occupy the same ecological niche. Concomitant with the benefits for the plant host (Sturz et al. 2000), endophytic *Streptomyces* may as well benefit by obtaining a living environment and nutritional supplementation via plant exsudates from the host (Sturz et al. 2000, Castillo et al. 2002, Tokala et al.2002).

Marine sponges belong to the oldest multicellular organisms, playing an important ecological role by filtering remarkable amounts of seawater. Moreover, they are well-known for the production of a diverse range of bioactive metabolites, a reason for the rapid and continuously increasing research interest towards this group of animals. Although surveys on sponge-bacteria interactions are mainly motivated by the possible discovery of microbe-mediated bioactive metabolites, ongoing research more and more emphasizes the significance of the high abundance of sponge associated bacteria with regard to the sponges´ ecology and functionality (Taylor et al. 2007a; Vogel 2008; Webster and Taylor 2012)
Many marine sponges host a vast variety of microorganisms, which can account for a third of the sponge´s total biomass in some species. Although, the bacteria mostly serve as a food source for marine sponges it has been hypothesized that some sponge-associated microbes may form permanent symbiotic interactions, ranging from parasitism to mutualism (Taylor et al. 2007b). For example, most sponges host photosynthesizing organisms, notably Cyanobacteria and Dinoflagellates, which are known to supply 50% to 80% of the sponge´s energy requirements (Ruppert et al. 2004), and Actinobacteria as well as different other antibiotic-producing bacteria have been suggested to provide protection against diseases (Selvin et al. 2009). In general, evidence for the contribution of symbiotic bacteria to survival and health of the sponge host is still scarce (Wilkinson 1979; Hoffmann et al. 2005; Thacker 2005; Steger et al. 2008; Freeman and Thacker 2011), however, Actinobacteria, particularly *Streptomyces,* have recently been hypothesized to aid in the protection of the sponge host via the production of antimicrobial compounds (Pimentel-Elardo et al. 2010). Although evidence for a defensive association is still lacking, the repeated isolation of *Streptomyces* strains from various sponge genera (Jiang et al. 2007; Taylor et al. 2007a; Zhang et al. 2008; Selvin et al. 2009; Khan et al. 2010; Schneemann et al. 2010; Khan et al. 2011) shaped the idea of stably associated sponge symbionts that offer beneficial traits for means of protection to their sponge host.

Marine cone snails of the genus *Conus* have also been demonstrated to live in close association with Actinobacteria, including several strains of the genus *Streptomyces* (Peraud et al. 2009). Cone snails are predatory sea snails, which got their name because of the snail´s colorful, cone shaped shell. *Conus* snails are venomous and prey on fish, marine worms or molluscs, by paralyzing them first before they devour their prey. The bacteria associated with these marine organisms have been shown to produce antimicrobial bioactive substances (Peraud et al. 2009; Lin et al. 2013), but their biological function for the host has not been identified yet.

In addition to the aforementioned examples of *Streptomyces* as putative partners in symbioses, there are numerous other representative associations between Actinobacteria and invertebrates.
Most notably insects have recently been found to engage in such symbiotic associations with Actinobacteria, gaining advantages for means of protection from this relationship (see paragraph 1.3). Taken together, there is increasing evidence that Actinobacteria not only comprise free-living soil-dwelling and marine bacteria, but also engage in symbiotic associations with other organisms, particularly plants and invertebrates. In fact, the multifaceted interactions of Actinobacteria with other organisms might be one reason for the evolution of the vast variety of secondary metabolites

produced by these bacteria, and provide a starting point for future research on host-microbe associations and their potential biotechnological and medical applications (Seipke et al. 2012b).

1.2 EVOLUTION AND MAINTENANCE OF BACTERIAL MUTUALISM

Symbioses are elementary constituents in most biological systems and driving forces of evolution in living organisms (Margulis 1998). Microorganisms can influence the ecology and evolution of their hosts in various ways, facilitating the formation of life-long mutualisms that can span all kingdoms of organisms, e.g. plants, animals, fungi and bacteria. It is known that approximately 90% of terrestrial plants are associated with mycorrhizal fungi, enhancing growth and performance of the host plant in nutrient deficient soils, and many herbivorous mammals depend on microorganisms that provide nutrients by degrading cellulose from plant material. Moreover, insects are associated with an amazing variety of symbiotic microorganisms (Buchner 1965; Maynard-Smith 1989), and their pervasiveness in symbiotic relationships is evident, as insects comprise the most diverse group of animals on earth, representing more than half of all known living animal species (May 1988; Chapman 2006).

Despite the prevalence of beneficial symbiotic associations, however, fundamental questions about the origin of mutualisms as well as underlying mechanisms, contributing towards the evolutionary fixation of such partnerships remains unsolved (Szathmary and Smith 1995). To evolve host associations the bacteria have to compete with antagonists on host surfaces as well as novel resources in and/or on the host (Sachs et al. 2011b). Furthermore, they have to develop strategies to evade the host´s immune responses and finally achieve transmission to the next host generation (Sachs et al. 2011b). However, the maintenance of beneficial long-term relationships requires both the symbiont and the host to confine adaptive interactions. While adaptations to a symbiotic life style in the endosymbionts are already well understood (Shigenobu et al. 2000; Toh et al. 2006; Pettersson and Berg 2007; Moran et al. 2009), interestingly, information on how the host´s immune system has evolved to tolerate cooperative microorganisms still remain scarce and are mainly restricted to extracellular associations with environmental and/or horizontal symbiont transmission (Round and Mazmanian 2009; Troll et al. 2010).

Earlier, Ewald (1987) proposed two potential scenarios of how bacterial mutualisms may have evolved in the first place. (i) The microbial partner may have originated from a parasitic ancestor and the loss of virulence and improvement of mutualistic traits were selected for by vertical transmission. (ii) The microbial mutualists evolved from environmental commensals, where a free-living ancestor exhibited traits that offered immediate benefits to the host (Ewald 1987). Phylogenentic studies demonstrated recently that there is evidence for both hypotheses, although they highlight that bacterial mutualism most often appear to evolve from environmental ancestors (Sachs et al. 2011b), mediated via beneficial traits provided by the ancestral bacteria or via horizontal gene transfer of symbiosis loci (Sullivan et al. 1995; Sachs et al. 2010).

Once bacterial mutualism has emerged, it can be stabilized by different selective mechanisms such as by-product cooperation, partner fidelity feedback, and partner choice (Axelrod and Hamilton 1981; Bull and Rice 1991; Sachs et al. 2004; Sachs et al. 2011b). By-product cooperation occurs when the symbiont exhibits a beneficial trait which not only benefits itself but coincidentially provides benefits to the host (Brown 1983; Connor 1995). It does not bear costs for the symbiont and, in additon, can

work in both directions. By-product cooperation has been discussed to be preferentially important for the origin of cooperative associations (Sachs et al. 2004). However, once the interaction has been established, the host should quickly evolve traits which promote the infection with a beneficial symbiont strain (Connor 1995; Foster and Wenseleers 2006), including mechanisms to favor beneficial symbionts (Vaishnava et al. 2008) or to facilitate symbiont transmission to their offspring (Wilkinson 1999; Turnbaugh et al. 2009). In partner fidelity feedback a symbiont-mediated fitness benefit to the host feeds back as returned benefits to the symbionts, rewarding beneficial strains while harmful symbionts experience reduced fitness (Bull and Rice 1991; Simms and Taylor 2002; Sachs et al. 2004; Sachs et al. 2011a). Partner fidelity is usually assumed to stabilize symbioses with a vertical transmission mode, since it strongly correlates the reproductive interests of the hosts and their symbionts (Sachs et al. 2004; Foster and Wenseleers 2006).
Finally, under horizontal transmission partner choice appears to select for beneficial associations when hosts particularly reward helpful symbionts and sanction cheaters (Bull and Rice 1991; Denison 2000; Sachs et al. 2004; Sachs et al. 2011a).

Despite the increasing research progress with regard to the evolution of cooperation, and the variety of evolutionary models available, however, it is still difficult to reconstruct the impact of individual models on the maintenance of cooperative symbioses in many systems. Although the described models can work independently, multiple mechanisms may operate in various mutualistic associations. Thus, an increasing understanding of the processes involved in the formation and persistence of mutualistic associations may provide new insights into the evolution of symbioses.

1.3 DEFENSIVE INSECT-ACTINOBACTERIA SYMBIOSES

Symbiotic Actinobacteria have been described from many different invertebrate species such as termites, heteropteran bugs, leaf-cutter ants, bark beetles, and from marine invertebrates. Mostly, the symbionts patronize the host´s metabolic potential by producing essential dietary supplements, promoting growth and survival (Baines 1956; Douglas 1998; Douglas 2006; Moya et al. 2008; Salem et al. 2012). For example, termites have been found to harbor *Streptomyces* and other actinomycete bacteria in the digestive tract, where they seem to aid in cellulose and hemicellulose degradation, thereby contributing to the termites' carbon metabolism (Bignell et al. 1991; Varma et al. 1994; Schafer et al. 1996; Husseneder 2010). *Rhodnius prolixus* (Hemiptera, Reduviidae) and *Pyrrhocoris apterus* (Pyrrhocoridae) harbor actinomycete bacteria of the genera *Rhodococcus* and *Coriobacterium*, respectively, that help to process B-complex vitamins and appear to be essential for the bug´s development and reproduction (Baines 1956; Harington 1960; Hill et al. 1976; Benyakir 1987; Kaltenpoth et al. 2009; Salem et al. 2012; Salem et al., in prep).

However, recent studies yield evidence that symbiotic microorganisms can also provide protection against pathogens to the host, its offspring, or its nutritional resources (Currie et al. 1999a; Kellner 2002; Kaltenpoth et al. 2005; Scott et al. 2008; Teixeira et al. 2008; Brownlie and Johnson 2009; Mattoso et al. 2012). To mediate this protection, the symbiotic microbes employ different mechanisms: (i) Symbionts can anticipate the successful establishment of pathogenic infections, competitively restraining pathogens by colonizing susceptible niches in and on their host (Dillon and Charnley 1995; Dillon and Dillon 2004; Forsgren et al. 2009; Koch and Schmid-Hempel 2011), (ii) they can produce chemical substances with the ability to harm or deter antagonists (Kellner 2002; Liu et al. 2009; Oh et al. 2009a; Oh et al. 2009b; Mattoso et al. 2012), and (iii) they can modulate the

insect immune system to prime the host immune defense against pathogenic attack (Evans and Lopez 2004; de Souza et al. 2009).
Although various bacterial taxa have been identified to play important roles in symbiotic relationships, it has become evident that species involved in defensive symbiosis oftentimes originate from the phylum Actinobacteria (Kaltenpoth 2009), which might be attributed to their ability to utilize a broad range of carbon sources, to survive inhospitable conditions by forming spores, and to produce secondary metabolites with antibiotic properties (Goodfellow et al. 1983; Goodfellow and Williams 1983).

1.3.1 Protection of the host´s nourishment

Fungus-farming ants of the tribe Attini (Hymenoptera, Formicidae) provide a well-known example for defensive symbiosis with Actinobacteria (Currie et al. 1999a; Currie 2001b; Haeder et al. 2009). Fungiculture has evolved several times in insects, but has been most extensively investigated in attine ants. Attine ants cultivate a mutualistic fungus from the family Lepiotaceae (Basidiomycota) on plant material inside their colonies, an association which dates back about 50 million years (Currie et al. 2003). The fungal cultivar is used as a source of nourishment for their larvae as well as for adult ants (Hölldobler and Wilson 1990; Chapela et al. 1994). However, the ants' fungus-gardens can be parasitized by several parasitic fungi, especially fungi of the genus *Escovopsis* (Ascomycota, Hypocreales). The pathogenic fungi of this genus show a high phylogenetic congruency with the ant´s fungal cultivars (Currie et al. 2003), highlighting the long co-evolutionary history of this association. *Escovopsis* fungi are strongly specialized on their fungal cultivars (Gerardo et al. 2004) and can seriously reduce the fitness of the attine ant colonies (Currie et al. 1999b; Currie 2001a; Reynolds and Currie 2004).

To protect the fungal cultivar against pathogens, ant workers use a combination of grooming and weeding (Currie and Stuart 2001), a social behavior also known from other ant species (Cremer et al. 2007; Ugelvig and Cremer 2007; Konrad et al. 2012), as well as a secretion with antimicrobial properties from their metapleural glands (Ortius-Lechner et al. 2000; Bot et al. 2002; Fernandez-Marin et al. 2006; Yek and Mueller 2011; Yek et al. 2012). Additionally, previous studies found evidence that the ants engage in a symbiotic association with actinomycete bacteria of the genus *Pseudonocardia* (Currie et al. 1999a; Cafaro and Currie 2005; Poulsen and Currie 2010; Cafaro et al. 2011), which produce antimicrobial substances (Oh et al. 2009a; Carr et al. 2012). These bacteria-produced antibiotics have been shown to efficiently inhibit the growth of the pathogenic fungus *Escovopsis*, providing protection to the ants´ fungus garden (Currie et al. 1999a). The symbiotic bacteria are cultivated in cuticular crypts, which have been modified in structure and complexity during the ants' evolutionary history (Currie et al. 1999a; Currie et al. 2006). It has been shown that the ants´ cuticular crypts are connected to gland cells, which might transfer nutrients from the host to the bacteria (Currie et al. 2006), promoting symbiont growth. In addition, the localization of the bacteria inside the crypts exhibits a novel ecological niche and may facilitate a successful transmission of the bacterial symbionts (Currie 2001b).

The symbiotic *Pseudonocardia* are vertically transmitted to offspring colonies via the reproductive females during their mating flights, providing the opportunity for co-cladogenesis of the bacterial symbiont with its host (Currie et al. 1999a). However, phylogenetic investigations of leaf-cutting ants and their symbionts revealed some degree of specificity for *Pseudonocardia* (Currie et al. 2006), but also horizontal uptake of different symbiotic bacteria (Poulsen et al. 2005; Mueller et al. 2008; Mueller et al. 2010; Cafaro et al. 2011). Moreover, recent studies suggested the association of attine ants with

several other Actionbacteria (Sen et al. 2009), such as *Streptomyces* and *Amycolatopsis*, which might be obtained via horizontal transfer or *de novo* acquisition from the environment (Barke et al. 2010; Barke et al. 2011). The lower attine ant species *Apterostigma denterigerum* has been found to harbor symbiotic *Pseudonocardia,* which produce an antifungal agent, dentigerumycin (Oh et al. 2009a). Moreover, there is evidence that *Acromyrmex* ants are closely associated with *Streptomyces* (Haeder et al. 2009; Barke et al. 2010), which can produce several antifungal compounds, such as candicidin (Haeder et al. 2009; Barke et al. 2010; Seipke et al. 2011), nystatin (Barke et al. 2010), antimycin (Schoenian et al. 2011; Seipke et al. 2011), and valinomycin (Schoenian et al. 2011). These antifungal compounds are well known from *Streptomyces* species and might aid in the protection of the insect host against antagonists. Taken together these surveys imply that actinobacterial symbioses are widespread among attine ants, providing evidence of the prevalence of defensive actinobacteria symbioses throughout the whole invertebrate kingdom.

The symbiotic interaction of Actinobacteria with a fungus-growing bark beetles depicts another possible example of defensive associations, where bacterial-produced antimicrobial substances may protect the host´s nourishment (Scott et al. 2008). *Dendroctonus frontalis*, the southern pine beetle, is a species of bark beetles native to the forests of the southern United States, Mexico and Central America. The beetles carve galleries into the bark of pine trees and cultivate a mutualistic fungus, *Entomocorticum*, which they rely on as a food source for their offspring as well as for adult beetles. The fungus is localized in special crypts of the beetle´s cuticle, called mycangia, which may aid in an efficient transmission of the fungus to new pine trees and, subsequently, the inoculation of new galleries (Klepzig and Wilkens 1997; Hofstetter et al. 2006). Previously, it has been shown that the beetle´s fungal cultivar can be parasitized by an antagonistic fungus, *Ophiostoma minus*, which overgrows the fungal nourishment and thereby perils the successful development of the beetle larva (Hofstetter et al. 2006). Recent observations, however, provide evidence that bacteria of the genus *Streptomyces* can provide protection against *Ophiostoma minus*, and most likely other pathogens, by producing an antimicrobial compound, called mycangimycin (Scott et al. 2008; Oh et al. 2009b). The compound belongs to the group of polyene antifungal agents and has been shown to efficiently ward off *Ophiostoma minus*, without affecting the *Entomocorticum* fungal cultivar (Scott et al. 2008).

Further evidence for the prevalence of symbiotic associations of bark beetles with Actinobacteria is provided by the spruce beetle, *Dentroctonus rufipennis,* which is a major forest pest and native to British Columbia, Newfoundland and Labrador, Nova Scotia, Ontario, Quebec, the Yukon, and Maine. The beetles have been found to use an oral secretion to deter antagonistic fungi that co-colonize the tree and thereby reduce spruce beetle survival, egg gallery construction, oviposition, and adult survival (Cardoza et al. 2006). Various bacterial strains, which might aid in the protective effect, have been identified in the oral secretion of the beetles (Cardoza et al. 2006; Cardoza et al. 2009), such as the Actinomycete *Micrococcus luteus*, which has been shown to strongly inhibit the antagonistic fungi (Cardoza et al. 2006).

Similar to attine ants, the genus *Allomerus* has recently been suggested to be associated with Actinobacteria of the genera *Amycolatopsis* and *Streptomyces*. *Allomerus* ants are an Amazonian ant species that can be found in the tropics of South America (Fernández 2007). In contrast to the fungus-growing tribe Attini, *Allomerus* species have specialized on complex predatory behaviors, involving symbioses with plant and fungal organisms for catching prey (Dejean et al. 2001; Dejean et al. 2005). The ants live in small pouches, called domatia, between leaves and stems of their host plant, *Hirtella physophora*. On the host plant, they use a mold fungus to construct galleries along the plant´s branches, subsequently hiding inside these galleries to ambush their prey (Dejean et al. 2001; Dejean et al. 2005; Ruiz-Gonzalez et al. 2011). Their primary diet includes large insects, but they also exploit

parts of the host plant, such as nectar and food bodies produced by *Hirtella* plants. However, the plant also benefits from the association with the ants. By preying on insects the ants defend their host plant against potential herbivores, which are immediately captured by the ants as soon as they enter the plant (Dejean et al. 2001; Dejean et al. 2005; Grangier et al. 2008; Leroy et al. 2008). Specialized structures on the plant reveal that the two species might share a long history of co-evolution. A previous survey by Leroy et al. (2008) provided strong evidence for adaptation of the plant towards supporting the *Allomerus* ants: (i) The domatia are located next to the stems which are used by the ants for hunting, (ii) the domatia contain extrafloral nectar and food bodies which the ants rely on as a food source if there is no other nourishment available, (iii) the domatia contain less chloroplasts, suggesting a reduced photosynthetic activity, (iv) the domatia contain more stomata, which might be an adaptation to use the ants´ respiratory carbon dioxide, and (v) the domatia contain more cellulose, leading to a stronger cell wall and, subsequently, a more stable domatium to support the additional weight of the ants. These findings provide evidence that areas designated to become domatia are a result of co-evolution and that they were produced to support this specific species of ants (Leroy et al. 2008).

Recently, an additional association of these ants with Actinobacteria of the genus *Amycolatopsis* and *Streptomyces* has been suggested by Seipke et al. (2012a). The Actinobacteria isolated from the *Allomerus* ants have been found to produce antimicrobial substances that may suppress growth of potentially pathogenic fungi, which otherwise might be able to out-compete the ants´ mutualistic fungus (Ruiz-Gonzalez et al. 2011). A screening of the ants´ traps revealed not only the mutualistic fungus, but spores of different, potentially pathogenic fungi as well. Direct evidence for an ant-bacteria mutualism is still lacking, however, the authors hypothesized that the system might be similar to the one described from attine ants and that the fact that competing fungi could be found only as spores, suggests mechanisms which may control the germination and growth of competing fungi (Seipke et al. 2012a).

1.3.2 Protection of the insect host and its offspring

A recent study elucidated the potential symbiotic association of *Streptomyces* bacteria with solitary wasps of the genera *Sceliphron* and *Chalybion (Poulsen et al. 2011),* which belong to the hymenopteran family Sphecidae and are commonly referred to as mud-dauber wasps. *Sceliphron* wasps construct their nests using mud collected from water puddles. The nests are usually located in shaded niches, often just inside of windows or vent openings, and it may take a female a day to construct a cell requiring dozens of trips carrying mud. The nests are partitioned into separate brood cells, which each contain a single egg as well as a paralyzed prey item that serves as nourishment for the wasp´s offspring. The provision generally consists of spiders such as crab spiders, orb-weaver spiders and jumping spiders. Females add new cells one by one to the nest after a brood cell is provisioned, and over their whole life span species can lay a modest average of 15 eggs. By contrast, *Chalybion* wasps re-utilize nests that have been established by other females. They empty the contents of the brood cells and supply them with a new prey item as well as their own egg (Poulsen et al. 2011). In a recent cultivation-based survey, more than 200 *Streptomyces* species were isolated from the mud-dauber wasps (Poulsen et al. 2011). Since *Streptomyces* are known to produce a vast variety of antimicrobial substances, Poulsen et al. (2011) analysed the bioactive compounds produced by some of the isolated strains and identified 11 different secondary metabolites, including one novel macrocyclic lactam that they called sceliphrolactam (Poulsen et al. 2011). Furthermore, they were able to show that the compounds produced by these *Streptomyces* strains efficiently inhibit the growth of entomopathogenic bacteria and, to a certain degree, pathogenic fungi (Poulsen et al. 2011). Although, it is not evident whether the

Streptomyces bacteria associated with mud-dauber wasps play a role as symbionts in this system, the bacteria-produced antimicrobial substances could be utilized by the wasps as defensive agents, which might provide a potential benefit to the wasp in the defense against antagonists.

To date, the only known defensive insect-Actinobacteria symbiosis that protects the insect host instead of its nutritional resources, however, is the beewolf-*Streptomyces* mutualism, in which the bacteria on the wasps´ cocoons protect their progeny against pathogenic microorganisms via the production of different antibiotic substances (Kaltenpoth et al. 2005; Kroiss et al. 2010) (for details see paragraph 1.3).
Nevertheless, besides the aforementioned expamples, several other insect species have recently attracted considerable attention due to the discovery of microbe-mediated secondary metabolites with antibacterial and antifungal properties. In dung beetle brood balls, a novel tricyclic lactam antibiotic has been found to be produced from *Streptomyces* sp. (Park et al. 2012), and a screening of fungus-growing termites revealed various actinobacterial strains, although specificity with the insect host appears to be lacking (Visser et al. 2012). In summary, this newly emerged attention towards defensive insect-bacterial symbioses demonstrates the great potential of this particular field of research for the discovery of novel sources of bioactive natural products, which in future could be utilized in human medicine to treat microbial infections.

1.4 THE BIOLOGY OF THE EUROPEAN BEEWOLF *PHILANTHUS TRIANGULUM* (HYMENTOPTERA, CRABRONIDAE)

1.4.1 Geographic distribution of *Philanthus triangulum* (Hymenoptera, Crabronidae)

Philanthus triangulum (Hymenoptera, Crabronidae), commonly known as European beewolf, is a solitary, ground-nesting digger wasps in warm and sandy regions, hunting arthropods as nourishment for their progeny. Currently, the genus *Philanthus* comprises 137 described species (Bohart and Menke 1976) with a wide distribution. With the exception of Australia and the South American continent, beewolves can be found in Europe, Central- and North America, and Asia (Pate 1947; Bohart and Grissel 1975; Ebrahimi 2005; Matthews and Matthews 2005; Jacobs 2007).
Together with the genus *Trachypus* (Alexander 1992), which occurs in Central and South America (Bohart and Menke 1976), and the rare and species-poor genus *Philanthinus,* they represent the tribe Philanthini, the sister group to a clade that comprises the two other tribes of digger wasps within the crabronid subfamily Philanthinae, the Cercerini and Aphilanthopini.
The distribution of the European beewolf, *Philanthus triangulum* ranges from Scandinavia in the north to South Africa in the south, with an eastern expansion to the Near and Middle east (Arnold 1925; Erlandsson 1962; Bohart and Menke 1976; Hansen 1997; Ebrahimi 2005; Ebrahimi 2008; Nemkov 2008).

1.4.2 Biology of female European beewolves

Females of the solitary digger wasp *Philanthus triangulum* (Hymentoptera, Crabronidae) construct their nests in sandy, sun-exposed soils, excavating tunnels that can extend up to one meter in depth. The nests consist of a main burrow, with several – up to 34 – side burrows, containing a terminal brood cell each (Figure 1.2) (Simon-Thomas and Simon-Thomas 1972; Simon-Thomas and Veenendaal 1978; Evans and O'Neill 1988; Strohm 1995). European beewolves exclusively prey on honey bee workers (*Apis melifera*) as a food source for their progeny. It has been shown that the hunting behavior of European beewolves is mediated by both visual (Tinbergen 1932) and olfactory cues (Herzner 2004; Herzner et al. 2005). After visually localizing a honey bee feeding on a flower, female beewolves have been observed to hover in a distance of about 10 cm before finally attacking and paralyzing the bee. This hovering behavior has been shown to be an important phase in the hunting sequence of European beewolves (Herzner et al. 2005), in which the honey bee appears to be chemically identified. The substance being essential for this olfactory identification of the bees by the female wasp has been elucidated to be a long-chained alcohol, (*Z*)-11-eicosen-1-ol (Herzner et al. 2005).

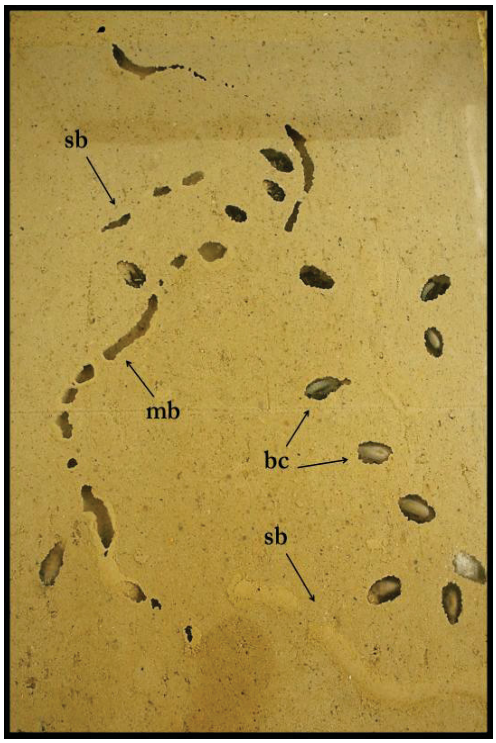

Fig. 1.2 Nest constructed by female beewolf, **mb** = main burrow, **sb** = side burrow, **bc** = terminal brood cell

Immediately after identification by the female beewolf, the honeybee is attacked, paralyzed by injection of a venom with a sting into the bee´s thorax (Rathmayer 1962a; Rathmayer 1962b; Piek 1966; Dunbar and Piek 1982), and carried back to the nest in flight (Figure 1.3). Female beewolves sequentially hunt up to six honey bees which are temporarily stored in the main burrow. Only after a sufficient number of bees have been brought into the nest, the female excavates a side burrow with a terminal brood cell and the honey bees are transferred into this brood chamber (Figure 1.2). The amount of bees provided for individual offspring depends on the sex of the offspring, on the prevailing temperature, the availability of honey bees, and the female´s individual "performance" (Strohm 1995; Strohm and Linsenmair 1997; Strohm and Linsenmair 1998; Strohm and Linsenmair 1999; Strohm and Linsenmair 2000; Strohm and Marliani 2002; Strohm and Daniels 2003). Once the female placed the prey items in the brood chamber it deposits an egg on one of the honey bees by fixing it to the bee´s thorax with a sticky secretion, and, subsequently, closes the brood cell. The brood chambers as well as the side burrows of the nest are always sealed with sand after completion of the brood cell, most likely for means of protection against parasitoids like cuckoo wasps and sarcophagid flies (Evans and O'Neill 1988). After eclosion from the egg, the larva feeds on the provisioned bees for several days before spinning a cocoon. Usually, beewolves hibernate in the larval stage inside the cocoon and emerge in spring the following year. The increasing temperatures in late spring induce pupation in the beewolf larvae, leading to a completion of development and eclosion of an adult beewolf from the cocoon (Figure 1.4a-e).

Fig. 1.3 Female beewolf hunting for a honey bee (*Apis melifera*), **a)** female beewolf with paralyzed bee on a flower, **b)** female beewolf carrying a paralyzed bee to the nest in flight, **c)** female beewolf with paralyzed prey entering the nest

Once emerged from the cocoon, adult beewolves have to cope with the problem of finding their way out of the subterranean burrow (Simon-Thomas and Veenendaal 1978; Evans and O'Neill 1988; Strohm 1995; Strohm and Linsenmair 1995). To facilitate this task, the female beewolf applies a white antennal gland secretion to the distal site of the brood chamber prior to oviposition (Strohm 1995; Strohm and Linsenmair 1995). The secretion contains spatial information for the beewolf larva to find its way out of the nest. During cocoon spinning, the beewolf larva detects the secretion inside the brood cell and, by using this directional information, attaches the cocoon to the distal side of the brood chamber (Strohm and Linsenmair 1995). Once the cocoon is completed, the beewolf larva faces the proximal side of the brood cell, directing to the main tunnel of the nest. After eclosure, the adult beewolf starts digging forward and by that reaches the main burrow, which in contrast to the

side burrows is not sealed with sand (Strohm and Linsenmair 1995). Using the tunnels of the main burrow, the newly emerged beewolf can easily leave its mother's nest.

Like many ground-nesting insects, beewolves have to cope with competing soil microorganisms that do not only threaten to devour the progeny´s provisions, but can also peril the developing offspring itself (Figure 1.4f). In the humid, subterranean brood cells, the developing beewolf larva is continuously threatened by pathogenic bacteria and fungi that may invade the brood cells from the surrounding soil as well as from the remains of the provisioned honeybee prey, which usually results in the death of the beewolf larva (Strohm and Linsenmair 1995; Strohm and Linsenmair 2000; Strohm and Linsenmair 2001). To counteract these threats, the female wasps invest in an at least three-fold chemical defense strategy, facilitating a successful development of their progeny. (i) The paralysation of the bees, instead of killing them, may offset pathogen infection, since the bee´s immune system still might combat pathogenic microbes. (ii) After deposition of the bees in the brood cell, the female wasps embalm the prey items with a postpharyngeal gland (PPG) secretion, which has been demonstrated to reduce water condensation and thereby delay the onset of fungal germination (Strohm and Linsenmair 2001; Herzner et al. 2007; Herzner and Strohm 2007; Herzner and Strohm 2008).

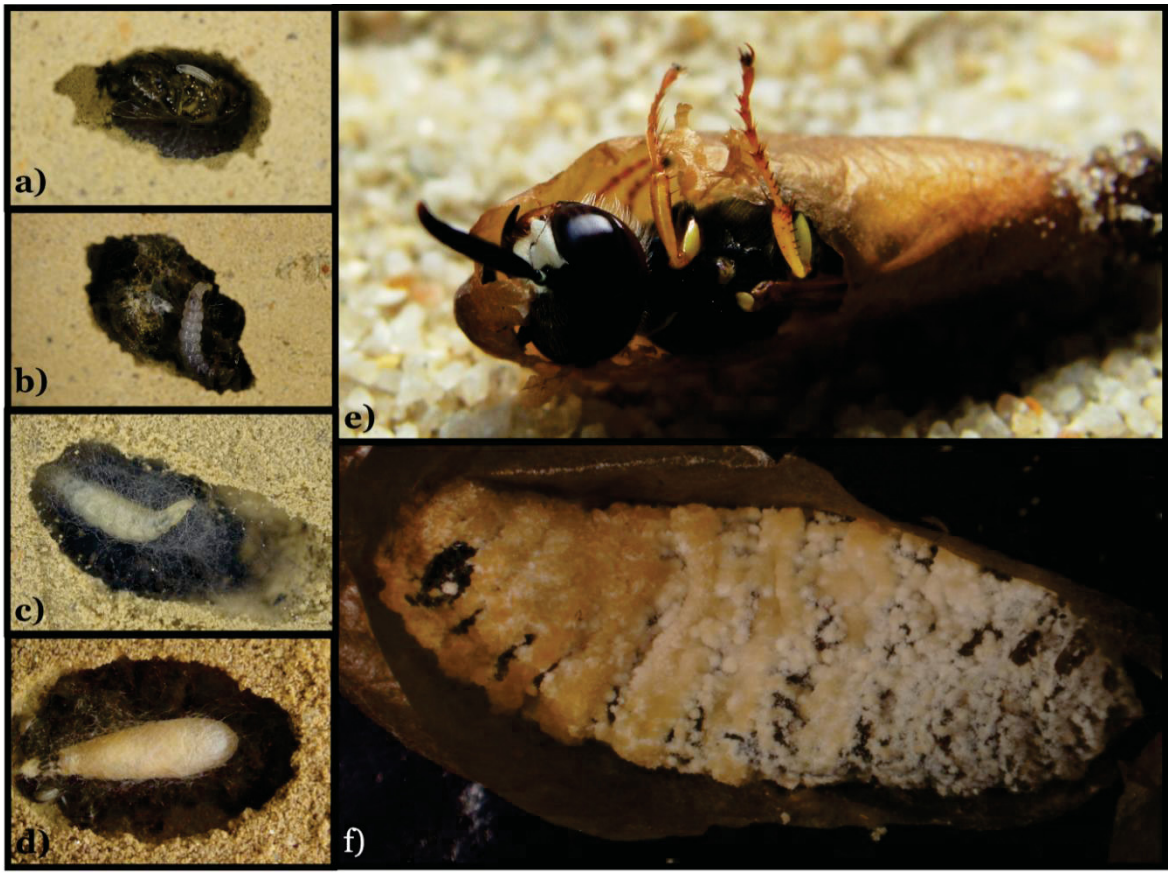

Fig. 1.4 Development of the European beewolf *Philanthus triangulum* inside the brood cell, **a)** Egg on paralyzed honey bee, **b)** Beewolf larva feeding on provisioned honey bees, **c)** Beewolf larva spinning a cocoon, **d)** Beewolf cocoon in a brood cell, **e)** Beewolf female emerging from the cocoon, **f)** Beewolf infested with fungi inside the cocoon

(iii) The beewolf egg releases a nitrous gas with a high oxidation potential (Engl 2011, Strohm & Engl et al, in prep), which has been shown to effectively inhibit the germination of fungal spores and delay the growth of potentially pathogenic fungi that might harm the beewolf larva during development

(Strohm 1995; Engl 2011, Strohm & Engl et al, in prep). (iv) Finally, recent studies elucidated that beewolves engage in a unique symbiotic association with Actinobacteria, providing antimicrobial substances for means of protection of the beewolf offspring during their development in the subterranean brood cells. (Kaltenpoth et al. 2005; Kaltenpoth et al. 2006).

1.4.3 The beewolf-*Streptomyces* symbiosis

As mentioned above, the female beewolf secretes a white substance into the subterranean brood cells prior to oviposion. In addition to the spatial information encoded (Strohm and Linsenmair 1995), this white substance has been demonstrated to contain antibiotic producing bacteria that aid in the protection of the beewolf larva during the long and vulnerable phase of larval development (Kaltenpoth et al. 2005; Kaltenpoth et al. 2006). The bacteria of the genus *Streptomyces* (´*Candidatus* Streptomyces philanthi`) are cultivated in specialized antennal gland reservoirs, which are localized in five segments of each female beewolf antenna (Kaltenpoth et al. 2005; Kaltenpoth et al. 2006; Goettler et al. 2007). The reservoirs are surrounded by glandular cells, which likely provide nourishment to the bacterial symbionts (Kaltenpoth et al. 2005; Goettler et al. 2007). During the process of cocoon spinning, the larva incorporates the symbiotic *Streptomyces* bacteria into the cocoon walls (Figure 1.5), where they produce a cocktail of at least nine different antibiotic substances that have been shown to ward off a wide range of pathogenic microorganisms (Kroiss et al. 2010) and thereby enhance the survival probability of the developing beewolf offspring during the unpredictable phase of hibernation (Kaltenpoth et al. 2005).

Recently, the presence of antennal symbionts has also been demonstrated in two other genera of Philanthini wasps that are closely related to the genus *Philanthus* (Kaltenpoth et al. 2010; Kaltenpoth et al. 2012). In two species of the genus *Trachypus* (Kaltenpoth et al. 2010), which is the closest relative to *Philanthus*, and in one species of the rare genus *Philanthinus* (Kaltenpoth et al. 2012) the antennal reservoirs as well as the symbiotic bacteria have been successfully detected, using morphological and molecular methods. Interestingly, the genera *Cerceris*, *Aphilanthops* and *Clypeadon* appear to lack the antennal symbionts (Kaltenpoth et al. 2006). Together with the occurrence of the antennal symbionts in the genera *Trachypus* and *Philanthinus* this suggests that the symbiosis of Actinobacteria with digger wasps may represent an apomorphy that evolved along the branch to the Philanthini wasps, and that may be much older and more widespread than has been previously recognized (Kaltenpoth et al. 2010; Kaltenpoth et al. 2012).

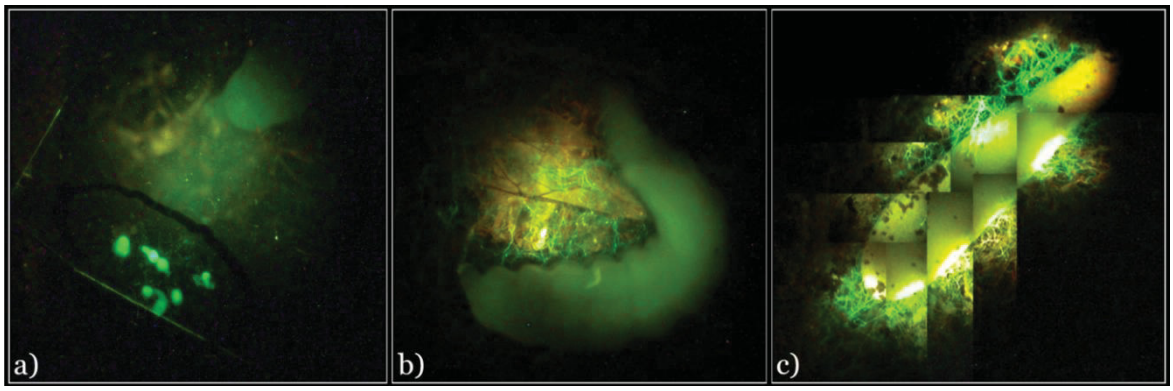

Fig. 1.5 Fluorescent staining of symbiotic *Streptomyces* bacteria from the antennal gland secretion of female European beewolves with EUB-784-FITC, **a)** White substance on the ceiling of the brood chamber, **b)** Larva spinning the first silk strings for a cocoon, **c)** Completed cocoon with fluorescent bacteria on the surface and the surrounding cocoon silk

1.5 THESIS OUTLINE

1.5.1 Ecology of *Streptomyces*-mediated antimicrobial defense

In the first part of this thesis (Chapters 2-4) we investigate the ecology of the unique symbiosis between beewolf wasps and their *Streptomyces* symbionts. As has been demonstrated before symbiont-produced antibiotic substances provide benefits to the beewolf host by protecting its offspring against pathogen infestation (Kaltenpoth et al. 2005; Kroiss et al. 2010). Though the protective effect is evident, knowledge on the ecology of the symbiont-mediated antibiotics with regard to their long-term efficiency and potential factors influencing the symbiont-produced substances in their natural environment so far remains limited.

Thus, the chapters 2-4 of this thesis investigate the dynamics of the *Streptomyces* population size as well as the production dynamics and stability of symbiont-mediated antibiotic substances on beewolf cocoons in order to elucidate mechanisms that allow for an efficient long-term defense against pathogens. In chapter 2 we provide evidence that the defensive antibiotics, though produced only within the first two weeks after cocoon spinning, project a high stability and remain on the cocoon surface for months of hibernation. Furthermore, the impact of environmental factors on the antimicrobial defense in the beewolf-*Streptomyces* symbiosis has been characterized in detail in chapter 3, demonstrating that the *Streptomyces*-mediated antibiotic production is not affected by current environmental conditions but rather independent of external effects and optimized to serve as a reliable long-term antimicrobial defense for the beewolf progeny. In addition, the results presented in chapter 4 show that antibiotic production in the *Streptomyces* symbionts is positively affected by the beewolf larva inside the cocoon.

1.5.2 Evolutionary history of the beewolf-*Streptomyces* symbiosis

In the second part of this thesis (Chapters 5-7) we investigate the evolutionary history of the symbiotic association of beewolf wasps with their *Streptomyces* symbionts. First, we characterize the transmission route of the symbiotic bacteria from mother to offspring, as well as the underlying mechanism and consequences of the severe bottleneck of the *Streptomyces* population during vertical symbiont transmission (Chapter 5).

In chapter 6 we provide evidence for a long co-evolutionary history of the beewolf-*Streptomyces* symbiosis. Using molecular clock approaches based on the available Philanthini fossil records the origin of this symbiosis was determined, revealing an ancient symbiotic relationship that dates back at least 68 million years.
The diffuse pattern of co-cladogenesis found in the beewolf phylogeny, however, indicates that despite their localization in specialized antennal gland reservoirs and their vertical transmission mode, the extracellular lifestyle as well as the external transmission via the cocoon surface allow for horizontal symbiont replacement or *de novo* uptake of bacterial strains (Chapters 6). However, the phylogetic trees show that only a distinct monophyletic clade of symbionts appears to successfully establish a long-term association with the beewolf host, suggesting mechanisms that promote the long-term stability of the mutualistic association with a specific clade of symbionts (Chapter 6).

Finally, chapter 7 yields insights into the specificity of this symbiotic association with regard to horizontal symbiont replacement. We show that heterospecific Actinobacteria can grow inside the female beewolf´s antennal reservoir, however, the transmission of these bacteria seems to be selectively hindered, resulting in a less efficient vertical transmission of the bacteria to the next wasp generation. These results allow us to draw some conclusions on possible mechanisms that mediate symbiont recognition and specificity in the symbiosis between beewolf wasps and their protective *Streptomyces* bacteria.

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CHAPTER 2

DYNAMICS OF SYMBIONT-MEDIATED ANTIBIOTIC PRODUCTION REVEAL EFFICIENT LONG-TERM PROTECTION FOR BEEWOLF OFFSPRING

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2.1 ABSTRACT

Background: Insects have evolved a wide range of mechanisms to defend themselves and their offspring against antagonists. One of these strategies involves the utilization of antimicrobial compounds provided by symbiotic bacteria to protect the host or its nutritional resources from pathogens and parasites. In the symbiosis of the solitary digger wasp, *Philanthus triangulum* (Hymenoptera, Crabronidae), the bacterial symbiont '*Candidatus* Streptomyces philanthi' defends the developing larvae against pathogens by producing a mixture of at least nine antimicrobial substances on the cocoon surface. This antibiotic cocktail inhibits the growth of a broad range of detrimental fungi and bacteria, thereby significantly enhancing the offspring's survival probability.

Results: Here we show that the production of antimicrobial compounds by the beewolf symbionts is confined to the first two weeks after cocoon spinning, leading to a high concentration of piericidins and streptochlorin on the cocoon surface. Expression profiling of housekeeping, sporulation, and antibiotic biosynthesis genes indicates that antibiotic production coincides with morphological differentiation that enables the symbionts to survive the nutrient-limited conditions on the beewolf cocoon. The antibiotic substances remain stable on the cocoon surface for the entire duration of the beewolf's hibernation period, demonstrating that the compounds are resistant against environmental influences.

Conclusions: The antibiotic production by the beewolf symbionts serves as a reliable protection for the wasp offspring against pathogenic microorganisms during the long and unpredictable developmental phase in the subterranean brood cells. Thus, the beewolf-*Streptomyces* symbiosis provides one of the rare examples of antibiotics serving as an efficient defense in the natural environment and may aid in devising new strategies for the utilization of antibiotic combination therapies in human medicine against increasingly resistant bacterial and fungal pathogens.

2.2 BACKGROUND

Predators, parasites and pathogens can significantly reduce the reproductive success of insects. Developmental stages are especially prone to pathogen infestation given their often limited mobility as well as trade-offs that lead to limited resources being allocated into growth instead of defense (Schmid-Hempel 2003; Soler et al. 2003; Vainio et al. 2004; Rantala and Roff 2005). Additionally, many insect species rear their offspring in subterranean nesting sites where the progeny is continuously threatened by soil pathogens (Janzen 1977; Strohm and Linsenmair 2001; Herzner and Strohm 2007). To counteract these threats, insects have evolved an intricate immune system as well as chemical and behavioral defenses, including brood care, nesting in habitats which are difficult to invade for predators or brood parasites, and the application of antimicrobial substances as defense against pathogen infestation (Batra 1968; Strohm and Linsenmair 2001; Bot et al. 2002; Degenkolb et al. 2011).

Sociality has been documented to provide insects with collective defensive behaviors aimed at eliminating parasites from group members and thereby limiting the spread of infections within the colony (Hughes et al. 2002; Cremer et al. 2007; Fefferman et al. 2007; Reber et al. 2011). Specifically, allogrooming can serve as an efficient strategy to mechanically remove fungal spores, nematodes, mites and other parasites (Schmid-Hempel 1998; Hamilton et al. 2011; Reber et al. 2011). By contrast, solitary species are usually lacking these behavioral defenses (Field and Brace 2004; Cremer et al. 2007; Ugelvig and Cremer 2007; Konrad et al. 2012), making other means of protection of their offspring against fungal and bacterial pathogens particularly important. Although nest hygienic behaviors have also been observed in solitary insects (Field and Brace 2004), there is often no or only limited contact between adult and developing individuals, so chemical mechanisms are likely to play a more important role for antimicrobial defense (Cane et al. 1983; Strohm and Linsenmair 2001; Kellner 2002; Kaltenpoth et al. 2005). Concordantly, the production and use of various defensive compounds for the protection of the developing offspring has been described for a number of solitary insect species (Marchini et al. 1997; Feld et al. 2001; Rozen et al. 2008; Burse et al. 2009; Discher et al. 2009; Cotter and Kilner 2010; Degenkolb et al. 2011).

In addition to the insect's own defenses, recent studies have demonstrated that several taxa team up with bacterial symbionts to protect the host, the offspring or its nutritional resources against pathogens, predators, parasites, or parasitoids (Currie et al. 1999b; Kellner 2002; Oliver et al. 2003; Kaltenpoth et al. 2005; Scott et al. 2008; Brownlie and Johnson 2009; Mattoso et al. 2012). This protection can be mediated by (i) competitive exclusion of pathogenic microorganisms (Dillon and Dillon 2004; Forsgren et al. 2009; Koch and Schmid-Hempel 2011), (ii) interaction with the host's immune system to enhance resistance against pathogenic infestation (Evans and Lopez 2004; de Souza et al. 2009; Konrad et al. 2012), or (iii) the production of chemicals that harm and/or deter antagonists (Currie et al. 1999a; Scott et al. 2008; Oh et al. 2009; Kroiss et al. 2010; Mattoso et al. 2012).

Interestingly, many of the mutualistic microorganisms involved in defensive partnerships with insects belong to the bacterial phylum Actinobacteria (Kaltenpoth 2009; Seipke et al. 2012). Members of this group appear to be predisposed towards engaging in defensive symbioses by their widespread distribution in the soil, the ability to subsist in nutritionally deficient environments, and, notably, their capacity to produce a wide variety of secondary metabolites with antimicrobial properties (Watve et al. 2001; Kaltenpoth 2009; Kroiss et al. 2010; Solecka et al. 2012). These compounds may provide an efficient way to defend the producer against pathogenic or competing microorganisms, making Actinobacteria particularly suitable partners for defensive mutualistic interactions with insect hosts. Especially species of the genus *Streptomyces* are efficient producers of antimicrobial substances, with over two-thirds of the clinically relevant natural product antibiotics originating from this genus

(Berdy 1980; Watve et al. 2001). Despite the common perception of antibiotics as agents of chemical warfare among competing microorganisms, evidence for the natural roles of these compounds and their defensive activity under *in vivo* conditions is still scarce (Linares et al. 2006; Dufour and Rao 2011). In fact, recent studies suggest that their primary effect in maintaining microbial communities may be the modulation of gene transcription at low concentrations rather than the elimination of competitors (Yim et al. 2007).

Fungus-farming ants represent a prime example of protective symbioses. The ants cultivate symbiotic fungi as a food source for their colony. To protect their source of nourishment from fungal infestation by the pathogenic fungus *Escovopsis*, the ants engage in a protective symbiosis with actinomycete bacteria which have been reported to produce antibiotic substances that inhibit the growth of *Escovopsis* (Currie et al. 1999a; Currie et al. 1999b; Haeder et al. 2009; Poulsen and Currie 2010; Cafaro et al. 2011), thereby protecting the fungal gardens without affecting the cultivar itself (Poulsen et al. 2010). Similarly, the fungus growing bark beetle *Dendroctonus frontalis* is associated with bacteria of the genus *Streptomyces* (Scott et al. 2008). The bacteria, present in the fungal galleries as well as in the mycangia of the beetle, produce the antifungal substance mycangimycin, which can protect the beetle's nutritional resources against the antagonistic fungus *Ophiostoma minus* (Scott et al. 2008; Oh et al. 2009).

Solitary digger wasps in the tribe Philanthini ('beewolves', Hymenoptera: Crabronidae) engage in a defensive symbiosis with bacteria of the genus *Streptomyces* for the protection of the developing offspring (Kaltenpoth et al. 2005; Kroiss et al. 2010; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). Female beewolves cultivate the symbiotic microorganisms in specialized antennal gland reservoirs (Goettler et al. 2007) and secrete them into the brood cells prior to laying an egg on one of the provisioned bees (Kaltenpoth et al. 2005). After oviposition, the female seals the brood cell with sand and subsequently does not provide any further brood care. As beewolf development can take up to nine months, including a long period of larval diapause during hibernation in the humid underground brood cells, the beewolf's offspring is continuously threatened by pathogenic bacteria and fungi that may invade the brood cells from the surrounding soil as well as from the remains of the provisioned honeybee prey (Strohm and Linsenmair 1995). As an efficient broad-spectrum defense, the larvae incorporate the symbiotic streptomycetes into the silken walls of their cocoons, where the symbionts produce at least nine different antibiotic substances that serve as an "antimicrobial combination prophylaxis" against pathogenic bacteria and fungi during the long and vulnerable period of hibernation (Kroiss et al. 2010).

The mutualistic association of beewolves and *Streptomyces* provides a unique opportunity to study the natural role of antimicrobial compounds in a symbiotic context (Kaltenpoth et al. 2005; Kroiss et al. 2010; Kaltenpoth et al. 2010a; Kaltenpoth et al. 2012). Here we investigated the dynamics of population size and antibiotic production of the symbiotic *Streptomyces* on the beewolf cocoon in order to elucidate the mechanisms that allow for the long-term defense against pathogens. The results yield insights into an efficient strategy for offspring protection in a solitary insect and provide a unique case study on the long-term efficacy of bacterial secondary metabolites directly in the natural environment.

2.3 METHODS

2.3.1 Beewolf rearing conditions

Female European beewolves, *Philanthus triangulum*, were obtained from a natural population in Berlin, Germany. The wasps were individually housed in observation cages (Strohm and Linsenmair 1995) situated in the greenhouse (14 h day, 10 h night; 23°C (+/− 3°C fluctuation) and provided *ad libitum* with honeybees *(Apis mellifera)* as prey and honey as food for the adult females within the cage.

2.3.2 Sample preparation

Male beewolf cocoons were obtained from the females' nests at different time points after cocoon spinning (day 0, 1, 2, 4, 8, and 16 after cocoon spinning). The cocoons were longitudinally cut to create an opening through which the larva was carefully removed using forceps. After dissection, the cocoon samples were directly stored at −80°C. Additionally, some cocoons with larvae were left to complete their development, and the cocoons were collected directly after the adult beewolf emerged from the cocoon. Some of these individuals developed directly within about four weeks ("emergence without hibernation"), while others went into diapause and emerged in the following spring ("emergence after hibernation"). All cocoon samples for emergence were transferred from the observation cages into open 1.5 ml Eppendorf cups approximately one week after cocoon spinning, placed in a box with moist sand and stored in an incubator at 26°C. Cocoons of individuals that developed directly and emerged within the same year were dissected immediately after emergence and frozen at −80°C. Individuals that had not entered the pupal stage four weeks after cocoon spinning were assumed to have entered diapause. These cocoons were transferred to the fridge and stored at 6°C for eight months over winter to mimic hibernating conditions. Subsequently, the samples were returned to the incubator at 26°C to induce pupation, so the cocoons in this group were about 9–10 months old upon sampling (eight months of diapause plus 4–8 weeks of development). After emergence of the adult beewolves, cocoons were stored at −80°C for subsequent analyses.

2.3.3 Methanol extraction

Frozen (−80°C) beewolf cocoons (N=157) were thawed on ice. The cocoons were transferred with forceps to GC-MS vials (1.5 ml vials, 6 mm jar opening, CZT, Kriftel, Germany), and 1 ml of methanol (>99.9%, Roth, Karlsruhe, Germany) was added to each sample. Forceps were cleaned in methanol and hexane in between samples to prevent contamination. Finally, the samples were placed on a shaker (Heidolph Vibramax 100) for 1 h at 350-400 rpm. After extraction, cocoons were taken out of the GC-MS vials, placed on a tissue to drain methanol and finally transferred to clean 1.5 ml cups (Eppendorf). Cocoon samples were placed under the hood for approximately 1 h to evaporate the remaining methanol for molecular analyses. Methanol extracts were stored at −20°C for subsequent GC-MS analysis.

2.3.4 GC-MS analysis of antibiotics

Methanol extracts were taken out of the freezer and evaporated to dryness under a gentle stream of Argon. Antibiotics were re-suspended in 50 µl of methanol and transferred to a 150 µl GC-µ-vial (CZT, Kriftel, Germany) for GC-MS analysis. An aliquot of 1 µl of each sample was injected into a Varian 450GC gas chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies, Böblingen, Germany) using a split/splitless injector at 250°C with the purge valve opened after 60 s. The GC was equipped with a DB5-MS capillary column (30 m x 0.25 mm diameter, film thickness: 0.25 µm, Agilent Technologies) and programmed from 150 to 300°C at 5°C/min with a 1 min. initial isothermal and a 5 min. final isothermal hold. Helium was used as carrier gas, with a constant flow rate of 1 ml/min. Mass spectra were recorded using electron ionization (EI-MS). Data acquisition and quantifications were achieved with MS Workstation Version 6.9.3 Software (Agilent Technologies). The five most abundant compounds on beewolf cocoons were quantified, i.e. streptochlorin (SC), piericidin A1 (PA1), piericidin B1 (PB1), piericidin A5 (PA5) and piericidin C1 (PC1) (Kroiss et al. 2010). A dilution series (500–0.1 ppm) of commercially available piericidin A1 was used as an external calibration standard for the four different piericidin derivatives, assuming similar ionization efficiencies based on the high structural similarity. For quantification of streptochlorin, we used a dilution series of a synthesized streptochlorin standard (for the synthesis of streptochlorin see Additional file 2: Supplementary methods). The peaks were identified by comparison of their mass spectra with the standard spectra or with published reference spectra (Kroiss et al. 2010), and peak areas were automatically integrated using the MS Workstation Software. Finally, the success of this integration was controlled manually for every peak.

2.3.5 DNA/RNA extraction

After methanol extraction, the dried beewolf cocoons were used for nucleic acid extraction. The cocoons were homogenized in liquid nitrogen followed by DNA/RNA extraction using the Epicentre MasterPure™ DNA extraction kit (Epicentre Technologies, Madison, USA). The kit is based on a precipitation reaction and can be used for combined DNA/RNA extraction. All solutions not included in the kit (TE buffer, water, 70% EtOH, Isopropanol) were prepared from RNase-free stock solutions. The protocol was adjusted as follows: the lysozyme treatment was omitted, and all centrifugation steps were done at room temperature. Finally, the DNA pellet was resuspended in 100 µl RNase-free low-TE buffer (1 mM Tris/HCl, 0.01 mM EDTA). Each sample was partitioned in two aliquots with 50 µl each and stored at −80°C.

2.3.6 Quantification of *Streptomyces* population size and gene expression

Quantitative real-time PCR (qPCR) with diagnostic primer pairs was used to quantify the population size (16S rRNA gene copy number) and the expression of housekeeping (*gyrB, EF-Tu and ftsZ*), sporulation (*whiB*) and antibiotic genes (*pks1*) of '*Ca.* S. philanthi'. Primers were obtained from the literature (16S and *gyrB*: (Kaltenpoth et al. 2010a)) or designed based on available whole genome shotgun sequencing data (Nechitaylo et al., unpubl. data) using Primer3 (v. 0.4.0, http://frodo.wi.mit.edu/; Table 2.1). The *pks1* gene is localized within the cluster that is predicted to be involved in piericidin biosynthesis, based on *in silico* prediction as well as by the high similarity to the published piericidin gene cluster of *Streptomyces piomogenus var. hangzhouwanensis* (Liu et al. 2012). The PCR conditions for each primer pair were optimized using gradient PCR with a template

extracted from female beewolf antennae, and specificity was confirmed by gel electrophoresis and sequencing.

To establish a standard curve for each gene of interest, DNA extracts of female beewolf antennae were thawed and used for PCR amplification. Amplification was performed on a VWR Gradient Thermocycler (UnoCycler, VWR, Darmstadt, Germany) in a total reaction volume of 12.5 µl, containing: 1 µl template DNA, 1 x PCR buffer (key buffer, Tris/HCl, $(NH_4)_2SO_4$ and 0.1% Tween 20), 2.5 mM $MgCl_2$ (including the 1.5 mM $MgCL_2$ in the buffer), 240 µM dNTPs, 0.8 µM of each primer and 0.5 U *Taq* DNA-Polymerase (VWR, Darmstadt, Germany). Cycle parameters were as follows: an initial denaturation step at 94°C for 3 min, 35 cycles of 94°C for 40 s, 65°C for 40 s and 72°C for 40 s, and a final extension step of 72°C for 4 min. PCR success was verified by gel electrophoresis using a GelRed™ (Biotium, Hayward, USA) stained 1.5% TBE agarose gel (150V, 30 min). The documentation of gel pictures was conducted using GeneSnap image acquisition software (GeneSnap 7.09.06, Syngene, Camebridge, United Kingdom).

Positive bands were excised from the gel and purified using PeqGold MicroSpin Gel extraction kit (Peqlab, Erlangen, Germany). The final DNA concentration of the purified PCR-product was obtained from NanoDrop measurements (ND1000 photo-spectrometer, PeqLab, Erlangen, Germany). Finally, the DNA samples were diluted to 1 ng/µl with RNase free water, and a serial dilution of 10^{-1} to 10^{-8} ng/µl was established to serve as a standard for qPCR quantification.

Table 2.1 Primers used for PCR and quantitative PCR amplification of '*Ca.* S. philanthi' genes

Primer	Sequence (5'-3')	Direction	Ampl. length	References
Strep_phil_fwd3mod	TGGTTGGTGGTGGAAAGC	Fwd	135	Kaltenpoth et al., 2010
Strep_16S_rt_rev	GTGTCTCAGTCCCAGTGTG	Rev		Kaltenpoth et al., 2010
PKS1-SPT-F1	TCTTCCGACAGTCGATAGCC	Fwd	139	This study
PKS1_SPT-R1	GAGATCATGACGGCGAAGAG	Rev		This study
Strep_phil_EF-Tu_rt_fwd	CGACTACACGCACAAGAAG	Fwd	108	This study
Strep_phil_EF-Tu_rt_rev	CACGGACGGGATGTACTC	Rev		This study.
Strep-phil-gyrB-rt_fwd	CGCCAACACGATCCACAC	Fwd	115	Kaltenpoth et al., 2010
Strep-phil-gyrB-rt_rev	GTCCTTCTCCCGCAGCAG	Rev		Kaltenpoth et al., 2010
FtsZ-Ptr_F1	GACCGACTGCTGTCCATTTC	Fwd	136	This study
FtsZ-Ptr_R1	CGAAGTCCAGGTTGATCAGG	Rev		This study
whiB(189)-F1	CGAGCTGTTCTTCCCCATC	Fwd	104	This study
whiB(189)-R1	ACTGCAGGCACTCCTCCAT	Rev		This study

QPCR was used to quantify the *Streptomyces* population size on the surface of beewolf cocoons. Thawed DNA extracts of whole beewolf cocoons were used for qPCR amplification with '*Ca.* S. philanthi' 16S rRNA gene-specific primers (Table 2.1) in a total reaction volume of 25 µl containing the following reagents: 6.5 µl RNase free water and 12.5 µl SYBR-Mix (Rotor-Gene SYBR Green RT-PCR kit, Qiagen, Hilden, Germany); 2.5 µl of each primer (10 µM), 1 µl template. Quantitative PCR was performed on a Rotor-Gene Q Cycler (Qiagen, Germany) using the following cycle parameters: 95°C for 10 min, 45 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 20 s, and a final

melting curve analysis was performed by increasing the temperature from 72 to 95°C with 1°C gain. Based on the standard curve, the total amount of DNA was calculated based on the qPCR threshold values using the absolute quantification method (Lee et al. 2006; Lee et al. 2008).
For quantification of the antibiotic gene expression as well as the expression of the housekeeping and sporulation genes, RNA extracts of whole beewolf cocoons were thawed and used for reverse transcription to obtain cDNA using the QuantiTect reverse transcription kit (Qiagen, Germany) and the specific primer pairs according to the manufacturer's instructions. Subsequently, these cDNA samples were used for qPCR amplification with the same primers, using the same amplification conditions as indicated above.

2.3.7 Statistical analysis

Statistical analyses were performed using SPSS 17.0 Software (IBM, New York, USA). The amount of antibiotics estimated from GC-MS analysis as well as the symbiont 16S copy numbers and expression levels of housekeeping, antibiotic production, and sporulation genes estimated by qPCR were compared across different time points using ANOVA with Tukey posthoc measures. Additionally, changes in the composition of the antibiotic cocktail over time were analyzed based on the most abundant and consistently detected compounds, i.e. streptochlorin, piericidin A1 and piericidin B1. Omitting the compounds that were not consistently detected (PC1, PA5) is an approach that is conservative with regard to the hypothesis tested, i.e. that the composition of the cocktail changes over time. Single peak areas were translated into relative peak areas and subsequently log-ratio transformed using the Aitchison transformation (Aitchison 1986). Finally, a discriminant analysis was used to elucidate differences in the composition of the antibiotic cocktail on beewolf cocoons over time (Figure 2.2b, Additional file 1: Figure S1)

2.4 RESULTS

2.4.1 *Streptomyces* population dynamics on beewolf cocoons

The number of *Streptomyces* bacterial cells was quantified for different time points after cocoon spinning (day 0 [N=11], 1 [N=7], 2 [N=7], 4 [N=25], 8 [N=27], 16 [N=9], emergence without hibernation [N=32], emergence after hibernation [N=39]) using quantitative real-time PCR (qPCR) analysis of the 16S rRNA gene. 'Emergence without hibernation' refers to beewolves that emerged from the cocoon about four weeks after cocoon spinning without entering diapause, whereas 'emergence after hibernation' designates cocoons of individuals that entered diapause and were kept for eight months at 6°C before completing development and emerging from the cocoon (for details see Methods section). Thus, the symbiont numbers on cocoons in the two 'emergence' groups represent the population sizes present after the beewolves completed development. The estimated 16S copy numbers (representing the total values per beewolf cocoon) indicate that the symbiont population size increases within the first two days after cocoon spinning followed by cessation of bacterial growth (Figure 2.1; ANOVA: df=7, P=0.02).

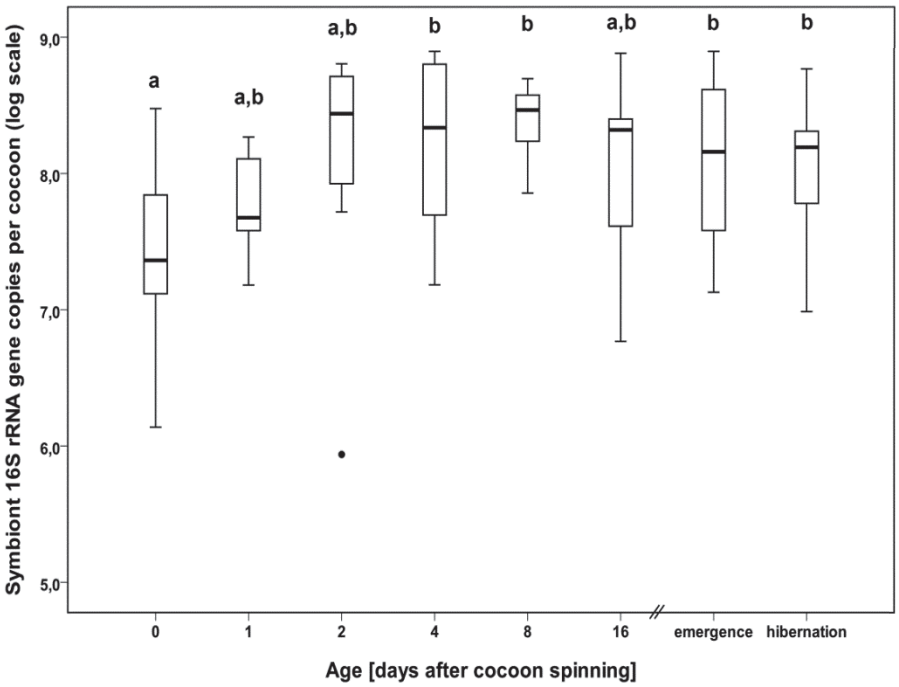


Fig. 2.1 Estimated 16S rRNA gene copy number of *Streptomyces* bacterial cells on male beewolf cocoons of different ages after cocoon spinning (on log scale; the different groups refer to the days after cocoon spinning). Bold lines represent medians obtained from qPCR of 16S rRNA, boxes comprise the interquartile range, and bars indicate minimum and maximum values, outliers are given as dots. Different letters above the boxes represent significant differences (ANOVA with Tukey post-hoc tests: df=7, P=0.02).

2.4.2 Dynamics of the antibiotic cocktail and piericidin gene expression

Gas chromatography – mass spectrometry (GC-MS) analyses of methanol extracted beewolf cocoons at different time points after cocoon spinning showed significant changes in the amount of the major antibiotics on the cocoon over time (Figure 2.2a, ANOVA: df=7, P<0.001). Directly after cocoon spinning (Day 0–2), there was already a low amount of antibiotics (0.6–4.5 µg/cocoon) present on the cocoon surface. Within the following 1–2 weeks, the amount of antibiotics slowly increased, with an average amount of 26.4 µg/cocoon at day 8 to 16, and subsequently decreased slowly over time. Nevertheless, there was still a considerable amount of antibiotics present on the surface of beewolf cocoons after hibernation for approximately eight months, with an average amount of 12.5 µg/cocoon.

The discriminant analysis based on the relative amounts of streptochlorin (SC), piericidin A1 (PA1), and piericidin B1 (PB1) showed a significant change in the composition of the antibiotic cocktail over time, suggesting temporal differences in the production and/or deterioration of the single compounds (Wilks' λ=0.432, χ^2=126.4, df=21, P<0.001; Figure 2.2b, Additional file 1: Figure S1). In particular, we detected a decline in the relative amount of PA1 as compared to SC and PB1 towards the later time points, suggesting that production of PA1 starts earlier than that of SC and PB1, and that PA1 may be less stable than the other two compounds.

The expression of the putative piericidin gene (*pks1*) revealed a similar pattern as the chemical analyses, albeit shifted to earlier time points. Expression levels peaked at day 2 and 4 and subsequently decreased to background levels (Figure. 2.2c, ANOVA: df=7, P<0.001).

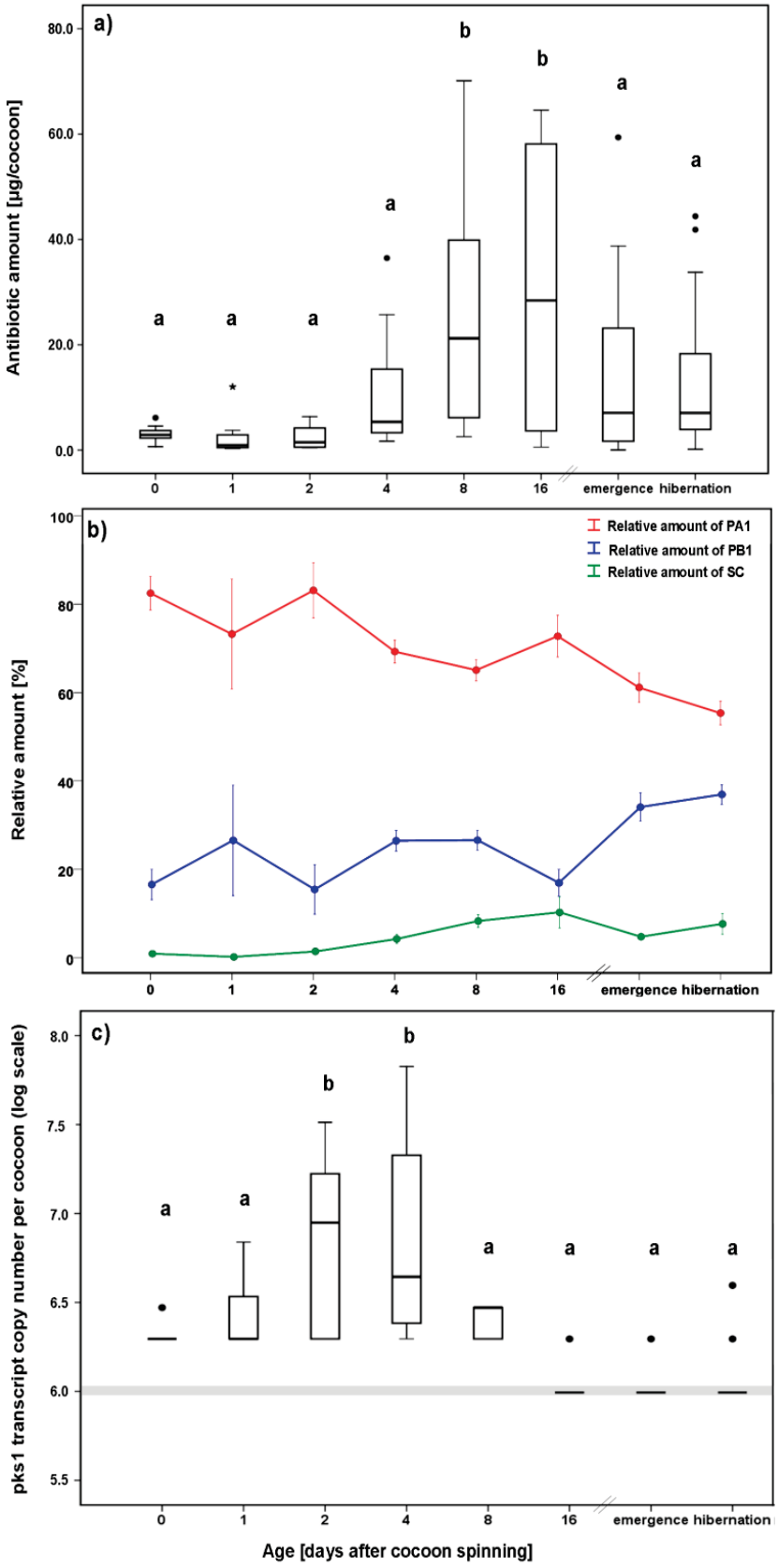


Fig. 2.2 Dynamics of antibiotic production on beewolf cocoons. **a)** Total amount [µg/cocoon] of antibiotic substances on beewolf cocoons at different time points after cocoon spinning quantified using GC-MS. Different letters above the boxes represent significant differences (ANOVA with Tukey post-hoc tests: df=7, P<0.001), **b)** Average relative amounts of the three most abundant antibiotic substances on beewolf cocoons (in %) at different time points after cocoon spinning quantified by GC-MS. A discriminant analysis based on the composition of the antibiotic cocktail correctly classified 20–72.2% of the originally grouped cases (40.1% on average, Wilks' λ: 0.432, χ^2: 126.4, df=21, P<0.001, see Additional file 1: Figure S1), **c)** Absolute number of putative piericidin transcripts (*pks1*) on beewolf cocoons at different time points after cocoon spinning, obtained from RT-qPCR assays (on log scale). Different letters above the boxes represent significant differences (ANOVA with Tukey post-hoc tests: df=7, P<0.001). The different groups refer to days after cocoon spinning; emergence: eclosion of adult beewolf from the cocoon (about four weeks after cocoon spinning), hibernation: eclosion after diapause (about 8 months after cocoon spinning). Bold lines represent medians, boxes comprise the interquartile range, bars indicate minimum and maximum value, outliers are given as dots; the grey bar indicates the level of unspecific amplification as obtained from the qPCR negative control.

2.4.3 Expression of housekeeping and sporulation genes

The expression of the housekeeping genes *ftsZ and EF-Tu* as well as the sporulation regulatory gene *whiB*, obtained from qPCR analyses, remained constant for the first four days after cocoon spinning (Fig. 2.3a-c). However, at day 8 the expression level of all three genes decreased by several orders of magnitude, indicating a reduced metabolism of the *Streptomyces* symbionts approximately one week after cocoon spinning, probably due to morphological differentiation. By contrast, the transcript level of *gyrB* decreased more slowly over time, suggesting a role of the gyrase topoisomerases during the onset of the dormant phase (Figure 2.3d).

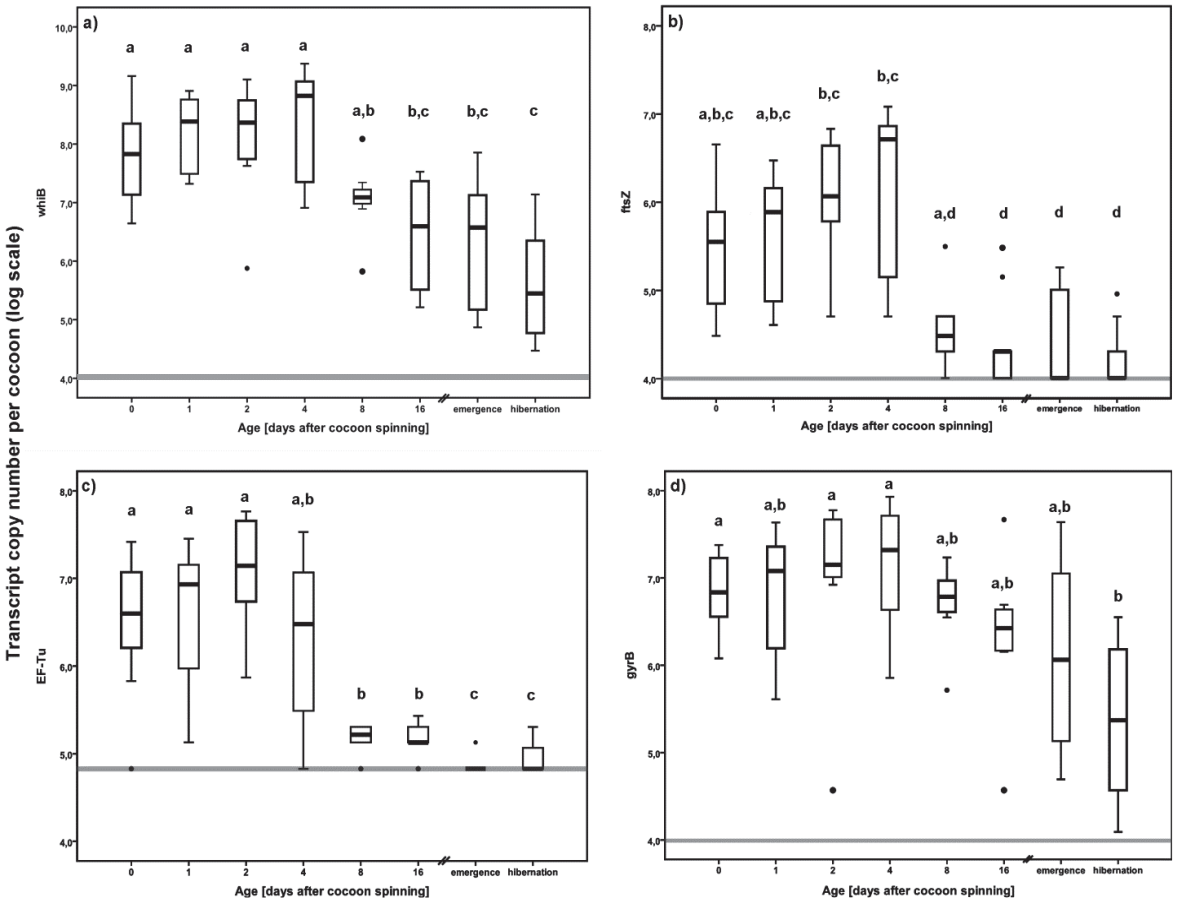


Fig. 2.3 Expression levels of different housekeeping genes and the *whiB* sporulation regulatory gene on beewolf cocoons at different time points after cocoon spinning, obtained from RT-qPCR assays (on log scale). **a)** *whiB*; **b)** *ftsZ*; **c)** *EF-Tu*; **d)** *gyrB*. The different groups refer to days after cocoon spinning; emergence: eclosion of adult beewolf from the cocoon (about four weeks after cocoon spinning), hibernation: eclosion after diapause (about 8 months after cocoon spinning). Bold lines represent medians, boxes comprise the interquartile range, bars indicate minimum and maximum values, outliers are given as dots. Different letters above the boxes represent significant differences (ANOVA with Tukey post-hoc tests: df=7, P<0.05 for all analyses), The grey bar indicates the level of unspecific amplification as obtained from the qPCR negative control.

2.5 DISCUSSION

An improved understanding of the production and ecology of antimicrobial substances in natural environments is important to complement our present knowledge on the evolution of antibiotic resistance and to acquire new perspectives for the utilization of antibiotics in human medicine. The beewolf-*Streptomyes* symbiosis represents a unique model system to study these factors *in vivo*. Our results indicate that an "antibiotic cocktail" is produced by the defensive *Streptomyces* symbionts on beewolf cocoons within the first two weeks of larval development and subsequently serves as a reliable long-term antimicrobial protection for the wasp offspring. The stability and long-term efficacy of the symbiosis is mediated by morphological differentiation of the bacteria to survive unfavorable conditions on beewolf cocoons during larval hibernation and by the production of environmentally resistant antibiotics that remain stable on the cocoon as an efficient protection until emergence of the adult beewolf.

2.5.1 Growth and morphological differentiation of beewolf symbionts on the cocoon

Species of the genus *Streptomyces* are well known for the production of numerous small molecules that are biologically active outside of the cell. Many of those substances are inhibitors of enzymes and cellular processes that act as antimicrobials (Linares et al. 2006; Yim et al. 2007; Chater et al. 2010; Kitani et al. 2011), which help the producers to compete with antagonistic microorganisms or act as signaling molecules affecting gene transcription in co-occurring microbes (Linares et al. 2006; Yim et al. 2007). In many species, the production of antimicrobial secondary metabolites has been found to be tightly linked to morphological differentiation into inactive spores (Berdy 1984; Miyadoh 1993; Champness and Chater 1994; Hopwood 2007; Chater et al. 2010; Dharmaraj 2010). In this context, the antimicrobial compounds may protect the cell's metabolites from competitors during the vulnerable phase of re-allocation of the resources into spores. Prior studies of '*Candidatus* Streptomyces philanthi' already proposed a morphological differentiation of the bacteria when applied to the surface of beewolf cocoons as a mechanism to survive the long period on the likely nutrient-limited cocoon surface during beewolf hibernation (Kaltenpoth et al. 2010a). Our results provide support for this hypothesis and indicate that the symbiont population only grows within the first two days following cocoon spinning and subsequently undergoes morphological differentiation (Figures 2.1 and 2.3). Concordantly, two of the three analyzed housekeeping genes (*ftsZ* and *EF-Tu*) as well as the sporulation regulatory gene *whiB* show high expression levels during the first week on the cocoon, but then their expression is reduced to background levels (Figure 2a-c). *WhiB*, in particular, is known to be essential for early morphological differentiation as well as for the regulation of later sporulation gene transcription (Chater 1972; Ryding et al. 1999). It closely interacts with *ftsZ,* which regulates septum formation in normal cell division as well as during sporulation (Flärdh et al. 2000; Willemse et al. 2012). Recent studies demonstrated that the expression of several regulatory *whi* genes, including *whiB*, is important for the correct timing of *ftsZ* and, as a result, for an optimal *FtsZ* protein level, which is essential for the formation of aerial biomass prior to morphological differentiation (Willemse et al. 2012). The early onset of *whiB* and *ftsZ* expression on the cocoon (already at day 0) suggests that at least some of the symbiont cells already initiate morphological differentiation prior to incorporation into the cocoon silk. This is supported by the observation that low amounts of antibiotics can already be detected in the antennal gland secretion of female beewolves within the brood cell (T. Engl, S. Koehler, and M. Kaltenpoth, unpublished data).
Surprisingly, *gyrB* showed a much slower decrease in expression levels after day 8 as compared to the other genes (Figure 2.3d), suggesting that gyrase may play an important role during the later stages of

morphological differentiation. Concordantly, previous studies in *Bacillus subtilis* (Vazquez-Ramos and Mandelstam 1981) and in *E. coli* (Goss et al. 1965; Smith and Davis 1967; Staudenbauer 1976) observed an inhibition of sporulation by gyrase inhibitors. Taken together, our gene expression analyses provide strong evidence for morphological differentiation of the beewolf symbionts into metabolically inactive spores about 1–2 weeks after cocoon spinning.

2.5.2 Dynamics of antibiotic production and PKS gene expression

As observed for free-living *Streptomyces* strains under *in vitro* conditions (Champness and Chater 1994; Hopwood 2007; van Wezel and McDowall 2011), morphological differentiation coincided with antibiotic production in '*Ca.* S. philanthi'. The expression profile of a gene within the putative piericidin biosynthesis gene cluster (*pks1*) (Nechitaylo et al., unpubl. data; Liu et al. 2012) showed up-regulation between day 1 and day 4 after cocoon spinning, followed by down-regulation from day 8 onwards (Figure 3.2c). Accordingly, the highest amounts of antibiotics could be detected by GC-MS following the peak in antibiotic gene expression, i.e. between day 8 and 16 (Figure 2.2a). By producing antibiotic substances within the first two weeks after cocoon spinning, the symbionts establish a high concentration of antimicrobials on the surface of the cocoons. These compounds remain stable for months during hibernation, with an average total amount of 12.5 µg still present when the adult beewolf emerges. Even on nutrient-rich agar plates, this concentration is high enough to effectively inhibit the growth of a wide range of soil fungi (Kroiss et al. 2010). Thus, the stability of the antibiotic substances allows them to serve as an efficient long-term defense throughout the beewolf's developmental phase.

2.5.3 Stability of antibiotics mediates long-term antimicrobial protection for wasp offspring

In natural habitats, antibiotic compounds are subject to inactivation and degradation by different physicochemical and biological processes, e.g. photolysis, sorption, hydrolysis, and biodegradation (Jefferys 1952; Kuehne et al. 2000; Huang et al. 2001). Consequently, the stability varies greatly across compound classes and is strongly affected by abiotic and biotic conditions. While some substances are stable for months under aerobic conditions (e.g. fluorquinolones, (Golet et al. 2002; Giger et al. 2003)), others (e.g. sulphonamides) experience rapid degradation and are cleared from the environment within 2–3 weeks (Kreuzig and Holtge 2005; Schmidt et al. 2008). Polyene antibiotics, the class containing the piericidins, have been described as compounds which in solution are decomposed upon exposure to air, heat and light (Oroshnik and Mebane 1963), while they show much higher stability (for up to several years) in dry state and in the absence of heat and light (Raab 1972; Bonner et al. 1975). In the beewolf symbiosis, the localization of the piericidins on the cocoon surface in the dark and moderate climate of the subterranean brood cell may explain how these compounds can stably subsist in the environment for a prolonged period of time. The long-term stability of the antibiotic cocktail is crucial for an efficient protection of the beewolf offspring during hibernation, and it is therefore strongly selected for on the holobiont level of the interacting partners.
In addition to the absolute amount of antibiotics, the composition of the cocktail changes on the cocoon surface over time (Figure 2.2b, Additional file 1: Figure S1). PA1 is overrepresented in the first days after cocoon spinning, while its relative amount declines later on in favor of a higher proportion of PB1 and SC, suggesting differences in the timing of production as well as differential stability of the compounds. Possibly, PA1 is converted into PB1 by methylation of the hydroxyl

group at the C16 position, which may explain the delayed increase and deterioration of PB1. The adaptive value of the changes in the antibiotic cocktail remains to be investigated. It is conceivable that the changing composition aids in preventing the emergence of resistant strains (Mouton 1999) or serves to efficiently ward off a succession of different soil fungi and bacteria (Challis and Hopwood 2003).

2.5.4 The importance of antibiotics for the wasp's offspring

The stability of the symbiont-produced antimicrobial cocktail is essential for beewolves considering their brood care strategy. While social insects generally supply their progeny progressively with fresh food, mass provisioning is by far the most common strategy among solitary taxa (Field and Brace 2004; Field 2005). In mass provisioning species, the adult insect supplies all of the food for each offspring prior to laying the egg and subsequently closes the brood cell. This strategy has the advantage that a closed brood cell is less prone to invasion by parasitoids, e.g. cuckoo wasps. However, the risk of pathogen infestation in subterranean nesting sites is severe, especially in combination with the storage of resources. In progressive provisioners such as social insect societies, this problem is counteracted by worker insects continuously cleaning off pathogens and contaminated materials from developing brood and nestmates (Hughes et al. 2002; Fefferman et al. 2007; Reber et al. 2011), or by applying antimicrobial compounds to limit the spread of pathogens (Bot et al. 2002). By contrast, mass-provisioning species like the European beewolf lack the possibility to continuously apply defensive chemicals or actively remove pathogens to protect their progeny. Therefore, the protection of the young with antimicrobial substances that are long lasting and resistant against environmental influences represents a particularly adaptive mechanism of parental care. In European beewolves, the symbiont-provided antibiotic treatment complements additional parental investments by the adult female to protect the offspring from pathogen infestation, such as embalming the prey bees with a postpharyngeal gland secretion to reduce fungal germination (Herzner et al. 2007; Herzner and Strohm 2007; Strohm et al. 2008b). Altogether, these mechanisms ensure a successful completion of larval development for the beewolf progeny over their unpredictable developmental phase and serve as a reliable long term prophylaxis against pathogen attack.

It seems likely that long-term defensive strategies including symbiosis with antibiotic-producing microorganisms are more common among the large number of solitary insects that mass-provision their offspring in subterranean brood cells. However, the often unusual localization of protective symbionts as well as the inherent context-dependency of defensive symbioses hinder the detection of such associations. The increasing affordability of high-throughput sequencing technologies for microbial community analyses may at least partly ameliorate this problem by providing detailed insights into insect-associated microbial symbionts and uncovering candidates with putative protective functions.

2.6 CONCLUSIONS

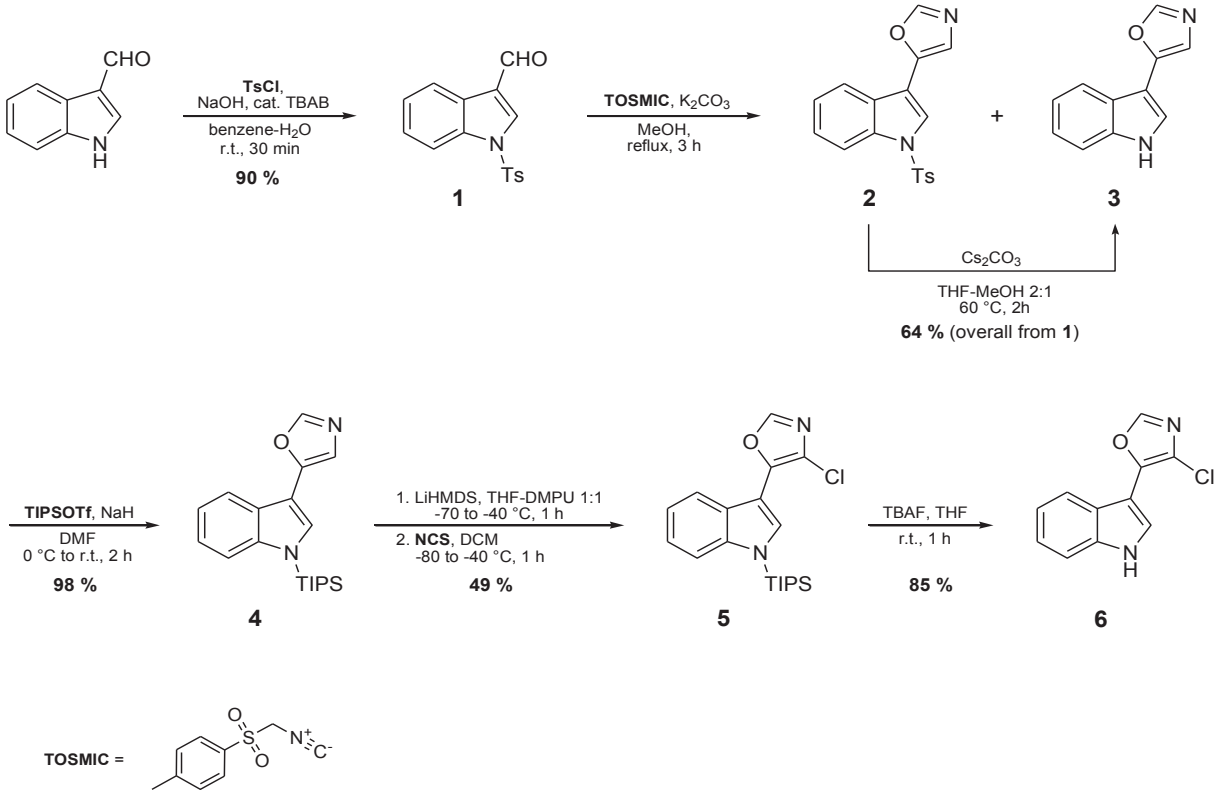
In the beewolf-*Streptomyces* mutualism, symbiont-produced antibiotic substances have previously been found to significantly enhance the survival probability of the wasp's offspring (Kaltenpoth et al. 2005). Here we show that these compounds are produced only within a short period after larval cocoon spinning but subsequently remain stable on the cocoon surface for up to eight months of beewolf hibernation, thereby providing a reliable long-term protection for the developing beewolf larva against pathogens. Changes in the composition of the "antibiotic cocktail" over time indicate different production and degradation dynamics for the antibiotic compounds, which may aid to prevent the evolution of resistant microorganisms. The production of antibiotics on the cocoon coincides with morphological differentiation of the symbionts, which enables them to survive the inhospitable conditions during beewolf hibernation and to be successfully acquired from the cocoon surface by the next generation of emerging wasps. Thus, this symbiotic system is finely tuned towards enhancing the efficiency of vertical symbiont transmission as well as providing long-term protection to the host offspring by warding off a broad range of pathogenic microorganisms. These findings provide new insights into the ecology of antibiotic production in the natural environment and may aid in exploring new strategies for the utilization of antibiotics in human medicine by using multi-drug combination therapies to counteract the increasing risk of resistant bacterial and fungal pathogens.

2.7 SUPPLEMENT

2.7.1 Supplementary methods

Synthesis of Streptochlorin

Commercially available chemicals were used without further purification, and solvents were dried as follows: dichloromethane and DMPU were dried by storage over molecular sieves 4Å for several days prior to use, dry methanol and THF (Extra Dry, AcroSeal®, Acros Organics), and absolute DMF (Fluka) were used as received. All reactions were carried out under an argon atmosphere. The preparative column chromatography (flash chromatography) was performed on Merck Kieselgel 60 (230-400 mesh), and TLC analysis on commercial Merck Kieselgel 60 F_{254} plates. Melting points were determined on a Büchi Melting Point apparatus B-540 and are uncorrected. ^{1}H-NMR and ^{13}C-NMR spectra were measured on a Bruker Avance DRX 500, AV-500, and 400 NMR spectrometers. Chemical shifts are reported in ppm downfield from TMS. Structures of all compounds were confirmed by signal assignment based on standard COSY, HSQC and HMBC spectra. HRMS and MS (EI) measurements (direct input mode) were performed with a MasSpec 2 instrument (Micromass) in positive ion mode using 70eV ionization energy. ESI-MS measurements were performed with a UltiMate 3000 UPLC (Dionex) coupled to an Orbitrap XL (Thermo-Fisher) equipped with an electrospray (ESI) source.



Scheme 1: TsCl = *p*-toluenesulfonyl chloride; TBAB = tetra-*n*-butylammonium bromide; TIPSOTf = triisopropylsilyl triflate; LiHMDS = lithium hexamethyldisilazane; DMPU = *N,N´*-dimethylpropylene urea; NCS = *N*-chlorosuccinimide; TBAF = tetra-*n*-butylammonium fluoride.

1-(Toluene-4-sulfonyl)-1*H*-indole-2-carbaldehyde (1)

According to a reported procedure (Waser et al. 2006), aqueous 30% NaOH (90 ml, 0.675 mol), tosyl chloride (5.01 g, 26.28 mmol) and tetra-*n*-butylammonium bromide (0.81 g, 10 mol%) were added to a suspension of indole-3-carboxaldehyde (3.63 g, 25.0 mmol) in 90 ml of benzene. After stirring vigorously for 30 min, the layers were separated and the aqueous layer was extracted with benzene (1× 50 ml). The combined organic layers were extracted with water, 10% aq. citric acid, and brine (1× 20 ml each), dried with $MgSO_4$, and, after addition of charcoal, passed through a short column of silica gel. The column was washed with 100 ml of benzene and the collected fractions were evaporated under reduced pressure. The crude product was purified by crystallization from a 2:1 *n*-hexane-benzene mixture affording 6.752 g (90%) of the aldehyde as an off-white crystalline solid.
^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.36 (s, 3H), 7.29 (m, 2H, *J* = 8.5 Hz), 7.35 (ddd, 1H, *J* = 7.8, 7.3 and 1.1 Hz), 7.40 (ddd, 1H, *J* = 8.3, 7.3 and 1.4 Hz), 7.85 (m, 2H, *J* = 8.5 Hz), 7.95 (ddd, 1H, *J* = 8.3, 1.1 and 0.7 Hz), 8.23 (s, 1H), 8.25 (ddd, 1H, *J* = 7.8, 1.4 and 0.7 Hz), 10.09 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 21.65, 113.24, 122.35, 122.59, 125.04, 126.28, 126.30, 127.22 (2C), 130.31 (2C), 135.22, 134.34, 136.23, 146.16, 185.32.

3-(Oxazol-5-yl)-1-(toluene-4-sulfonyl)-1*H*-indole (2) and 3-(oxazol-5-yl)-1*H*-indole (3)

The compounds were prepared according to a reported procedure (Chakrabarty et al. 2005) with minor modifications.

Method A: A solution of 300 mg of the aldehyde **1** (1 mmol), 215 mg of TOSMIC (1.1 mmol), and 155 mg of anhydrous K_2CO_3 (1.1 mmol) was refluxed in 10 ml of methanol for 1 hour. The reaction mixture was allowed to cool down to room temperature, poured into brine (50 ml), and extracted with EtOAc (4× 20 ml). The combined organic extracts were dried with $MgSO_4$ and evaporated. The residue was purified by column chromatography on silica gel (25-50% EtOAc/*n*-hexane) to furnish 164 mg (48%) of the N-Ts-derivative **2** as off-white crystals, and 14 mg (8%) of the deprotected product **3** as pale beige crystals. [Due to the content of other byproducts and similar R_F values, the purification of both products **2** and **3** (R_F = 0.26 and 0.20 in 50% EtOAc/hexane) was quite tedious and led only to an incomplete separation, providing thus diminished yields. Therefore, the pre-purified reaction mixture was used directly in the next step (*Method B*)].

Method B: Following *Method A*, 3.0 g of the aldehyde (10 mmol), 2.15 g of TOSMIC (11 mmol), and 1.52 g of K_2CO_3 were refluxed for 3 hours. After the workup, the reaction mixture was passed through a short column of silica gel and eluted with 35% EtOAc/*n*-hexane. The fractions containing products **2** and **3** were collected and evaporated to afford 2.63 g of yellow oil.

The crude material was dissolved in 90 ml of a 2:1 mixture of THF/MeOH, 7.59 g of Cs_2CO_3 (3 equiv.) (Bajwa et al. 2006) was then added, and the mixture was heated to 60°C for 2 hours. After cooling to room temperature, the reaction mixture was poured into water (200 ml) and extracted 4× with 50 ml of EtOAc. The combined organic fractions were washed once with 5% aq. citric acid (50 ml), once with brine (50 ml), dried with $MgSO_4$, and evaporated under reduced pressure. The crude mixture was purified by column chromatography on silica gel (0-2% MeOH/$CHCl_3$) to provide 1.176 g (64% based on the starting aldehyde) of the 3-(oxazol-5-yl)-1*H*-indole **3**.

2: ^{1}H NMR (500 MHz, $CDCl_3$): δ = 2.34 (s, 3H), 7.24 (m, 2H, *J* = 8.5 Hz), 7.35 (ddd, 1H, *J* = 7.8, 7.4 and 1.1 Hz), 7.38 (s, 1H), 7.41 (ddd, 1H, *J* = 8.2, 7.4 and 1.2 Hz), 7.77 (d, 1H, *J* = 7.8 Hz), 7.81 (m, 2H, *J* = 8.5 Hz), 7.92 (s, 1H), 7.95 (s, 1H), 8.04 (d, 1H, *J* = 8.2 Hz). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 21.60, 110.91, 113.88, 120.52, 122.28, 123.06, 124.03, 125.53, 126.89, 126.96 (2C), 130.07 (2C), 134.86, 135.10, 145.43, 145.67.

3: ^{1}H NMR (500 MHz, *d_6*- acetone): δ = 7.19 (ddd, 1H, *J* = 7.9, 7.0 and 1.2 Hz), 7.23 (ddd, 1H, *J* = 8.1, 7.0 and 1.3 Hz), 7.38 (s, 1H), 7.52 (m, 1H, *J* = 8.1 Hz), 7.79 (d, 1H, *J* = 2.4 Hz), 7.91 (m, 1H, *J* = 7.9 Hz), 8.11 (s, 1H), 10.71 (bs, 1H). ^{13}C NMR (125 MHz, *d_6*- acetone): δ = 105.56, 112.83, 119.95, 120.45, 121.25, 123.30, 123.94, 125.07, 137.72, 149.11, 149.97.

3-(Oxazol-5-yl)-1-(triisopropylsilyl)-1*H*-indole (4)

The bulky triisopropylsilyl (TIPS) group was chosen to protect the indole moiety, before the next step, because *N*-TIPS substitution is known to prevent deprotonation at C(2). Thus, contrary to some other silyl groups, TIPS group is also resistant toward rearrangements upon treatment with strong bases.

To a stirred solution of 3-(oxazol-5-yl)-1*H*-indole **3** (925 mg, 5.02 mmol) in 25 ml of anhydrous DMF was slowly added 60% dispersion of NaH in mineral oil (220 mg, 5.5 mmol) at 0°C. After 20 minutes, 1.49 ml of triisopropylsilyl triflate (5.52 mmol) was added dropwise and the reaction mixture was left to stir for additional 15 minutes at 0°C, and then at room temperature for 1 hour. The reaction mixture was quenched with 5 ml of saturated aq. $NaHCO_3$, poured into a 1:1 mixture of brine and

water (150 ml), and extracted 4× with 50 ml of 25% EtOAc/hexane. The combined organic fractions were dried with Na_2SO_4, evaporated, and the crude product was purified by column chromatography on silica gel (0-20% EtOAc/*n*-hexane) affording thus 1.674 g (98%) of the title compound **4** as colorless crystals.

Mp 65.5°C. ^{1}H NMR (500 MHz, $CDCl_3$) [Similarly poor resolution of the aromatic proton signals was observed when spectra measured either in d_6-acetone or d_6-benzene]: δ = 1.17 (d, 18H, *J* = 7.5 Hz), 1.75 (septet, 3H, *J* = 7.5 Hz), 7.24-7.26 (m, 2H), 7.30 (s, 1H), 7.54-7.56 (m, 1H), 7.59 (s, 1H), 7.83-7.85 (m, 1H), 7.89 (s, 1H). ^{13}C NMR (125 MHz, $CDCl_3$): δ = 12.87 (3C), 18.07 (6C), 107.41, 114.32, 119.52, 119.73, 120.93, 122.47, 127.41, 129.08, 141.34, 147.94, 148.76. MS (EI); *m*/*z* (%): 340 (100) [M^+], 297 (55), 255 (12), 227 (7), 168 (6), 115 (11). HRMS *m*/*z* 340.1956 ($C_{20}H_{28}N_2OSi$ requires 340.1971).

3-(4-Chlorooxazol-5-yl)-1-(triisopropylsilyl)-1*H*-indole (5)

The procedure for regioselective chlorination of the oxazole **4**, described herein, has been adapted from a method developed for iodination of oxazoles at C(4) (Vedejs and Luchetta 1999). An approach based on the use of DMF as a participating solvent (Li et al. 2007) was also tested but gave only 17% yield.

A solution of the oxazole **4** (1.221 g, 3.585 mmol) in a mixture of 10 ml of dry THF and 10 ml of dry DMPU was cooled to -70°C, and 1M solution of lithium hexamethyldisilazane in THF (4.3 ml, 1.2 equiv.) was added dropwise. The reaction mixture was allowed to warm to -40°C within 1 hour and then cooled again to -80 °C. A solution of 708 mg of N-chlorosuccinimide (5.3 mmol, 1.48 equiv.) in 10 ml of dry DCM was added slowly at this temperature via syringe. The resulting orange slurry was stirred (with occasional shaking) at -60 to -40°C for an additional 1 hour, quenched at -40°C by addition of 20% aq. solution of NH_4Cl (2 ml), and allowed to warm to room temperature. The mixture was poured into a vigorously stirred mixture of 200 ml of water, containing 0.5 g of $Na_2S_2O_3$, and 50 ml of 10% EtOAc/*n*-hexane. The organic layer was separated and the aqueous phase was extracted 3× with 10% EtOAc/*n*-hexane. The combined organic fractions were dried with MgSO4 briefly and evaporated to give 1.68 g of brown oil which was purified by column chromatography on silica gel (0-5% EtOAc/*n*-hexane). The pure product (685 mg, 49% yield) was obtained as yellow viscous oil which solidifies upon standing to give ochre crystals.

Mp 84-86°C. ^{1}H NMR (400 MHz, C_6D_6): δ = 0.96 (d, 18H, *J* = 7.5 Hz), 1.42 (septet, 3H, *J* = 7.5 Hz), 7.03 (s, 1H), 7.26 (ddd, 1H, *J* = 7.9, 7.1 and 1.4 Hz), 7.31 (ddd, 1H, *J* = 7.7, 7.1 and 1.1 Hz), 7.48 (dd, 1H, *J* = 7.9 and 1.1 Hz), 7.96 (s, 1H), 8.27 (dd, 1H, *J* = 7.7 and 1.4 Hz),. ^{13}C NMR (100 MHz, C_6D_6): δ = 12.93 (3C), 18.04 (6C), 106.62, 114.37, 121.64, 121.71, 122.71, 123.18, 128.59, 130.55, 141.29, 143.48, 147.80. MS (EI); *m*/*z* (%): 374 (100) [M^+], 331 (56), 289 (13), 261 (8), 137 (12), 115 (17). HRMS *m*/*z* 374.1582 ($C_{20}H_{27}{}^{35}ClN_2OSi$ requires 374.1581).

3-(4-Chlorooxazol-5-yl)-1*H*-indole – Streptochlorin (6)

A solution of the TIPS-protected chlorooxazole **5** (684 mg, 1.82 mmol) in 15 ml of dry THF was treated with 1M solution of TBAF in THF (3.7 ml, 2 equiv.) at room temperature for 1 hour. The reaction mixture was poured into a 1:1 mixture of saturated aq. $NaHCO_3$ and brine (50 ml), and extracted with dichloromethane (4× 15 ml). The combined organic fractions were washed 1× with water (10 ml) and 1× with brine (10 ml), dried with $MgSO_4$, and evaporated under reduced pressure to give light brown oil. Column chromatography on silica gel (10-50% EtOAc/*n*-hexane) afforded

336 mg (85%) of pure streptochlorin as pale beige crystals. An analytical sample was obtained by further crystallization from benzene.
LC-MS data, including MS-MS of the $[M+H]^+$ ion, and NMR spectra (d_4-MeOH) were in accordance with those reported previously (Kroiss et al. 2010).

Mp 170.8°C. ^{1}H NMR (500 MHz, d_4-MeOH): δ = 7.15 (ddd, 1H, J = 8.1, 7.0 and 1.0 Hz), 7.22 (ddd, 1H, J = 8.1, 7.0 and 1.0 Hz), 7.46 (d, 1H, J = 8.1 Hz), 7.83 (s, 1H), 8.00 (d, 1H, J = 8.1 Hz), 8.18 (s, 1H). ^{1}H NMR (500 MHz, d_6-acetone): δ = 7.19 (ddd, 1H, J = 8.0, 6.9 and 0.9 Hz), 7.25 (ddd, 1H, J = 8.1, 6.9 and 1.0 Hz), 7.55 (d, 1H, J = 8.1 Hz), 7.97 (bd, 1H, J = 2.3 Hz), 8.04 (d, 1H, J = 8.0 Hz), 8.23 (s, 1H), 10.86 (bs, 1H). ^{13}C NMR (125 MHz, d_6-acetone): δ = 103.5, 112.87, 121.12, 121.48, 121.78, 123.62, 125.20, 125.50, 137.25, 144.17, 149.44. MS (EI); m/z (%): 218 (100) $[M^+]$, 189 (13), 183 (9), 163 (11), 155 (36), 144 (16), 128 (17). HRMS m/z 218.0251 ($C_{11}H_7{}^{35}ClN_2O$ requires 218.0247).

2.7.2 Supplementary figures

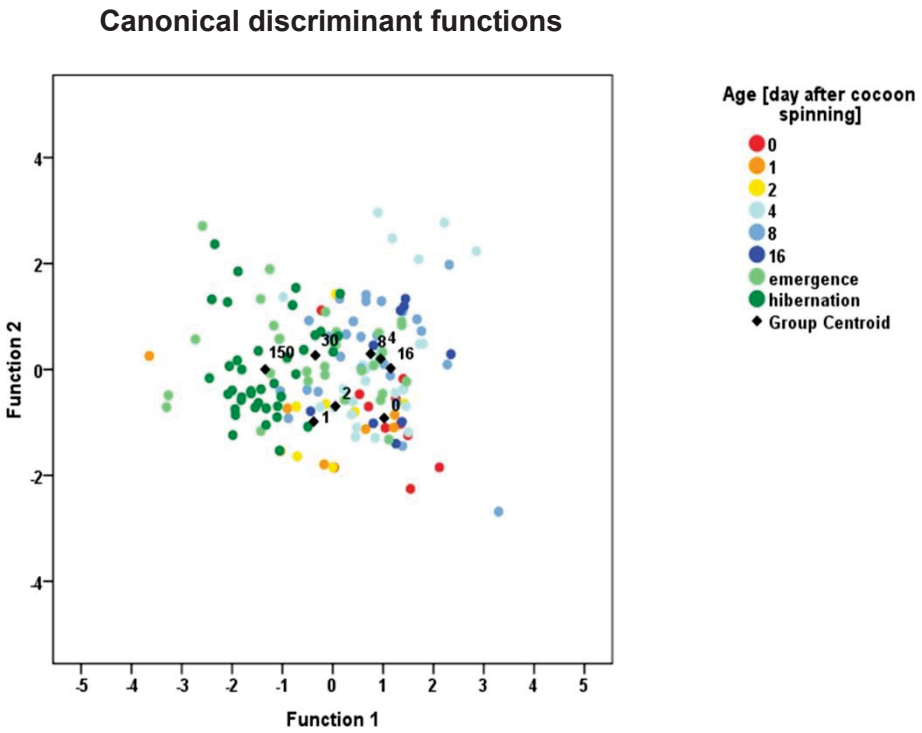


Fig. S1 Multivariate discriminant analysis of the relative amounts of the three most abundant antibiotics on beewolf cocoons (SC, PA1 and PB1) based on GC-MS data from Fig.2.2b (Wilks´ λ: 0.432, χ2: 126.4, df=21, P<0.001).

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CHAPTER 3

MATERNAL AND ENVIRONMENTAL EFFECTS ON SYMBIONT-MEDIATED ANTIMICROBIAL DEFENSE

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3.1 ABSTRACT

Bacteria produce a remarkable diversity of bioactive molecules with antimicrobial properties. Despite the importance of such compounds for human medicine, little is known about the factors influencing antibiotic production in natural environments. Recently, several insects have been found to benefit from symbiont-produced antimicrobial compounds for defense against pathogenic microbes. In the European beewolf, *Philanthus triangulum* (Hymenoptera, Crabronidae), bacteria of the genus *Streptomyces* provide protection against pathogens by producing antimicrobials on the larval cocoon during hibernation, thereby significantly enhancing the survival probability of the beewolf larva. To investigate the effects of abiotic and biotic factors on antibiotic production, we exposed beewolf cocoons to different environmental conditions and quantified the amount of *Streptomyces*-produced antibiotics using gas chromatography – mass spectrometry (GC-MS). The results revealed no significant influence of temperature, humidity or pathogen load on the antibiotic amount, indicating that antibiotic production is not affected by current environmental conditions but may rather be optimized to serve as a reliable long-term protection during the unpredictable phase of beewolf hibernation. However, the amount of antibiotics was positively correlated with the symbiont population size on the cocoon, which in turn is affected by the number of *Streptomyces* cells provided by the mother into the brood cell. Additionally, we found a positive correlation between the amount of hydrocarbons and the number and length of bacterial cells in the antennal gland secretion, suggesting that maternal investment affects symbiont growth and, thus, antibiotic production on the larval cocoon.

3.2 INTRODUCTION

Since the discovery of penicillin, secondary metabolites with antibiotic properties produced by soil-dwelling bacteria and fungi have revolutionized human medicine (Demain and Sanchez 2009). Despite the importance of antibiotics for human welfare, little is known about their ecological function and the factors influencing antibiotic production in natural environments (Dillon et al. 2000; Hurst and Hutchence 2010). Several studies support the hypothesis that bacteria can gain selective advantages over competitors in nutrient-limited habitats by the production of antimicrobial compounds (Rasool and Wimpenny 1982; Thomashow et al. 1990; Howie and Suslow 1991; Roughley et al. 1992; Turpin et al. 1992; Wiener 1996; Slattery et al. 2001; Aminov 2009). Especially when present in low concentrations, however, microbe-produced antibiotics can also act as inter- and intracellular signaling molecules (Goh et al. 2002; Tsui et al. 2004; Linares et al. 2006; Dufour and Rao 2011), affecting gene expression, orchestrating microbial growth (Hoffman et al. 2005; Linares et al. 2006; Lopez et al. 2009), and initiating morphological differentiation (Miall 1971; Vanek and Mikulik 1978; Gräfe 1983; Chater 1989).

In vitro, the production of antibiotics and other secondary metabolites is affected by the abiotic and biotic conditions that the producing microorganism is exposed to. Several important influencing factors have been identified, e.g. the availability of different carbon sources (Barratt and Oliver 1994; Hassan et al. 2001), pH and temperature (Higashide 1984; Hassan et al. 2001), and the exposure to competing microorganisms (Wiener 1996; Slattery et al. 2001). However, little is known about environmental effects on secondary metabolite production in their natural habitats (Dillon et al. 2000; Hurst and Hutchence 2010).

In nature, antibiotics do not only mediate microbial interactions, they can also play key roles for mutualistic associations with macroorganisms (Hurst and Hutchence 2010). Notably, insects are a rich source of microbial symbionts (Buchner 1965), and recent studies indicate that symbiotic bacteria provide defense to several insects or their nutritional resources by the production of bioactive secondary metabolites (Currie et al. 1999a; Kellner 2002; Kaltenpoth et al. 2005; Scott et al. 2008; Haeder et al. 2009; Kaltenpoth and Engl 2013). Species of the phylum Actinobacteria seem to be especially suitable partners in such defensive symbiotic relationships, which can at least partly be attributed to the large diversity of secondary metabolites they produce (Kaltenpoth 2009; Oh et al. 2009a; Chater et al. 2010; Poulsen et al. 2011; Seipke et al. 2012; Solecka et al. 2012). In leaf-cutting ants, Actinobacteria of the genus *Pseudonocardia* protect the ants' fungus garden as well as the worker ants themselves, against pathogenic fungi (Currie et al. 1999b; Oh et al. 2009a; Poulsen et al. 2010; Mattoso et al. 2012). Other actinobacterial genera, notably *Streptomyces* and *Amycolatopsis*, have also been implicated in the defense of the fungal garden by producing antimicrobial compounds (Haeder et al. 2009; Barke et al. 2010; Schoenian et al. 2011). Similarly, in fungus-growing bark beetles (*Dentroctonus frontalis)*, a *Streptomyces*-produced compound, mycangimycin, may provide protection of the beetle´s fungus garden against the competitor *Ophistosoma minus* (Scott et al. 2008; Oh et al. 2009b).

Female solitary digger wasps of the genera *Philanthus, Trachypus,* and *Philanthinus* cultivate symbiotic Actinobacteria that provide a chemical defense to the wasp offspring against soil-dwelling pathogens (Kaltenpoth et al. 2005; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). The bacteria of the genus *Streptomyces* (`*Candidatus* Streptomyces philanthi`) are cultivated in specialized antennal reservoirs of female beewolves, which are surrounded by gland cells that probably provide nutrients to the bacterial

symbionts (Kaltenpoth et al. 2005; Goettler et al. 2007). Prior to oviposition, an antennal gland secretion (AGS hereafter) containing the bacteria is secreted into the subterranean brood cells by the female beewolf. During cocoon-spinning, the larva incorporates the symbiotic *Streptomyces* bacteria into the cocoon walls, where they produce a cocktail of at least nine different antibiotic substances that provide protection against a wide range of pathogenic microorganisms (Kroiss et al. 2010) and thereby significantly enhance the offspring's chances to survive until adulthood (Kaltenpoth et al. 2005). Although antibiotic production by the symbionts is limited to a short phase of 1-2 weeks after cocoon spinning, the long-term stability of the antimicrobial compounds ensures protection for the beewolf offspring during the long phase of hibernation in the cocoon (Koehler et al. 2013).

Here we investigated the influence of environmental and maternal effects on antibiotic production by the symbiotic *Streptomyces* bacteria on beewolf cocoons. We hypothesized that the production of antibiotics is buffered against current environmental influences to ensure efficacy of the antibiotic cocktail during the long and unpredictable phase of beewolf hibernation. Furthermore, we tested whether female beewolves differ in the allocation of symbiotic bacteria to the brood cell and whether such differences could affect antibiotic production on the cocoon.

3.3 METHODS AND MATERIALS

3.3.1 Beewolf rearing conditions

Female European beewolves, *Philanthus triangulum*, were collected from natural populations in Würzburg, Erlangen, and Berlin (Germany). The females were individually housed in observation cages consisting of a flight chamber and a compartment containing a thin layer (10 mm) of sand for nest building as described previously (Strohm and Linsenmair 1995), with one modification: between the sand and the glass cover, a layer of plastic foil was introduced to allow the complete removal of the AGS applied to the ceiling of the brood cell by the female beewolf. The observation cages were situated in a greenhouse (14 h day, 10 h night; 23°C, +/- 3°C) at the Max Planck Institute for Chemical Ecology in Jena, Germany, or in the laboratory at the University of Würzburg, Germany. Female beewolves were provided with honey bee workers *(Apis melifera)* and honey *ad libitum*. In addition to the field-collected individuals, their female offspring were also kept in observation cages and used for the experiments.

3.3.2 Cocoon collection and experimental conditions

In order to investigate environmental effects on symbiont-mediated antibiotic production, beewolf cocoons were collected from brood cells two days after cocoon spinning and subjected to various experimental conditions. Only cocoons from brood cells that had been supplied with 1-2 honeybees and/or that were produced by unfertilized females were chosen in order to limit the study to males (Strohm and Linsenmair 1999). The cocoons were measured in length and width before individually placing them in 37 ml plastic cups (Solo® Cup Company, Highland Park, IL, USA), resting on two parallel preparation needles (0.5 x 38 mm, Bioform, Nuremberg, Germany). The jars were equipped with a filter paper (Roth, Karlsruhe, Germany), as well as a sponge on the bottom to control humidity (Figure S1), and closed with a paperback lid. The lid was penetrated with a needle, introducing a small

hole to ensure sufficient oxygen supply, as well as to allow for the addition of water over the whole period of the experiment. After 16 days, all samples were frozen at -80°C for subsequent analyses.
To investigate the influence of abiotic and biotic factors on antibiotic production, we exposed the beewolf cocoons to different environmental conditions, including different temperatures, humidities and pathogen loads, and subsequently quantified the amount of antibiotics using GC-MS. To study the effect of temperature and humidity, the samples were placed in incubators (APT.line KB (E3.1), Binder GmbH, Tuttlingen, Germany) at 15°C and 25°C under humid and dry conditions (full-factorial design with four treatment groups, N=16 per group). Humid conditions were achieved by adding a wet sponge and filter paper to the experimental cups and by supplying water every other day via the penetrated lid, as described above. For samples under dry conditions this step was omitted, but the cups were also removed from the incubator and subsequently placed back to mimic the processing of samples under humid conditions. The humidity inside the cups was tested before the experiment using a Data Logger (Lascar Electronics Ltd., Salisbury, UK). At 15°C, atmospheric humidity inside the cups varied from 48.5-52% under dry conditions and 94.0-96.5% under humid conditions. Similarly, at 25°C, atmospheric humidity varied from 60-65% under dry conditions and 90-94% for humid conditions. To limit variation due to genetic or maternal effects, we used male cocoons originating from the same mother (four brothers) for the different treatment groups. For the analysis, 64 male beewolf cocoons from 11 different females were collected (for some females, several replicates of four cocoons each were available).
In addition to the constant temperature set-up, we tested the influence of changing temperatures on antibiotic production. Cocoon samples were placed in the incubator under humid conditions and the following temperature regime: 2 h ramp from 15°C to 25°C, 10 h of 25°C, 2 h ramp from 25°C to 15°C, and 10 h of 15°C, etc. A control was kept under humid conditions at a constant temperature of 20°C.
The effect of pathogens on the symbiont-mediated production of antibiotics was tested by adding spore suspensions of *Aspergillus tamarii* (570 spores/µl) and *Paecilomyces lilacinus* (840 spores/µl) in 0.05% Triton X to the samples. Both fungal species were previously isolated from beewolf brood cells as competitors and opportunistic pathogens, overgrowing the remains of the larval provisions and subsequently threatening to infest and kill the beewolf larva in the cocoon (Engl et al. submitted). Samples as well as controls were prepared as indicated above, and either 5µl of each spore suspension or 10 µl 0.05% Triton X (control treatment) was added directly onto the cocoon, respectively, before placing them in the incubator at 25°C (+/- 2°C). As for the temperature and humidity experiments, brothers were used to reduce maternal effects. Every other day the samples were checked and 500-1000 µl milipore water was added to the filter paper via the penetrated lid to assure humid conditions in the samples.

3.3.3 Quantification of antibiotics on beewolf cocoons by GC-MS

For the GC-MS analyses of the symbiont-produced antibiotics, cocoons from which the larva had been removed were placed individually in glass vials and extracted with 1 ml of methanol for 1 h. The methanol extracts were evaporated to dryness under a gentle stream of Argon and subsequently re-suspended in 50 µl of methanol. An aliquot of 1 µl was injected into the GC-MS using a split/splitless injector at 250°C, with the purge valve opened after 60 s. Analysis of the methanol samples was done using a Varian 450GC gas chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies, Böblingen, Germany). The GC was equipped with a DB5-MS capillary column (30 m x 0.25 mm, film thickness: 0.25 µm, Agilent Technologies), programmed from 150 to 300°C at 5°C/min with a 1 min. initial and a 5 min. final isothermal hold. Helium was used as a carrier gas with a constant flow rate of 1 ml/min. Mass spectra were recorded using electron ionization (EI-MS), and

data acquisition as well as quantification were done using MS Workstation Version 6.9.3 Software (Agilent Technologies). For absolute quantification, we used a dilution series (500-0.1 ppm) of commercially available piericidin A1 as an external calibration standard for the different piericidin derivatives. Based on the high structural similarity of the piericidin derivatives to piericidin A1, we assumed similar ionization efficiencies (Kroiss et al. 2010). The quantification of streptochlorin was achieved by using a dilution series of a synthesized streptochlorin standard (Koehler et al. 2013). We quantified the five most abundant antibiotics on beewolf cocoons, i.e. streptochlorin (SC), piericidin A1 (PA1), piericidin B1 (PB1), piericidin A5 (PA5) and piericidin C1 (PC1), as the other derivatives could not reliably be detected due to low concentrations and/or low volatility (Kroiss et al. 2010). The peaks were identified based on their mass spectra (Kroiss et al. 2010), and peak areas were automatically integrated using the MS Workstation Software. The success and accuracy of this integration was controlled manually for every peak.

3.3.4 Quantification of *Streptomyces* bacterial cells on the cocoon using quantitative real-time PCR (qPCR)

After methanol extraction, the male beewolf cocoons were homogenized in liquid nitrogen followed by DNA extraction using the Epicentre MasterPure™ DNA extraction kit (Epicentre Technologies, Madison, USA), with the following adjustments: the RNase and the lysozyme steps were omitted, and all centrifugation steps were accomplished at room temperature. All solutions not included in the kit (TE buffer, water, 70% EtOH, isopropanol) were prepared from stock solutions using diethylpyrocarbonate- (DEPC-) treated, RNase-free water. After successful extraction, the DNA pellet was re-suspended in 100 µl RNase free low-TE buffer (1 mM Tris/HCl, 0.01 mM EDTA), and two aliquots of 50 µl each were stored at -80°C.
Subsequently, qPCR with '*Ca.* S. philanthi' 16S rRNA gene-specific primer pairs (Kaltenpoth et al. 2010a) was used to quantify the population size (16S rRNA gene copy number) of the *Streptomyces* symbionts. For quantification of the total bacterial 16S gene copy number, we used a standard dilution series (10^{-1} to 10^{-7}) as described previously (Koehler et al. 2013). QPCR amplification was performed on a Rotor-Gene Q Cycler (Qiagen, Hilden, Germany) in a total reaction volume of 25 µl, including: 6.5µl RNase-free water and 12.5 µl SYBR-Mix (Rotor-Gene SYBR Green RT-PCR kit, Qiagen, Hilden, Germany); 2.5 µl of each primer (10 µM), 1 µl template. Cycle parameters were as follows: 95°C for 10 min, 45 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 20 s. Finally, a melting curve analysis was conducted by increasing the temperature from 72°C to 95°C with 1°C gain. Based on the standard curve, the total amount of DNA was calculated from the qPCR threshold values using the absolute quantification method (Lee et al. 2006; Lee et al. 2008).

3.3.5 Collection of AGS

In order to investigate whether maternal effects could influence cell number and growth of the symbionts, the AGS from 118 brood cells of ten different beewolf females were collected for chemical and morphological analyses. To this aim, the observation cages were checked daily for new brood cells, and the AGS was obtained by cutting out the piece of plastic foil containing the secretion from freshly constructed brood cells. Subsequently, the AGS was placed in glass vials (4 ml, Hartenstein, Würzburg, Germany) and frozen at -20°C for subsequent analysis. To obtain another correlate of maternal investment, the number of provisioned honey bees was recorded for each brood cell from which the AGS was sampled (Strohm and Marliani 2002).

3.3.6 Chemical analysis of the AGS by GC-MS

For GC-MS analysis, the plastic foils with AGS from the brood cells were extracted individually in glass vials with about 1 ml of distilled hexane for 5min, after adding 2 μg of *n*-octadecane as internal standard. After removal of the AGS, the sample volumes were reduced to about 50-200 μl by a gentle flow of nitrogen, and 1 μl was analyzed by coupled capillary GC-MS. The extracts of the AGS were analysed with an Agilent 6890N Series gas chromatograph (Agilent Technologies, Böblingen, Germany) coupled to an Agilent 5973 inert mass selective detector. The GC was equipped with a RH-5ms+ fused silica capillary column (30 m x 0.25 mm ID; df=0.25 μm; temperature program: from 60°C to 300°C at 5°C/min and held for 1 min at 60°C and for 10 min at 300°C). Helium was used as the carrier gas with a constant flow of 1 ml/min. A split/splitless injector was installed at 250°C in the splitless mode for 60 s. The electron impact mass spectra (EI-MS) were recorded with an ionisation voltage of 70eV, a source temperature of 230°C and an interface temperature of 315°C. Peaks were identified by their retention times and mass spectra according to Strohm et al. (2008) and Kaltenpoth et al. (2009). The chemical composition of the AGS from beewolf brood cells has been analyzed previously (Kaltenpoth et al. 2009). In most of the samples several of the described components were below the detection limit due to the low concentration of the compounds in individual samples (Table S1). All of the peaks that could be unambiguously identified by their retention times and mass spectra were manually integrated with MSD ChemStation software (Agilent Technologies). For each sample the peak areas of all substances were combined, and the total absolute amount of hydrocarbons was calculated by comparison with the peak area of the internal standard (Table S1).
Previous studies have shown that there is a distinct dimorphism in the chemical composition of the cuticular hydrocarbons (Strohm et al. 2010), the postpharyngeal gland secretion (PPG) (Strohm et al. 2008) and the AGS (Kaltenpoth et al. 2009) of female European beewolves: Some individuals have higher amounts of (*Z*)-9-pentacosene (C_{25}-type), whereas others have (*Z*)-9-heptacosene (C_{27}-type) as their major compound. To avoid any possible confounding effects of the chemical type, all analyses were performed separately for the two morphs.

3.3.7 Analysis of symbiont cell number and length in the AGS

After hexane extraction, two brood cells of each female were randomly selected for measuring the number and length of bacterial cells in the AGS. Since only one brood cell was available for three of the females, 17 AGS samples were analyzed. Plastic foils containing the AGS were placed individually in Eppendorf tubes, and the bacterial cells were suspended by adding 100 μl of DNA-A (10 mM Tris/HCl pH 7.5, 60 mM NaCl, 10 mM EDTA) and 100 μl of DNA-B (0.2 mM Tris/HCl pH 9.0, 30 mM EDTA, 2.0% SDS). The suspensions were vortexed vigorously to break up cell clusters. Bacterial suspensions were diluted 100-fold with 50% ethanol for further analysis. For each sample, four replicates of 1 μl each were pipetted onto a microscope slide, air-dried, heat-fixated, and then stained with a saturated solution of crystal violet in 50% ethanol for two minutes. Excess stain was washed off with distilled water, and the slide was allowed to dry. All *Streptomyces* cells on the slide were counted with a manual counter. The total number of cells in the AGS was calculated as the cell count multiplied by 20,000 (original volume of AGS suspension: 200 μl; dilution factor 1:100). Mean and standard error of the four replicates were calculated for each sample.
For the determination of the bacterial cell length, 20 cells were randomly selected for each sample. Digital pictures were taken with a Nikon DS-2Mv camera attached to a Zeiss Axioplan microscope, using a 200-fold magnification and a resolution of 1600 x 1200 pixels. The length of the bacterial cells in pixels was measured using Amira® software and converted into millimeters by calibration with an

object micrometer scale. Since the obtained values for the cell length deviated significantly from a normal distribution for most samples, the median cell length was determined for each sample.

3.3.8 Statistical analysis

The total amount of antibiotics on beewolf cocoons as revealed by GC-MS analysis was compared across treatments to test for environmental effects on symbiont-mediated antibiotic production (temperature, humidity, pathogen load). The experiment testing for an influence of temperature and humidity in a full-factorial setup with four different treatment groups was analyzed using repeated measures ANOVA to account for the dependent nature of the samples (because brothers were used). For the experiments testing for an influence of fluctuating temperatures over time and pathogen load, respectively, the analyses were performed using T-tests for dependent samples.
Furthermore, differences in the composition of the antibiotic cocktail across treatments were analyzed based on the three predominant and consistently detected compounds, i.e. streptochlorin, piericidin A1 and piericidin B1. The areas of single peaks were translated into relative peak areas with subsequent log-ratio transformation according to Aitchison (1986). Finally, a discriminant analysis was used to test for differences in the composition of the antibiotic cocktail on beewolf cocoons with regard to treatments.
The number of symbiont cells on the cocoon was compared across treatments for all three experimental setups using repeated measures ANOVA and T-tests for dependent samples, respectively. Since cocoons from different treatments did not differ significantly in either antibiotic amount or symbiont cell number, a Spearman correlation between the amount of antibiotics and the number of *Streptomyces* bacterial cells was conducted for all cocoons (combined). The cocoon size was calculated from the length (*h*) and width (*d=2r*) using the mathematical equation for a cone´s surface as the closest approximation:

(1) $$A_O = r\pi \cdot r + \left(\sqrt{h^2 + r^2} \right)$$

Pairwise correlations between the cocoon size and the amount of antibiotics as well as the *Streptomyces* cell number were conducted using Spearman correlations.
The total amount of hydrocarbons in the AGS was calculated and compared among different beewolf females by ANOVA. Likewise, an ANOVA was used to test for an effect of bee number and chemical morph on the amount of AGS hydrocarbons. To investigate whether the AGS from brood cells of different females can be separated on the basis of their chemical composition, a multivariate discriminant analysis was performed (Supplementary Methods and Figures S3-S4). The mean number of bacteria per brood cell and the median bacterial cell length in the AGS were compared among females. Pairwise correlations between the amount of hydrocarbons in the AGS and the number and length of bacterial cells in the AGS were performed by Pearson correlations and reduced major axis regressions using PAST (Hammer et al. 2001).

3.4 RESULTS

3.4.1 Environmental effects on symbiont growth and antibiotic production

To elucidate the influence of environmental effects on antibiotic production in the beewolf-*Streptomyces* symbiosis, we exposed male beewolf cocoons to different abiotic and biotic conditions and measured the amount of antibiotics using GC-MS. Within the variability of conditions that likely occur in natural beewolf brood cells in the soil, neither different temperatures (15°C or 25°C) nor humidity had a significant effect on antibiotic production on beewolf cocoons in a constant environment (Figure 3.1a, Wilks´ λ=1.0, F<0.001, P=0.993). Similar results were obtained for the experiment with fluctuating temperatures over a 24 h day-night-cycle (changing environment), as the amount of antibiotics did not differ between the fluctuating treatment (15-25°C) and the stable control group at 20°C (Figure 3.1b, t-test for dependent samples: t=0.083, df=9, P=0.936). Furthermore, the GC-MS analysis of male beewolf cocoons that were enriched with spores of mold fungi on the cocoon surface revealed no differences in the amount of antibiotic substances as compared to untreated controls (Figure 3.1c, t-test for dependent samples: t=0.594, df=12, P=0.563). To elucidate potential effects of environmental factors on the composition of the antibiotic cocktail, discriminant analyses were conducted for all three experiments. The analyses revealed no significant differences (at α=0.05) in the relative amounts of the three predominant antibiotic substances on beewolf cocoons (SC, PA1 and PB1) with regard to the different treatments (stable environment: Wilks´ λ=0.768, χ^2=15.69, df=9, P=0.074; changing temperature: Wilks´ λ=0.939, χ^2=1.100, df=3, P=0.777; pathogen load: Wilks´ λ=0.915, χ^2=1.990, df=3, P=0.574).
Additionally, the *Streptomyces* population size was quantified and compared among treatments for all three experiments to correct for the number of bacteria incorporated into the cocoon walls. However, there were no significant differences in the numbers of bacteria on the cocoon surface among the different treatments (stable environment: Wilks´ λ=0.999, F=0.022, P=0.883; changing temperature: t-test for dependent samples: t=-0.062, df=9, P=0.952; pathogen load: t-test for dependent samples: t=-0.319, df=12, P=0.755), suggesting a stable *Streptomyces* population size independent of biotic and abiotic environmental factors.
To test for an effect of symbiont cell number on antibiotic production, we compared the amount of antibiotics with the *Streptomyces* population size on the cocoon surface. Since the number of bacterial cells did not change with regard to different environmental factors, the correlation was conducted using all cocoon samples across the three experiments. The amount of antibiotics varied between 0.0002 and 15.9 µg/cocoon. The *Streptomyces* 16S total copy number varied between 2.41 x 10^7 and 6.45 x 10^9 copies/cocoon. The antibiotic amount was significantly correlated with the number of *Streptomyces* bacterial cells on the cocoon surface (Figure 3.2, Spearman´s ϱ=0.703, N=111, P<0.001).
The cocoon size was calculated and compared with the amount of antibiotics and the bacterial cell number on the surface of the cocoon across treatments. There was no significant correlation between the cocoon size and the antibiotic amount (Spearman´s ϱ=0.146, N=111, P=0.126) or the number of symbiotic *Streptomyces*, although larger cocoons showed a tendency towards higher numbers of symbiont cells (Spearman´s ϱ=0.179, N=111, P=0.06).

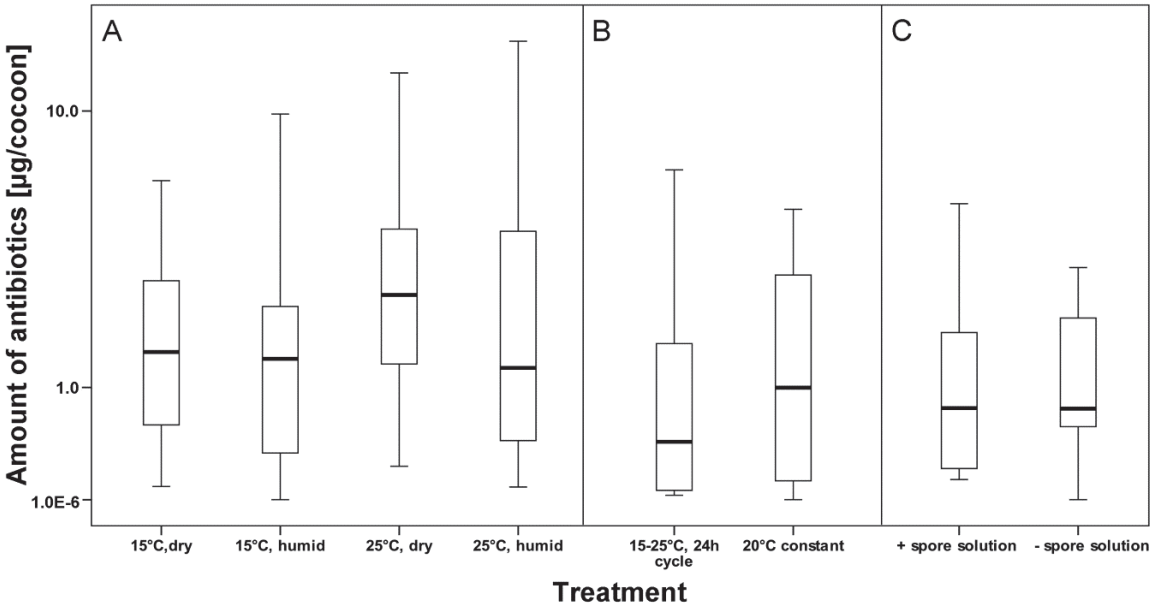

Fig. 3.1 Effect of abiotic and biotic environmental factors on the total amount of antibiotic substances produced by *Streptomyces* symbionts on European beewolf cocoons 16 days after cocoon spinning, quantified using GC-MS. **(A)** Influence of temperature and humidity on the amount of antibiotics (Wilks´ λ=1.0, F<0.001, P=0.993); **(B)** influence of fluctuating temperatures on the amount of antibiotics, under humid conditions (T-test for dependent samples: t=0.083, df=9, P=0.936); **(C)** effect of increased pathogen load on antibiotic production, by adding a spore solution of two different fungi (*Aspergillus tamarii* and *Paecilomyces lilacinus* in 0.05% TritonX) to the beewolf cocoons (T-test for dependent samples: t=0.594, df=12, P=0.563). Bold lines represent medians, boxes comprise the interquartile range, bars indicate minimum and maximum values.

3.4.2 Maternal investment: AGS

The amount of AGS hydrocarbons extracted from 118 beewolf brood cells varied between 0 and 9.59 µg (mean ± SD = 2.37 ± 1.72 µg) (Table S1). Individual female beewolves differed significantly in the total amount of AGS hydrocarbons supplied into the brood cells (ANOVA: $F_{7,108}$=14.8, P<0.001), as well as in the composition of the AGS hydrocarbon profile (Figures S3-S4, discriminant analyses, P<0.001 for both C_{25}-type females only and C_{25}-/C_{27}-type females combined). Neither the number of bees in the brood cell nor the chemical type had a significant influence on the total amount of AGS hydrocarbons (ANOVA, $F_{3,114}$=0.922, P=0.433). Furthermore, the amount of AGS hydrocarbons was not correlated with the brood cell number (consecutive numbering for each female according to the brood cell construction date) for either all females combined or each single female (Pearson correlations, P>0.05 for all analyses), indicating that the amount of AGS hydrocarbons does not change with the age of female beewolves.

The total number of *Streptomyces* cells in the AGS ranged from 4.65 x 10^3 to 6.21 x 10^6 (mean ± SD = 3.40 x 10^6 ± 1.84 x 10^6 cells). The number of cells as well as the cell length were significantly correlated with the total amount of hydrocarbons in the AGS (Figure 3.3: cell number, Pearson correlation: N=17, R^2=0.487, P=0.002; Figure S2: cell length, Pearson correlation: N=17, R^2=0.284, P=0.028). The chemical morph had no significant effect on the number or length of bacterial cells (two-way ANOVAs, bacterial cell number: $F_{3,13}$=1.49, P=0.263; cell length: $F_{3,13}$=0.959, P=0.441), although there was a tendency towards higher numbers of bacteria in C_{25}-type as compared to C_{27}-type brood cells.

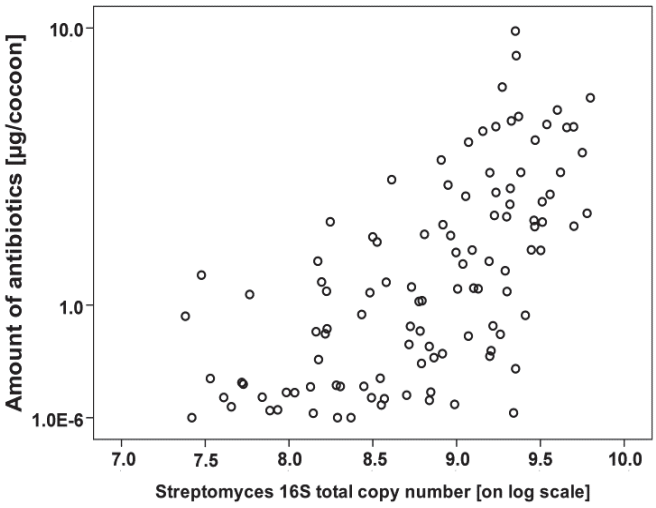

Fig. 3.2 Correlation of the estimated 16S rRNA gene copy number of *Streptomyces* symbiont cells and the total amount of antibiotics on male European beewolf cocoons (Spearman´s ϱ=0.703, N=111, P<0.001).

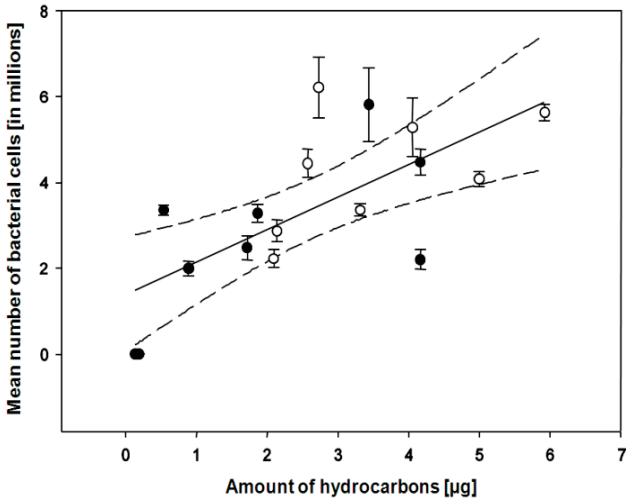

Fig. 3.3 Correlation between the mean number of bacterial cells (in millions) and the total amount of hydrocarbons (in µg) in the antennal gland secretion of female European beewolves (Pearson correlation: N=17, R^2=0.487, P=0.002). The circles indicate the different hydrocarbon profiles of the females (open circles=C_{25}-type, filled circles=C_{27}-type).

3.5 DISCUSSION

3.5.1 Effect of temperature and humidity on antibiotic production

Actinobacteria are potent producers of a wide variety of antimicrobial compounds, which can provide a competitive advantage over antagonists in nutrient-limited habitats (Rasool and Wimpenny 1982; Turpin et al. 1992; Sanchez and Brana 1996; Slattery et al. 2001). However, little is known about the factors influencing antibiotic production in natural environments. *In vitro*, the production of antibiotic substances can be affected by various factors, such as different carbon and nitrogen sources, pH, and temperature (Higashide 1984; James et al. 1991; Barratt and Oliver 1994; Nanavaty et al. 1998; Hassan et al. 2001; Oskay 2011). However, in the context of the defensive symbiosis between beewolf wasps and '*Ca.* Streptomyces philanthi', we found no significant effect of temperature or humidity on the production of antibiotics *in situ* (Figure 3.4). Within the range of environmental conditions likely occurring in beewolf brood cells in the first weeks after cocoon spinning, neither amount nor composition of the symbiont-produced antibiotic cocktail changed significantly regardless of whether conditions were constant or fluctuating (Figure 3.1a,b).

Given the direct influence of temperature on most metabolic processes, it seems surprising that antibiotic production is not affected by abiotic factors in '*Ca.* S. philanthi'. Ecologically, however, it makes sense that the production of antibiotic substances by the symbionts does not depend on the current conditions in the brood cells. Since the developing beewolves are exposed to unpredictable environmental conditions during hibernation, it appears to be adaptive that antibiotic production is buffered against external effects during the short period of symbiont metabolic activity in order to serve as an efficient long-term protection (Koehler et al. 2013). On the physiological level, the symbionts may achieve constant levels of antibiotic production by adjusting the duration of the metabolically active phase on the cocoon before undergoing morphological differentiation.

3.5.2 Effect of pathogen load on antibiotic production

Consistent with the hypothesis that antibiotics are produced to ward off competitors, prior studies on various *Streptomyces* strains found increasing antibiotic production with higher densities of competing microorganisms (Wiener 1996; Slattery et al. 2001). In the humid, subterranean brood cells, a wide variety of fungi can overgrow the remains of the beewolf´s provisions, threatening the developing larva inside the cocoon (Engl et al. submitted). However, our analysis revealed no increase in the production of antibiotics by the beewolf symbionts after adding a spore solution of two different fungi from beewolf brood cells to the cocoon (Figure 3.1c).

As opposed to the abiotic conditions discussed above, the presence of fungal spores on the cocoon during the phase of antibiotic production could provide valuable information of the future pathogen pressure to the symbiotic bacteria. Thus, both beewolves and their symbionts could benefit from increased amounts of antibiotics in the presence of pathogens, and it is surprising that our experiments do not show such an effect. Two mutually non-exclusive hypotheses could explain these observations: (i) Although the fungi used for the experiment have been isolated repeatedly from beewolf brood cells (Engl et al. submitted), they may not be pathogenic to the beewolf larva inside the cocoon. It is conceivable that the beewolf symbionts up-regulate antibiotic production only in the presence of specific pathogenic microorganisms. Concordantly, antibiotic production in a marine streptomycete is only induced upon exposure to specific competitors (Slattery et al. 2001). However, beewolf brood cells can be infested by a variety of detrimental fungi (Engl et al. submitted), and the symbiont-produced antibiotics inhibit a broad range of microorganisms (Kroiss et al. 2010), suggesting a general rather than specialized defense. The mechanisms of how *Streptomyces* could sense competing microbes is not yet understood, but gamma-butyrolactones as important signaling molecules among various bacterial taxa may constitute promising candidates (Khokhlov et al. 1967; Bachofen and Schenk 1998; Chhabra et al. 2005; Ohnishi et al. 2005; Takano 2006; Antunes and Ferreira 2009). (ii) The symbionts produce the maximum possible amount of antibiotics regardless of the presence of competitors or pathogens. Given the nutrient-limited conditions on beewolf cocoons, it seems likely that the symbionts are constrained in the allocation of resources to antibiotic production. A high investment in antibiotic production may be adaptive by ensuring competitive superiority over future antagonists, thereby increasing the chances of successful transmission of the symbionts to the next host generation. However, further experiments are necessary to disentangle these hypotheses by identifying specific beewolf pathogens and testing the response of the symbionts towards their presence, and/or by scrutinizing the bioactive potential of the symbionts on the cocoon in the presence of compounds stimulating antibiotic production.

3.5.3 Maternal effects on antibiotic production

To test for maternal effects on the symbiont-mediated antibiotic production, we analysed several factors that are influenced by the female beewolf during brood cell construction and may represent maternal investments, such as the amount of hydrocarbons and the number and length of symbiont cells in the AGS, and the offspring´s cocoon size, which is affected by the number of provisioned honeybees (Strohm 2000) (Figure 3.4). Our results revealed that the amount of antibiotics produced by the *Streptomyces* symbionts is positively correlated with the number of bacterial cells on the cocoon (Figure 3.2). Furthermore, beewolf females differed significantly in the amount of AGS hydrocarbons provided to the brood cell, which is strongly correlated with the number of symbiont cells (Figure 3.3, Figure S2). Thus, it seems likely that the differences in symbiont cell numbers on the cocoon are due to differences in maternal allocation of AGS to the brood cell. This conclusion is supported by previous studies demonstrating that (i) a large proportion of the symbiont cells secreted into the brood cell (50% on average) are incorporated into the cocoon by the beewolf larva (Kaltenpoth et al. 2010a), and (ii) the symbiont population shows only very limited growth on the cocoon surface (Koehler et al. 2013).

Surprisingly, our analyses revealed no significant correlations between cocoon size and symbiont cell number or the amount of antibiotics on the cocoon surface. The size of the beewolf larva, and consequently, the size of the cocoon, is mainly influenced by the amount of provisions supplied to the brood cell by the female beewolf (Strohm 2000). To account for the larger surface area, larger cocoons should be expected to contain higher numbers of symbionts, and, as a result, higher amounts of antibiotics in order to ensure an efficient protection against pathogens. However, we found only a weak, non-significant tendency towards larger cocoons having more symbiont cells, and antibiotic amount and concentration were independent of cocoon size. Thus, the amount of AGS and the number of symbiont cells that the female can allocate to a brood cell appears to be limited, suggesting that symbiont allocation may represent a costly component of maternal investment.

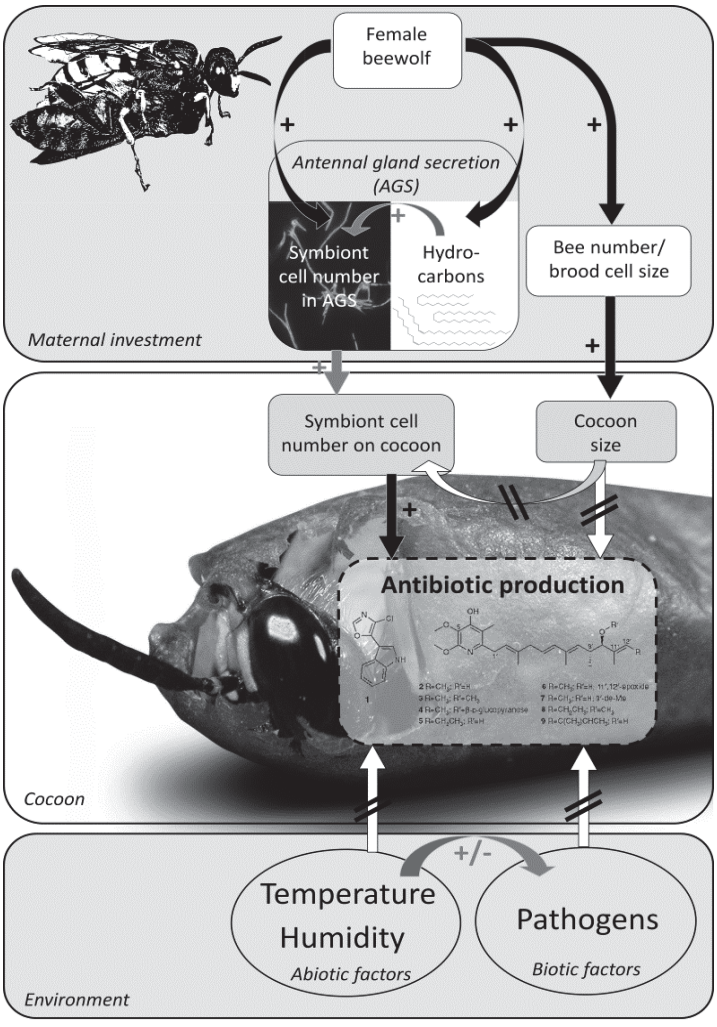

Fig. 3.4 Factors affecting symbiont-mediated antibiotic production by *Streptomyces* symbionts on European beewolf cocoons. Black arrows denote significant positive effects, grey arrows indicate putative effects, and blocked white arrows correspond to the absence of a significant influence.

As all organisms have to balance their allocation of resources to current and future reproduction in order to maximize their fitness, reproductive investment can be complex and multifaceted (Rosenheim et al. 1996), and individuals often differ in their ability to provide resources to the offspring. In *P. triangulum,* prey hunting is an extremely costly behavior for the females (Strohm and Marliani 2002), and a recent study provided evidence that the embalming of the prey items with a hydrophobic secretion from the postpharyngeal gland that reduces fungal germination on the provisions entails costs as well (Herzner et al. 2011). Since female beewolves likely supply the symbiotic bacteria in the antennal gland reservoirs with nutrients from the surrounding gland cells (Goettler et al. 2007) or via the hemolymph (Goettler et al. 2007; Kaltenpoth et al. 2009), the symbiont cultivation might add to the costs of parental investment. Hence, differences in the number of bacterial cells provided to the brood cell may reflect a female's quality to allocate resources for the offspring and ensure an efficient protection by the symbiont-produced antibiotics during hibernation. Alternatively, the secretion of bacterial cells into the brood cell may be subject to time constraints due to the low growth rate of the symbionts (Kaltenpoth et al. 2010a). After brood cell construction, the antennal reservoirs are diminished of bacterial symbionts (Kaltenpoth et al. 2010a), presumably leaving a suboptimal number of bacteria available for secretion into the following brood cell. Thus, the female can either allocate less bacterial secretion to the following brood cell, risking a higher fungal infestation rate of its offspring, or postpone the next brood cell until the symbiont cells have replenished the reservoirs (Kaltenpoth et al. 2010a), leading to a reduced rate of reproduction. Both metabolic costs and delays in the production of brood cells could constitute factors that contribute to resource allocation and parental investment in European beewolves.

In summary, contrary to previous *in vitro* experiments on free-living *Streptomyces* strains, antibiotic production does not seem to be affected by environmental factors in the beewolf-*Streptomyces* symbiosis. Rather, it may be optimized to ensure the long-term protection for the beewolf progeny in the cocoon during the unpredictable phase of hibernation. However, the number of symbiont cells provided into the brood cell by the mother influences antibiotic production on the cocoon and may thus reflect a costly maternal investment, due to the need to nourish the symbiont population in the antennal gland reservoirs. The beewolf-*Streptomyces* symbiosis constitutes an excellent model system to study the ecology of antibiotic production in the natural environment, providing the potential to unravel the complex network of factors influencing microbe-mediated antibiotic production *in situ*.

3.6 SUPPLEMENT

3.6.1 Supplementary methods

Discriminant analysis of beewolf AGS

To investigate whether the AGS from brood cells of different females can be separated on the basis of their chemical composition, a multivariate discriminant analysis was performed. For this analysis, the dataset was reduced to contain only brood cells with ≥ 1 μg of hydrocarbons (because the measurement of peak areas in samples with very low concentrations is increasingly inaccurate), and only females with more than six brood cells were included. For each sample, the relative peak areas for all substances were calculated (Table S1). Because the relative amounts constitute compositional data, they were transformed to log-contrasts prior to analysis (Aitchison 1986; Reyment 1989). The number of variables was reduced by a principle component analysis. The PCA factors were then subjected to a discriminant analysis (DA). Since it is known that there is a distinct chemical dimorphism in the hydrocarbon composition of the cuticle, the PPG, and the AGS of female beewolves (Herzner et al. 2007; Strohm et al. 2008; Kaltenpoth et al. 2009) that can seriously confound the results of DA, the PCA and DA were performed not only based on the complete dataset (Figure S3), but also with the reduced dataset containing only C_{25}-type females (Figure S4). Due to the low number of C_{27}-type females with more than six brood cells, no discriminant analysis was done with females of this morph only.

3.6.2 Supplementary tables

Table S1 Cuticular hydrocarbon profiles of antennal gland secretions (AGS) within 118 brood cells of 10 different beewolf females. Some peaks were not always clearly separated by GC-MS and were therefore combined for the analysis. Labels refer to the following compounds: C21=*n*-heneicosane; C22=*n*-docosane; C23:1=Δ-9- and Δ –x-tricosenes; C23=*n*-tricosane; M-C23=11-, 9-, 7-, and 5-methyltricosanes; C24:1= Δ-9-tetracosene; C24=*n*-tetracosane; C25:1=(*Z*)-9- and Δ-7-pentacosenes and *n*-pentacosane; M-C25=11-, 9-, 7-, and 5-methylpentacosanes; C26:1=(*Z*)-9-hexacosene; C26=*n*-hexacosane; C27:1=(*Z*)-9-heptacosene, *n*-heptacosane and Δ-16-pentacosen-8-one; C29:1=(*Z*)-9-nonacosene and Δ-18-heptacosen-10-one; C29=*n*-nonacosane.

	Brood	Number	Hydrocarbon	Total amount of	Relative amounts of hydrocarbons (in %)												
Female #	cell #	of bees	type	hydrocarbons [mg]	C21	C22	C23:1	C23	M-C23	C24:1	C24	C25:1	M-C25	C26:1	C26	C27:1	C29:1	C29
3	2	2	C25	0,09	0,00	0,00	0,00	16,18	0,00	0,00	0,00	83,82	0,00	0,00	0,00	0,00	0,00	0,00
3	3	1	C25	5,17	0,16	0,20	0,87	17,99	0,27	2,39	0,18	75,45	0,00	0,00	0,00	2,03	0,00	0,46
3	4	2	C25	4,05	0,17	0,22	0,86	18,51	0,27	2,36	0,21	75,11	0,00	0,00	0,00	1,86	0,00	0,43
3	5	2	C25	6,14	0,10	0,20	0,98	17,36	0,24	2,42	0,23	75,67	0,00	0,00	0,00	2,24	0,00	0,58
3	6	1	C25	2,95	0,05	0,15	0,72	17,44	0,41	2,10	0,25	76,97	0,00	0,00	0,00	1,62	0,00	0,30
3	7	1	C25	2,70	0,13	0,20	1,04	17,42	0,30	2,40	0,26	76,07	0,00	0,00	0,00	1,88	0,00	0,30
3	8	1	C25	4,88	0,12	0,24	1,38	16,78	0,31	2,56	0,24	75,86	0,18	0,00	0,00	2,05	0,00	0,28
3	9	1	C25	2,62	0,15	0,21	1,03	17,92	0,29	2,22	0,19	75,75	0,00	0,00	0,00	2,24	0,00	0,00
3	10	2	C25	5,58	0,07	0,15	1,09	16,35	0,22	2,39	0,26	77,17	0,00	0,00	0,00	1,99	0,00	0,30
3	11	2	C25	3,51	0,07	0,16	0,98	17,53	0,30	2,18	0,29	76,22	0,00	0,00	0,00	1,88	0,00	0,39
3	12	2	C25	4,99	0,10	0,18	1,38	17,10	0,30	2,44	0,24	76,01	0,00	0,00	0,00	2,03	0,00	0,22
3	13	1	C25	2,32	0,11	0,20	1,23	18,12	0,43	2,19	0,27	75,79	0,00	0,00	0,00	1,66	0,00	0,00
3	14	1	C25	2,23	0,23	0,28	1,26	18,76	0,34	2,26	0,30	74,62	0,00	0,00	0,00	1,96	0,00	0,00
4	1	1	C27	0,89	0,24	0,37	0,00	23,18	0,00	0,28	0,37	14,51	0,00	1,80	0,00	56,47	1,41	1,37
4	3	1	C27	0,18	0,00	0,00	0,00	23,05	0,00	0,00	0,00	13,80	0,00	0,00	0,00	63,15	0,00	0,00
4	4	1	C27	0,54	0,00	0,00	0,00	21,07	0,00	0,00	0,00	12,63	0,00	1,82	0,00	64,49	0,00	0,00
4	5	2	C27	0,55	0,00	0,00	0,00	22,22	0,00	0,00	0,00	12,07	0,65	2,01	0,00	63,05	0,00	0,00
4	6	2	C27	0,45	0,00	0,00	0,00	21,45	0,00	0,00	0,00	12,83	0,00	1,75	0,00	61,60	0,79	1,59

4	7	1	C27	0,39	0,00	0,00	0,00	20,18	1,44	0,00	0,00	12,94	0,00	1,76	0,00	63,68	0,00	0,00
9	8	1	C25	0,24	0,00	0,00	0,00	17,69	0,00	1,35	0,00	80,96	0,00	0,00	0,00	0,00	0,00	0,00
9	9	1	C25	1,23	0,20	0,31	0,48	18,25	0,41	2,17	0,30	75,04	0,00	0,00	0,00	2,43	0,00	0,41
9	10	1	C25	1,34	0,21	0,28	0,48	17,88	0,41	2,27	0,30	75,24	0,00	0,00	0,00	2,61	0,00	0,32
9	11	1	C25	2,09	0,19	0,27	0,49	17,58	0,36	2,25	0,26	75,10	0,00	0,00	0,00	3,17	0,00	0,32
9	12	1	C25	3,31	0,16	0,25	0,50	17,26	0,32	2,35	0,27	75,89	0,00	0,00	0,00	2,64	0,00	0,37
9	14	1	C25	1,48	0,22	0,27	0,41	17,65	0,45	2,01	0,26	76,27	0,00	0,00	0,00	2,26	0,00	0,20
9	15	2	C25	1,93	0,16	0,24	0,44	17,16	0,43	2,24	0,33	76,50	0,00	0,00	0,00	2,24	0,00	0,27
9	16	2	C25	1,95	0,19	0,24	0,45	17,29	0,48	2,20	0,26	75,72	0,00	0,00	0,00	2,60	0,00	0,57
9	17	1	C25	1,30	0,25	0,28	0,41	17,54	0,55	2,00	0,28	76,48	0,00	0,00	0,00	2,04	0,00	0,18
9	18	1	C25	2,32	0,13	0,21	0,39	16,97	0,59	1,98	0,29	77,37	0,00	0,00	0,00	2,08	0,00	0,00
9	19	2	C25	3,00	0,14	0,19	0,36	16,55	0,42	1,99	0,24	77,94	0,00	0,00	0,00	1,93	0,00	0,24
9	20	1	C25	1,14	0,19	0,21	0,35	16,40	0,53	1,45	0,38	80,49	0,00	0,00	0,00	0,00	0,00	0,00
9	21	2	C25	2,82	0,14	0,17	0,38	16,44	0,22	2,05	0,21	78,06	0,00	0,00	0,00	1,99	0,00	0,33
9	22	1	C25	2,60	0,09	0,14	0,39	15,89	0,43	2,04	0,28	78,57	0,00	0,00	0,00	1,87	0,00	0,31
9	23	1	C25	0,94	0,12	0,30	0,34	16,59	0,90	1,51	0,29	78,54	0,00	0,00	0,00	1,42	0,00	0,00
9	24	1	C25	1,36	0,17	0,31	0,44	17,87	0,65	2,03	0,30	76,22	0,00	0,00	0,00	2,02	0,00	0,00
9	25	1	C25	2,21	0,10	0,23	0,37	17,05	0,73	1,97	0,32	77,31	0,00	0,00	0,00	1,93	0,00	0,00
9	26	1	C25	0,09	0,00	0,00	0,00	19,55	0,00	0,00	0,00	80,45	0,00	0,00	0,00	0,00	0,00	0,00
9	27	1	C25	1,58	0,16	0,24	0,39	18,82	0,54	2,05	0,33	74,82	0,00	0,00	0,00	2,30	0,00	0,34
9	28	1	C25	1,07	0,23	0,24	0,37	18,52	0,87	1,84	0,40	75,19	0,00	0,00	0,00	2,34	0,00	0,00
9	29	1	C25	2,40	0,18	0,20	0,35	18,79	0,54	1,91	0,30	75,79	0,00	0,00	0,00	1,93	0,00	0,00
9	30	1	C25	2,49	0,14	0,21	0,38	18,11	0,37	2,06	0,25	75,91	0,00	0,00	0,00	2,13	0,00	0,43
10	2	1	C25	2,57	0,18	0,28	0,74	17,51	0,30	2,30	0,24	75,74	0,00	0,00	0,00	2,40	0,00	0,31
10	3	1	C25	0,42	0,00	0,00	0,00	19,74	0,00	1,35	0,00	78,91	0,00	0,00	0,00	0,00	0,00	0,00
10	5	1	C25	2,57	0,18	0,29	0,91	18,25	0,26	2,33	0,26	73,95	0,33	0,00	0,00	2,78	0,00	0,46
10	6	1	C25	6,07	0,15	0,27	1,07	16,89	0,25	2,45	0,27	74,50	0,18	0,24	0,00	3,44	0,00	0,28
10	7	2	C25	2,40	0,19	0,28	0,98	19,10	0,35	2,34	0,27	74,54	0,00	0,00	0,00	1,72	0,00	0,22
10	8	2	C25	3,93	0,16	0,25	0,94	19,46	0,32	2,21	0,24	74,39	0,00	0,00	0,00	1,68	0,00	0,36
10	9	2	C25	2,71	0,17	0,27	1,01	18,94	0,29	2,14	0,23	74,96	0,00	0,00	0,00	1,79	0,00	0,21
10	10	1	C25	5,02	0,19	0,30	1,34	17,82	0,35	2,47	0,26	74,23	0,44	0,15	0,00	2,11	0,00	0,34
10	11	1	C25	4,45	0,15	0,27	1,32	17,89	0,31	2,45	0,30	74,44	0,32	0,00	0,00	2,17	0,00	0,39
10	12	1	C25	5,92	0,20	0,31	1,57	19,94	0,26	2,49	0,28	72,21	0,00	0,00	0,00	2,35	0,00	0,39
10	13	1	C25	0,41	0,00	0,00	0,70	21,92	0,00	1,48	0,00	75,90	0,00	0,00	0,00	0,00	0,00	0,00
10	14	1	C25	2,19	0,24	0,26	1,18	20,44	0,57	2,00	0,30	73,68	0,00	0,00	0,00	1,32	0,00	0,00
10	15	1	C25	1,15	0,22	0,33	1,13	19,94	0,68	1,86	0,36	75,48	0,00	0,00	0,00	0,00	0,00	0,00
10	16	1	C25	0,26	0,00	0,00	0,00	21,15	0,00	0,00	0,00	78,85	0,00	0,00	0,00	0,00	0,00	0,00
12	1	2	C25	2,73	0,15	0,24	0,53	19,61	0,24	2,15	0,25	75,01	0,00	0,00	0,00	1,47	0,00	0,33
12	2	2	C25	2,16	0,13	0,27	0,66	19,35	0,41	2,28	0,22	75,02	0,00	0,00	0,00	1,65	0,00	0,00
12	3	1	C25	1,45	0,15	0,21	0,55	19,19	0,51	1,77	0,30	76,03	0,00	0,00	0,00	1,28	0,00	0,00
12	4	2	-	0,00														
12	5	2	C25	1,30	0,31	0,31	0,77	18,53	0,72	1,83	0,35	75,98	0,00	0,00	0,00	1,20	0,00	0,00
12	6	2	C25	2,14	0,05	0,09	0,28	15,79	0,35	1,81	0,28	79,38	0,00	0,00	0,00	1,97	0,00	0,00
12	7	2	C25	1,79	0,09	0,12	0,35	16,67	0,48	1,89	0,37	78,20	0,00	0,00	0,00	1,82	0,00	0,00
12	8	2	C25	3,66	0,06	0,13	0,37	16,28	0,34	2,15	0,34	77,91	0,00	0,00	0,00	2,00	0,00	0,43
13	1	1	C27	1,26	0,14	0,28	0,21	20,02	0,36	0,24	0,33	16,13	0,21	2,45	0,16	56,91	1,78	0,79
13	2	2	C27	3,44	0,23	0,22	0,14	20,67	0,24	0,21	0,26	15,04	0,16	2,34	0,00	58,35	1,47	0,68
13	3	1	C27	2,88	0,27	0,26	0,16	19,55	0,20	0,22	0,28	14,63	0,16	2,69	0,05	59,22	1,62	0,70
13	4	1	C27	1,72	0,24	0,26	0,12	20,49	0,32	0,17	0,26	13,93	0,21	2,54	0,13	58,63	1,86	0,84
13	5	1	C27	4,05	0,24	0,25	0,08	20,44	0,23	0,17	0,24	13,43	0,10	2,48	0,11	60,21	1,28	0,75
13	6	1	C27	3,90	0,37	0,31	0,09	22,10	0,32	0,21	0,29	13,98	0,12	2,70	0,10	56,58	2,16	0,67
13	7	2	C27	5,38	0,26	0,26	0,08	19,37	0,26	0,21	0,28	13,53	0,12	2,65	0,08	60,16	1,89	0,85
13	8	2	C27	4,67	0,30	0,31	0,09	19,88	0,19	0,23	0,31	13,56	0,12	2,69	0,05	59,32	2,28	0,67
13	9	1	C27	3,48	0,31	0,24	0,04	20,73	0,26	0,16	0,28	14,50	0,11	2,46	0,09	58,45	1,35	1,02
13	10	2	C27	2,71	0,24	0,27	0,07	19,35	0,27	0,20	0,33	13,70	0,15	2,79	0,09	59,45	2,29	0,78
13	11	1	C27	5,14	0,26	0,29	0,07	19,97	0,32	0,20	0,31	13,64	0,17	2,74	0,10	59,27	1,90	0,74
13	12	1	C27	3,80	0,24	0,24	0,06	19,30	0,32	0,19	0,26	13,15	0,20	2,67	0,11	60,64	1,85	0,77
13	13	1	C27	2,90	0,25	0,25	0,02	22,54	0,28	0,14	0,31	13,93	0,18	2,71	0,00	56,80	1,58	1,01

19	1	2	C27	1,14	0,16	0,19	0,20	17,28	0,28	0,18	0,58	13,03	0,21	2,41	0,08	61,14	3,46	0,78
19	2	1	C27	3,65	0,24	0,24	0,15	19,62	0,31	0,20	0,30	12,08	0,17	2,46	0,06	60,54	2,85	0,78
19	3	2	C27	4,16	0,30	0,25	0,10	20,03	0,25	0,20	0,33	11,06	0,17	2,58	0,09	60,90	3,11	0,63
19	4	2	C27	0,48	0,00	0,00	0,00	19,20	0,00	0,00	0,00	9,11	0,00	3,56	0,00	63,66	2,92	1,55
19	5	1	C27	4,16	0,25	0,22	0,06	18,92	0,23	0,16	0,30	10,04	0,13	2,68	0,07	62,53	3,69	0,72
19	6	1	C27	2,92	0,22	0,21	0,03	20,79	0,28	0,14	0,23	10,50	0,14	2,40	0,00	61,80	2,26	1,00
19	7	2	C27	3,55	0,22	0,20	0,03	19,38	0,27	0,15	0,28	10,37	0,14	2,58	0,09	62,08	3,14	1,08
19	8	2	C27	3,18	0,25	0,22	0,06	20,33	0,20	0,17	0,27	10,50	0,14	2,39	0,00	61,74	2,78	0,96
19	9	1	C27	3,00	0,27	0,26	0,04	21,21	0,39	0,20	0,31	11,57	0,14	2,58	0,13	59,38	2,30	1,22
19	10	2	C27	2,50	0,32	0,28	0,06	20,90	0,40	0,21	0,34	12,01	0,17	2,57	0,00	58,76	2,77	1,23
19	11	1	C27	2,74	0,30	0,29	0,04	20,55	0,38	0,20	0,33	12,49	0,17	2,64	0,12	58,86	2,65	0,99
19	12	1	C27	3,39	0,38	0,31	0,09	21,64	0,37	0,29	0,28	15,74	0,18	2,68	0,07	55,33	1,87	0,78
19	13	1	C27	1,45	0,38	0,31	0,13	21,06	0,48	0,27	0,28	15,97	0,22	2,60	0,00	55,64	1,92	0,75
19	14	1	C27	3,58	0,37	0,34	0,11	21,11	0,30	0,27	0,30	15,91	0,17	2,70	0,10	55,10	2,47	0,75
19	15	2	C27	3,42	0,36	0,32	0,10	20,04	0,38	0,28	0,28	15,04	0,17	2,67	0,08	57,76	1,73	0,79
19	16	1	C27	3,43	0,24	0,25	0,05	20,34	0,37	0,26	0,26	14,19	0,16	2,54	0,11	58,47	1,83	0,93
19	17	1	C27	3,44	0,27	0,25	0,07	20,93	0,36	0,22	0,29	14,21	0,16	2,62	0,12	57,57	1,92	1,01
19	18	2	C27	3,81	0,25	0,23	0,04	19,89	0,35	0,23	0,27	13,94	0,17	2,49	0,09	59,55	1,66	0,85
19	19	1	C27	2,10	0,25	0,23	0,04	20,50	0,51	0,28	0,27	14,46	0,22	2,19	0,00	58,91	1,22	0,93
19	20	2	C27	2,35	0,25	0,22	0,07	21,90	0,48	0,29	0,30	18,94	0,27	2,59	0,00	52,57	1,02	1,11
19	21	1	C27	2,01	0,22	0,23	0,15	21,39	0,62	0,33	0,35	18,71	0,29	2,24	0,21	53,21	0,84	1,23
19	22	1	C27	4,08	0,24	0,23	0,14	20,30	0,36	0,32	0,31	20,30	0,22	2,65	0,12	52,79	1,03	0,97
19	23	2	C27	2,35	0,26	0,22	0,14	21,46	0,61	0,31	0,29	21,02	0,35	2,37	0,00	51,44	0,51	1,01
19	24	1	C27	2,38	0,27	0,23	0,27	20,15	0,55	0,47	0,27	29,43	0,49	2,03	0,00	44,71	0,42	0,71
19	25	1	C27	1,53	0,27	0,28	0,27	19,79	0,77	0,54	0,29	32,06	0,67	1,90	0,22	41,72	0,43	0,80
19	26	1	C27	3,49	0,25	0,24	0,22	21,06	0,57	0,53	0,35	29,71	0,47	2,11	0,19	43,30	0,39	0,62
19	27	1	C27	2,79	0,31	0,24	0,18	21,97	0,65	0,44	0,30	26,53	0,43	2,05	0,17	45,62	0,43	0,66
21	5	2	C27	0,14	0,00	0,00	0,00	27,15	0,00	0,00	0,00	16,76	0,00	0,00	0,00	56,09	0,00	0,00
21	6	2	C27	0,19	0,00	0,00	0,00	22,35	0,00	0,00	0,00	16,14	0,00	1,57	0,00	59,93	0,00	0,00
21	8	2	C27	0,17	0,00	0,00	0,00	21,80	0,00	0,00	0,00	11,39	0,00	1,86	0,00	64,95	0,00	0,00
21	9	1	C27	0,08	0,00	0,00	0,00	27,68	0,00	0,00	0,00	29,19	0,00	0,00	0,00	43,13	0,00	0,00
21	10	1	C27	0,54	0,00	0,00	0,00	31,59	0,00	0,00	0,51	29,14	0,00	1,21	0,00	37,54	0,00	0,00
21	13	1	-	0,00														
21	14	1	C27	0,03	0,00	0,00	0,00	33,67	0,00	0,00	0,00	7,48	0,00	0,00	0,00	58,84	0,00	0,00
21	15	1	C27	0,05	0,00	0,00	0,00	29,74	0,00	0,00	0,00	22,51	0,00	0,00	0,00	47,75	0,00	0,00
21	16	1	-	0,00														
21	17	2	C27	0,08	0,00	0,00	0,00	31,02	0,00	0,00	0,00	21,93	0,00	0,00	0,00	47,05	0,00	0,00
21	18	1	-	0,00														
21	19	1	-	0,00														
21	20	1	-	0,00														
16	10		C25	9,59	0,36	0,35	0,90	17,26	0,27	2,52	0,18	74,26	0,00	0,00	0,00	3,26	0,00	0,63
31	6		C27	1,86	0,10	0,14	0,10	18,38	0,56	0,23	0,41	12,17	0,27	2,57	0,00	62,08	1,37	1,64

3.6.3 Supplementary figures

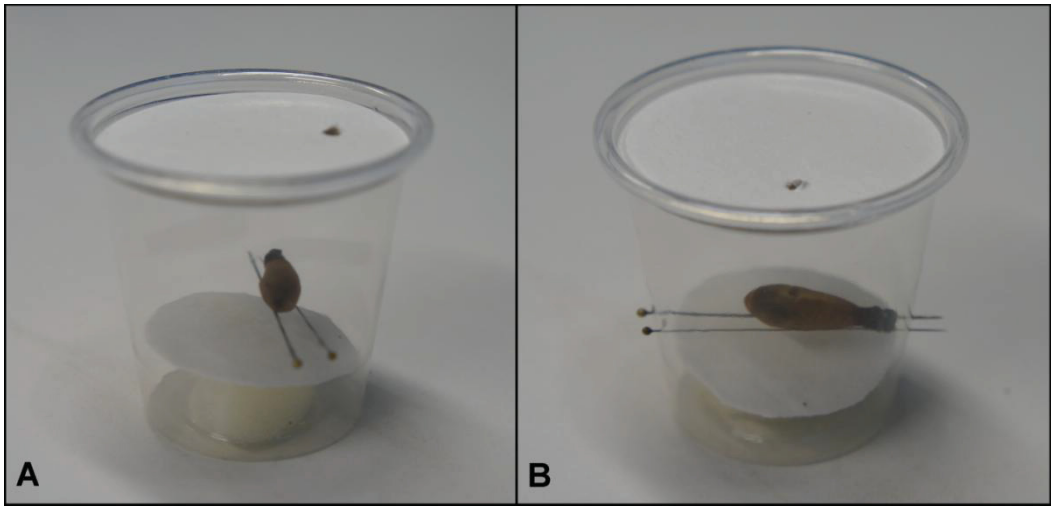

Fig.S1 Male beewolf cocoons, including larva, placed within experimental jars for all experiments testing for the influence of biotic and abiotic environmental factors on symbiont-mediated antibiotic production; **(a)** horizontal view; **(b)** vertical view.

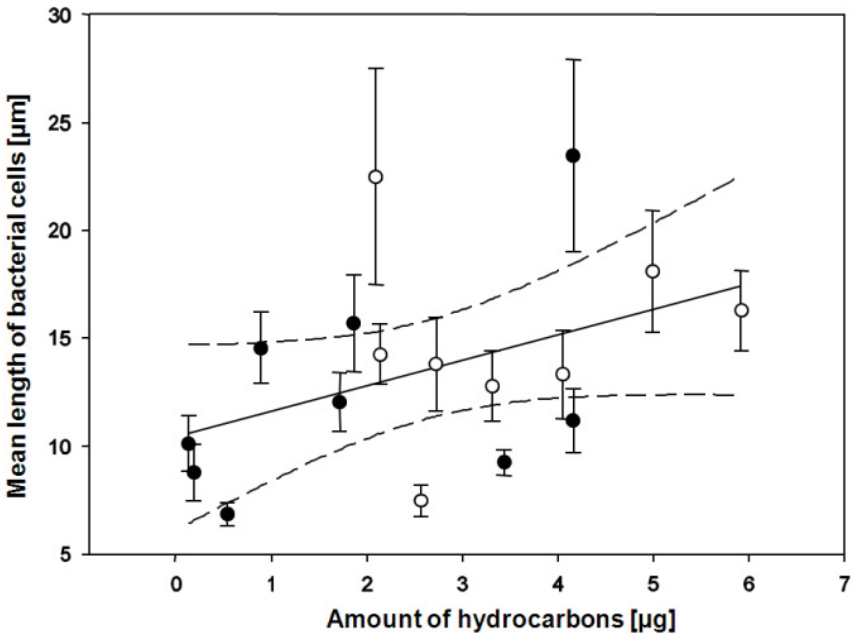

Fig.S2 Correlation between the mean length of bacterial cells (µm) and the total amount of hydrocarbons (in µg) in the AGS of female European beewolves (Pearson correlation: N=17, R^2=0.284, P=0.028). The circles indicate the different hydrocarbon profiles of the females (open circles=C_{25}-type, filled circles=C_{27}-type).

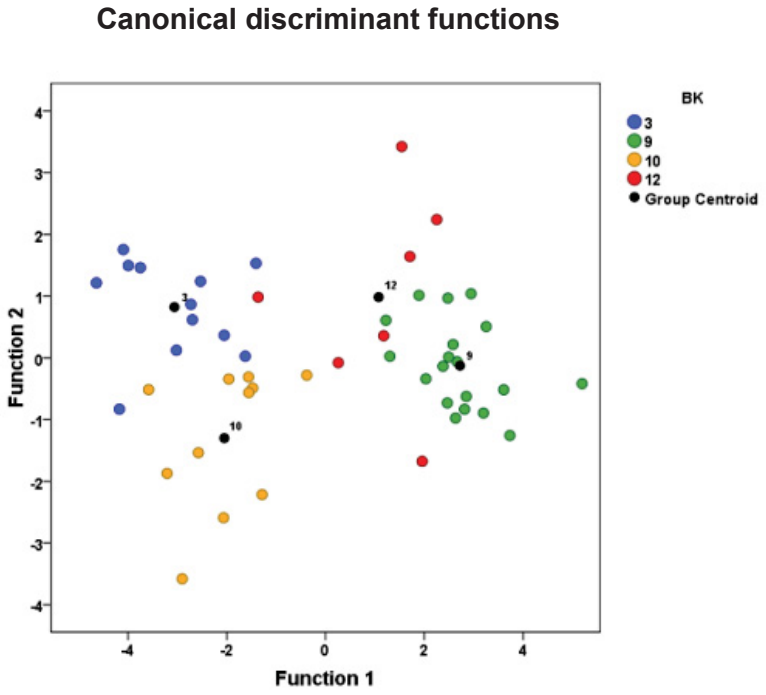

Fig.S3 Multivariate discriminant analysis of the AGS hydrocarbon profile composition of C_{25}- and C_{27}-type female beewolves based on GC-MS data (Wilks´ λ: 0.014, χ^2: 350.743, df=20, P<0.001).

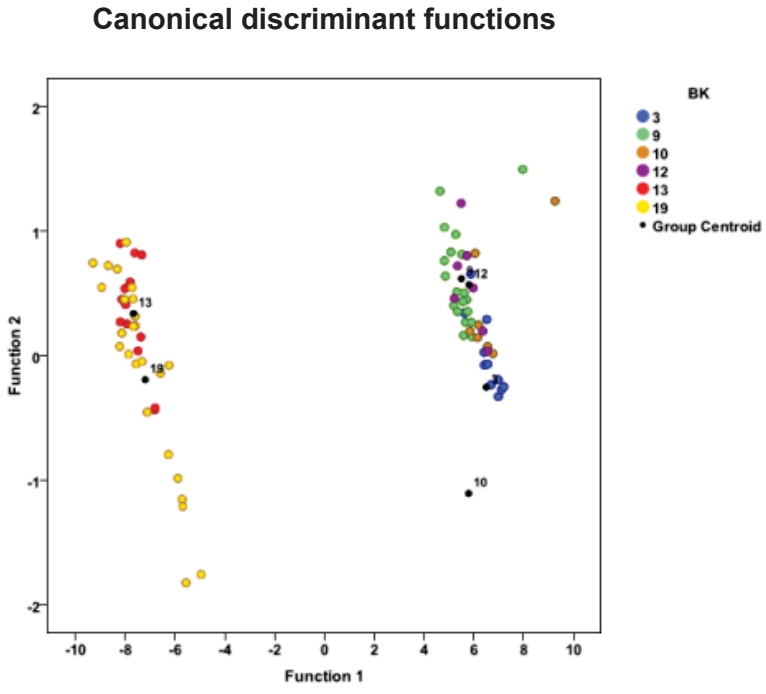

Fig.S4 Multivariate discriminant analysis of the AGS hydrocarbon profile composition of C_{25}-type female beewolves based on GC-MS data (Wilks´ λ: 0.066, χ^2: 118.172, df=15, P<0.001).

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CHAPTER 4

GUT EXCRETIONS OF EUROPEAN BEEWOLF LARVAE STIMULATE SYMBIONT-PRODUCED ANTIMICROBIAL PROTECTION

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4.1 ABSTRACT

Symbiotic microorganisms can provide a broad range of benefits to their insect hosts. Particularly associations for the exchange of essential nutrients between the host and its bacterial symbionts are most common and intensively studied. Moreover, microorganisms can affect the host´s hydrocarbon profiles which are essential for nestmate recognition, and they can provide compounds that are used by the host as pheromone components or defensive agents to protect the host, the host's nutritional resources and it´s offspring against predators, pathogens and parasitoids. In the solitary digger wasp, *Philanthus triangulum* (Hymenoptera, Crabronidae), symbiotic Actinobacteria of the genus *Streptomyces* provide protection against pathogenic microorganisms by producing various antimicrobial substances on larval cocoons during hibernation. The symbiont-produced substances appear to be highly stable and independent of environmental changes, providing a reliable long-term antimicrobial prophylaxis and, thereby, significantly enhancing the progeny´s survival probability.

Using GC-MS and RT-qPCR analyses, here we demonstrated that the beewolf larva inside the cocoon positively affects the amount of antibiotics, suggesting that the developing beewolf larva can actively enhance its own protection by the *Streptomyces* symbionts. Subsequent experiments suggested a role of the larval gut content in triggering antibiotic production upon its excretion onto the cocoon. Thus, host control over symbiont-mediated antibiotic production in the beewolf-*Streptomyces* symbiosis may be optimized towards enhancing the efficiency of an antimicrobial protection for the host progeny against pathogenic microorganisms, and thus, provides a unique model system to study fundamental questions on the regulation of symbiont-mediated secondary metabolite production in defensive insect-bacteria symbioses.

4.2 INTRODUCTION

Symbiotic microorganisms can provide a wide range of beneficial traits to their hosts (Margulis and Fester 1991; Douglas 1994; Moran and Baumann 2000; Bourtzis and Miller 2003; Douglas 2003; Kaltenpoth 2009) and recently significant progress has been made to understand molecular aspects of particularly mutualistic host-microbe associations. The most common and best-studied symbioses are interactions for the exchange of essential nutrients between the host and its symbionts (Margulis and Fester 1991; Schafer et al. 1996; Douglas 1998; Moran and Baumann 2000; Douglas 2003; Dillon and Dillon 2004; Zientz et al. 2004; Douglas 2006; Sabree et al. 2009; Salem et al. 2012), however, microorganisms have also been found to provide compounds that are used by the host as pheromone components (Dillon et al. 2000; Dillon et al. 2002), and can affect hydrocarbon profiles that are important for nestmate recognition (Matsuura 2003). Moreover, the symbionts can defend the host, the host's nutritional resources or its offspring against pathogens (Currie et al. 1999a; Currie et al. 2003; Kaltenpoth et al. 2005; Haine 2008; Brownlie and Johnson 2009; Kaltenpoth 2009; Hurst and Hutchence 2010; Kroiss et al. 2010) or parasitoids (Oliver et al. 2003; Moran et al. 2005), and the unique role of defensive characteristics in associations between hosts and their symbiotic bacteria has gained eminent scientific interest (Brownlie and Johnson 2009; Kaltenpoth 2009; Oliver and Moran 2009; Crotti et al. 2010; Ferrari and Vavre 2011), utilizing symbiotic systems as novel sources for natural product research (Bull and Stach 2007; Hochmuth and Piel 2009; Dharmaraj 2010; Poulsen et al. 2011; Qin et al. 2011; Park et al. 2012).

Especially symbiotic gut microorganisms have long been demonstrated to improve the host's resistance to pathogenic microorganisms in various insect species either by outcompeting pathogens (colonization resistance), exploiting the limited nutrients in the gut more efficiently (Godfray et al. 1999; Dillon and Dillon 2004), or via the production and release of chemical compounds with antibiotic properties (Dillon and Charnley 1995). Furthermore, it has been shown that the defensive symbionts can not only colonize the insect´s gut, but can be found in numerous other locations throughout the insect´s body. In leaf-cutting ants, Actinobacteria of the genus *Pseudonocardia* have been demonstrated to protect the ants' fungus garden against pathogenic fungi of the genus *Escovopsis* (Currie et al. 1999b; Oh et al. 2009a; Poulsen et al. 2010; Cafaro et al. 2011; Mattoso et al. 2012), and recent studies on this unique multipartite mutualism revealed several additional actinobacterial genera, notably *Streptomyces* and *Amycolatopsis*, that may also aid in the defense of the ants´ fungal cultivar by producing antimicrobial substances (Haeder et al. 2009; Barke et al. 2010; Schoenian et al. 2011). Likewise, fungus-growing bark beetles (*Dentroctonus frontalis)* have been shown to harbor *Streptomyces* bacteria in specialized organs, so-called mycangia. *In vitro* a symbiont-produced compound, mycangimycin, isolated from these bacteria has been shown to inhibit the growth of the pathogenic fungus *Ophiostoma minus*, suggesting that it provides protection to the beetle´s fungus garden against this antagonist (Scott et al. 2008; Oh et al. 2009b). Moreover, in a staphylinid beetle, *Paederus sabaeus,* symbiotic bacteria of the genus *Pseudomonas* produce a chemical substance, pederin, which has been shown to efficiently ward off the beetle´s predators (Kellner 2002; Liu et al. 2009), and aphids do not only live in a nutritional symbiosis with *Buchnera* bacteria (Douglas 1998), but they engage in various associations with secondary symbionts, enhancing the aphids' resistance to parasitoid wasps as well as pathogenic fungi (Oliver et al. 2003; Ferrari et al. 2004; Scarborough et al. 2005; Oliver et al. 2006; Oliver and Moran 2009; Oliver et al. 2010; Lukasik et al. 2013).

Female European beewolves of the genus *Philanthus* cultivate symbiotic Actinobacteria, facilitating a chemical defense to the wasp´s offspring against soil-dwelling microorganisms (Kaltenpoth et al. 2005; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). As any ground-nesting and mass-provisioning

insect, beewolves have to cope with soil microbes that not only threaten to decay the provision of the wasp´s progeny, but can also compromise the beewolf larva itself. Symbiotic bacteria of the genus *Streptomyces* (`*Candidatus* Streptomyces philanthi`) are cultivated in specialized antennal reservoirs of female beewolves and secreted into the brood cell as a white substance prior to oviposition (Kaltenpoth et al. 2005; Goettler et al. 2007). After the larva has hatched from the egg, it feeds on the remaining provision for several days before spinning a cocoon. During cocoon spinning, the larva incorporates the symbiotic *Streptomyces* into the cocoon walls, where they produce a cocktail of different antibiotic substances that provide protection against a wide range of pathogenic microorganisms (Kroiss et al. 2010), significantly enhancing the survival probability of the wasps progeny (Kaltenpoth and others 2005). Although antibiotic production by the symbionts is limited to a short phase of 1-2 weeks after cocoon spinning, the long-term stability of the antimicrobial compounds ensures protection for the beewolf offspring during the long phase of hibernation in the cocoon (Koehler et al. 2013).

In the present study we demonstrate that, in the larval stage, the beewolf host can influence the symbiont-mediated antibiotic production and thereby enhance its own protection against pathogenic microorganisms during the long and unpredictable phase of development. Two hypotheses may explain how the beewolf host influences antibiotic production in the *Streptomyces* symbionts. (i) The larva could apply compounds during the process of cocoon spinning, e.g. as a secretion from their oral glands, which could either serve as a nutritional resource or as a trigger for antibiotic production in the bacteria. (ii) Once inside the cocoon the larva could as well trigger antibiotic production in the symbionts, e.g. via excretions from their gut. Elevated amounts of antibiotics, however, may ward off soil pathogens more efficiently, and thus, ensure a successful development of the beewolf offspring. Additionally, high levels of antibiotics may provide the bacteria with competitive superiority over future antagonists on the cocoon, thereby increasing the chances for the next beewolf generation to successfully acquire the symbiotic bacteria. Thus, host control over antibiotic production may be essential to guarantee an efficient antimicrobial protection for the host progeny against pathogenic microoranisms, and to facilitate the successful transmission of the bacterial symbionts to the next generation of beewolf wasps.

4.3 MATERIALS AND METHODS

4.3.1 Sample preparation

Influence of the beewolf larva

To investigate a potential role of the developing beewolf larva inside the cocoon on symbiont-mediated antibiotic production, two treatment groups were established containing cocoons with and without beewolf larva, respectively. Male beewolf cocoons were taken from the females´ nests at day 2 (N=16) and 4 (N=22) after cocoon spinning, respectively, and prepared according to Koehler and Kaltenpoth (2013). Half of the cocoons were longitudinally cut, and the larvae were carefully taken out using forceps. After removing the beewolf larvae, the empty cocoons were placed in a 37 ml plastic jar (Solo® Cup Company, Highland Park, IL, USA), transferred to the incubator and kept at 25°C (+/- 2°C) under humid conditions until day 8 after cocoon spinning, since high amounts of antibiotics were previously detected on beewolf cocoons after eight days (Koehler et al. 2013). In the control group, the cocoons were kept intact, with the larva inside, but were otherwise treated the same way. Subsequently, the samples were frozen at -80°C for future analyses.

Influence of larval gut content

Observing a significantly higher amount of antibiotics on cocoons with larva as compared to cocoons without larva, we experimentally tested the influence of the pure larval gut excretion as well as several fractions of the larva´s gut content on the production of symbiont-mediated antibiotic substances. First, male cocoons (Koehler and Kaltenpoth 2013) were taken from the females´ nests two days after cocoon spinning, and the larva was removed from the cocoons as described above. To reduce the variance in the samples and to establish equal conditions, we then partitioned the cocoons in two pieces and subsequently used one piece each for treatment and control, respectively (Table 4.1). The cocoon pieces were placed in experimental jars (Koehler and Kaltenpoth 2013) in the incubator at 25°C (+/- 2°C). Subsequently, the larval gut was dissected from the removed larva and kept in the fridge at 6-7°C. After 3 days (i.e. at day 5 after cocoon spinning), the gut content was taken out of the fridge, re-suspended in 10 µl millipore water and applied as a whole (approx. 20 µl) to the attachment site of one cocoon half. The second half was kept as a control group alongside with the treatment group, but instead of the gut content, 20 µl of millipore water was added. Finally, samples and controls were frozen at -80°C at day 8 after cocoon spinning.

In addition to the whole gut content of the beewolf larva, individual fractions as well as other solutions/suspensions were experimentally tested (Table 4.1) in order to elucidate potential factors in the larval gut excretion that might affect symbiont-mediated antibiotic production. The different solutions/suspensions were added as follows: (i) pelleted fraction of the larva´s gut content after centrifugation and removal of the supernantant, (ii) supernatant of the larva´s gut content after centrifugation and removal of the pellet, iii) heat treated gut content of beewolf larvae, (iv) whole gut content of adult honey bee workers (*Apis melifera*), (v) supernatant of adult honey bee gut, and (vi) a *Streptomyces* culture medium. For the preparation of the different solutions and suspensions and the application to the cocoons refer to Table 4.1.

The cellular fraction was used to test whether competition may trigger antibiotic production in the *Streptomyces* symbionts as has been suggested for different *Streptomyces* spp. *in vitro* (Wiener 1996; Slattery et al. 2001). Also, dissolved compounds in the larva´s gut content, either host derived or produced by the gut microbiota, could influence the production of antibiotics, and given the strong effect of the complete gut content the fractionation into pellet and supernatant should retain the effect in one of the fractions.
To further elucidate potential components of the gut excretion that may be responsible for the observed effect we used heat treatment of the larval gut content to inactivate heat labile compounds such as proteins. Here we partitioned the cocoons in three pieces and added heat treated and untreated larval gut content to a single piece of beewolf cocoon, respectively. The third cocoon piece was used as a water control as described before (Table 4.1).

By adding whole gut as well as the gut supernatant of adult honey bee workers, we analyzed whether the effect on antibiotic production in the *Streptomyces* symbionts is beewolf specific or could as well be induced by the gut content of other insect species. Honey bees are comparatively closely related to beewolf wasps, and recent results on the beewolf gut microbiota indicated that the microbial community in the gut of beewolf larvae is highly similar to the honey bee microbiota (Koehler and Rani, unpublished data). Thus, the results would allow us to draw some preliminary conclusions on whether the causing agent for the observed effect on symbiont-mediated antibiotic production in the *Streptomyces* bacteria might derive from the host or from its gut microbiota.

Finally, the addition of the medium to beewolf cocoons could help to discriminate whether the detected increase in antibiotics is due to a growth effect of the *Streptomyces* symbionts, resulting in an

elevated amount of antibiotics, or results from a trigger stimulating antibiotic production in the bacteria.

Table 4.1 Different experimental treatments to elucidate host influence on symbiont-mediated antibiotic production on beewolf cocoons.

Substance added	Treatment – Cocoon piece 1	Treatment – Cocoon piece 2 (Control)	Treatment - Cocoon piece 3
Whole larval beewolf gut (N=12)	• gut suspension with 20µl millipore H_2O • 20µl of suspension added to the cocoon	20µl millipore water	-
Cellular fraction of the larval beewolf gut (N=9)	• gut suspension with 20µl millipore H_2O • centrifugation with 14.000 rpm for 1 min. • wash pellet 2-3 times with millipore H_2O (same centrifugation intensity) • re-suspend pellet in 20µl millipore H_2O • add 20µl of suspension to beewolf cocoon	20µl millipore water	-
Dissolved fraction of the larval beewolf gut (N=9)	• gut suspension with 20µl millipore H_2O • centrifugation with14.000 rpm for 1 min. • Transfer supernatant to new cup • Centrifuge again, 14.000 rpm • Add 20µl of supernatant to beewolf cocoon	20µl millipore water	-
Heat-treated whole larval beewolf gut (N=10)	• gut suspension with 40µl millipore H_2O • partition in two aliquots • aliquot 1: 95°C, 10 min. (heat treatment) • add 20µl to cocoon after cool down	20µl millipore water	• aliquot 2: untreated gut • add 20µl to cocoon
Whole adult honey bee gut (N=7)	• suspension of honey bee gut with 20µl millipore H_2O • 20µl of suspension added to the cocoon	20µl millipore water	-
Dissolved fraction of adult honey bee gut (N=11)	• gut suspension with 20µl millipore H_2O • centrifugation with14.000 rpm for 1 min. • Transfer supernatant to new cup • Centrifuge again, 14.000 rpm • Add 20µl of supernatant to beewolf cocoon	20µl millipore water	-
Medium (N=12)	• Add 20µl of medium to cocoon	20µl millipore water	-

4.3.2 Extraction of antibiotic substances from beewolf cocoons

Frozen (−80°C) cocoon pieces were thawed on ice and subsequently transferred to GC-MS vials (1.5 ml vials, 6 mm jar opening, CZT, Kriftel, Germany) using forceps. Forceps were cleaned with methanol and hexane in between samples to prevent contamination. Afterwards, 500 µl of methanol (>99.9%, Roth, Karlsruhe, Germany) were added to each sample, and the samples were placed on a shaker (Heidolph Vibramax 100) for 1 h at 350-400 rpm to extract antibiotics (Koehler et al. 2013). Subsequently, the cocoons were taken out of the GC-MS vials, placed on a tissue to drain remaining methanol, and finally transferred to clean 1.5 ml cups (Eppendorf). Cocoon samples were then placed under the hood for approximately 1 h to evaporate the remaining methanol, and subsequently frozen at -20°C for molecular analyses. The methanol extracts were stored at −20°C for subsequent GC-MS analysis.

4.3.3 Quantification of antibiotics using GC-MS

Methanol extracts of the antibiotic substances from beewolf cocoons were evaporated to dryness under a gentle stream of Argon, re-suspended in 50 µl of methanol, and subsequently, transferred to a 150 µl GC-µ-vial (CZT, Kriftel, Germany) for gas chromatography – mass spectrometry (GC-MS) analysis as described previously (Koehler et al. 2013, Koehler and Kaltenpoth 2013). Briefly, an aliquot of 1 µl per sample was injected into a Varian 450GC gas chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies, Böblingen, Germany) using a split/splitless injector at 250°C with the purge valve opened after 60 s. The GC was equipped with a DB5-MS capillary column (30 m x 0.25 mm diameter, film thickness: 0.25 µm, Agilent Technologies) and programmed from 150 to 300°C at 5°C/min with a 1 min. initial isothermal and a 5 min. final isothermal hold. As carrier gas, Helium was used, with a constant flow rate of 1 ml/min. Mass spectra were recorded using electron ionization (EI-MS). Data acquisition and quantifications were conducted using MS Workstation Version 6.9.3 Software (Agilent Technologies). Using this method, the three most abundant antimicrobial compounds on beewolf cocoons were quantified, i.e. streptochlorin (SC), piericidin A1 (PA1), piericidin B1 (PB1) (Kroiss et al. 2010). As an external calibration standard, a dilution series (500–0.1 ppm) of commercially available piericidin A1 was used for the two different piericidin derivatives, assuming similar ionization efficiencies based on the high structural similarity. For the quantification of streptochlorin, a dilution series of a synthesized streptochlorin standard (for the synthesis of streptochlorin see Koehler et al. (2013)) was used. The peaks were identified by comparison of their mass spectra with the standard spectra or with published reference spectra (Kroiss et al. 2010), and peak areas were automatically integrated using the MS Workstation Software. After automated integration of the antibiotic peaks, the success of this integration was controlled manually for every peak.

4.3.4 Quantification of *Streptomyces* bacterial cells

In order to control for differences in symbiont population sizes across cocoon pieces, we extracted total DNA from cocoon samples for quantification of symbiont 16S rRNA gene copy numbers via qPCR. Therefore, the beewolf cocoon pieces were homogenized under liquid nitrogen followed by DNA extraction using the Epicentre MasterPure™ DNA extraction kit (Epicentre Technologies, Madison, USA). The protocol was adjusted as follows: the lysozyme treatment was omitted, and all centrifugation steps were performed at room temperature. The DNA pellet was re-suspended in 100

µl nuclease-free low-TE buffer (1 mM Tris/HCl, 0.01 mM EDTA) and subsequently stored at −80°C.
We used quantitative PCR (qPCR) analyses with diagnostic primer pairs to quantify the *Streptomyces* population size on beewolf coccon pieces (16S rRNA gene copy number, Kaltenpoth et al. 2010a). QPCR was performed on a Rotor-Gene Q cycler (Qiagen, Germany) in a total reaction volume of 25µl containing the following reagents: 6.5 µl nuclease-free water and 12.5 µl SYBR-Mix (Rotor-Gene SYBR Green RT-PCR kit, Qiagen, Hilden, Germany); 2.5 µl of each primer (10 µM), 1 µl of template. The following cycle parameters were used: 95°C for 10 min, 45 cycles of 95°C for 10 s, 65°C for 20 s, and a final melting curve analysis was performed by increasing the temperature from 65 to 99°C with 1°C gain. Using a dilution series of 10^{-1} to 10^{-7} ng/µl as a standard curve, the total amount of DNA was calculated based on the qPCR threshold values using the absolute quantification method (Lee et al. 2006; Lee et al. 2008).

4.3.5 Statistical analysis

The amount of antibiotics measured by GC-MS analysis as well as the symbiont 16S copy numbers obtained from the qPCRs were compared across treatments using Wilcoxon signed rank tests, since the samples did not follow a normal distribution. To investigate the effect of the larval gut excretion and other suspensions/solutions on symbiont-mediated antibiotic production, we partitioned individual cocoons in two (or three, for the heat-treated gut content experiment) parts. Given this experimental setup with dependent samples, repeated measures ANOVA (heat treatment experiment) or paired t-tests (all other experiments) were used to compare antibiotic amounts and symbiont population sizes across treatments. To account for the variability in size of the different cocoon parts and the ensuing effect of differences in symbiont population sizes on antibiotic production, the amount of antibiotics was normalized with the *Streptomyces* 16S copy number for each cocoon piece. All statistical analyses were performed using SPSS 17.0 Software (IBM, New York, USA).

4.4 RESULTS

4.4.1 Influence of the beewolf larva on symbiont-mediated antibiotic production

To elucidate the influence of the host larva on symbiont-mediated antibiotic production in the beewolf-*Streptomyces* symbiosis, we dissected the larva out of beewolf cocoons at two time points after cocoon spinning and quantified the amount of antibiotics as well as the number of symbiont 16S gene copies on the cocoons. When dissected out of the cocoon at day 2 after cocoon spinning, the amount of antibiotics was significantly higher (83% on average) in the presence of the larva as compared to cocoons without larva (Figure 4.1a, Wilcoxon signed ranks test: Z=-2.12, P=0.03). However, when the larva was removed from the cocoon at day 4 after cocoon spinning, the effect vanished (Figure 4.1b, Wilcoxon signed ranks test: Z=-0.47, P=0.64). The number of *Streptomyces* cells on the cocoon did not differ significantly between cocoons with larva as compared to the cocoons without for both experiments (day 2: Wilcoxon signed ranks test: Z=-0.97, P=0.33; day 4: Wilcoxon signed ranks test: Z=-1.41, P=0.16).

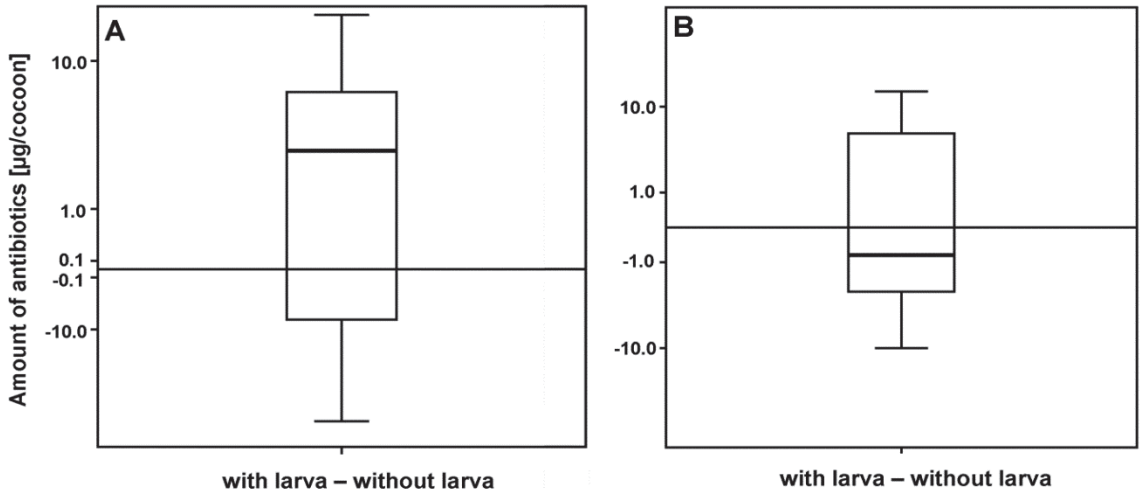

Fig. 4.1 Difference in antibiotic amount of cocoons with larva minus cocoons without larva, displayed on log scale, **A)** dissection of the larva from the cocoon 2 days after cocoon spinning (Wilcoxon signed ranks test: Z=-2.12, P=0.03), **B)** dissection of the larva from the cocoon at day 4 after cocoon spinning (Wilcoxon signed ranks test: Z=-0.47, P=0.64). Bold lines represent medians, boxes comprise the interquartile range and bars indicate the minimum and maximum values.

4.4.2 Influence of the beewolf larva´s gut content on symbiont-mediated antibiotic production

To investigate which factors are responsible for the increase of symbiont-mediated antibiotic production in the presence of the beewolf larva, we first focused on the larval gut content, as it is excreted onto the cocoon about three to four days after cocoon spinning, thus coinciding with the larval effect on antibiotic production (Kaltenpoth and Koehler, personal observation). To account for the variability in size of the different cocoons and cocoon parts and the effect of differences in

symbiont population sizes on antibiotic production, the amount of antibiotics was normalized with the *Streptomyces* 16S copy number for each cocoon and cocoon part, respectively.

Our analysis revealed that the amount of antibiotics (standardized with the *Streptomyces* 16S copy number) was significantly higher on cocoons with larval gut content as compared to the water treated control (Figure 4.2, t-test for dependent samples: t=-3.07, df=11; P=0.01). Furthermore we tested the effect of different fractions of the larval gut excretion in order to elucidate potential factors responsible for the observed effect. The cellular fraction of the gut content, containing the larva´s gut microbiota as well as remaining cells of the larva´s gut wall, did not affect the production of antibiotics in the *Streptomyces* symbionts (Figure 4.3a, t-test for dependent samples: t=-0.43, df=8, P=0.68). Although the supernatant of the beewolf larva´s gut, containing dissolved compounds such as proteins, also did not show a significant difference between cocoon halves that received the treatment as compared to the control halves, there was a strong tendency towards an increased antibiotic amount after application of the beewolf gut supernatant (Figure 4.3b, t-test for dependent samples: t=2.17, df=8, P=0.06).

Moreover, we tested the effect of heat-treated beewolf gut contents on the symbiont-mediated antibiotic production. The analysis revealed a significant difference between the heat-treated beewolf gut content and the non-treated gut content. By contrast, there was no difference between the heat treated gut content and the control (Figure 4.3.c, Wilks´ λ=0.18, F=18.2, df=2, P<0.01). Thus, heat treatment abolished the positive effect of the larval gut content on antibiotic production.

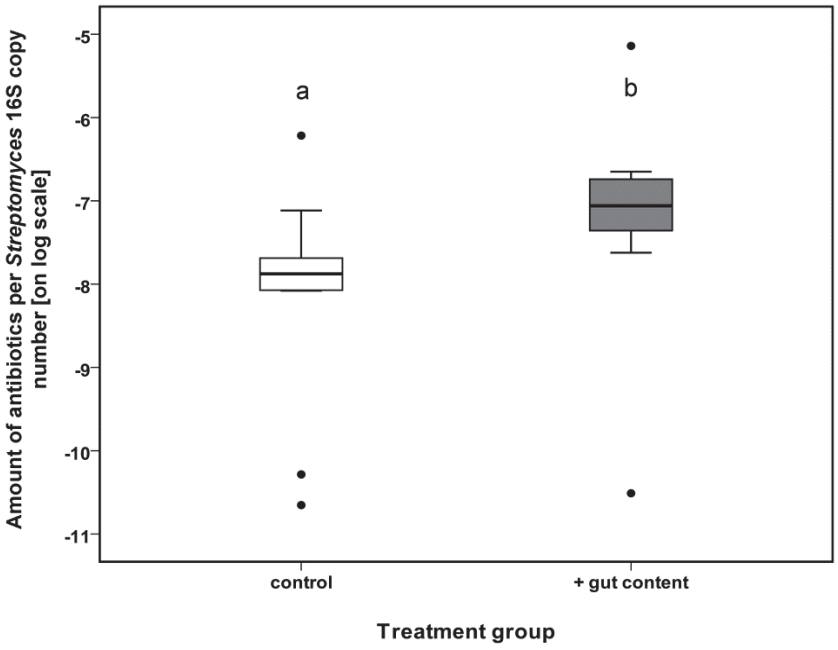

Fig. 4.2 Influence of the beewolf gut content on symbiont-mediated antibiotic production. The amount of antibiotics was normalized with the *Streptomyces* 16S copy numbers, data shown on log scale. (t-test for dependent samples: t=-3.07, df=11, P=0.01). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers. Different letters above boxes denote significant differences (P<0.05).

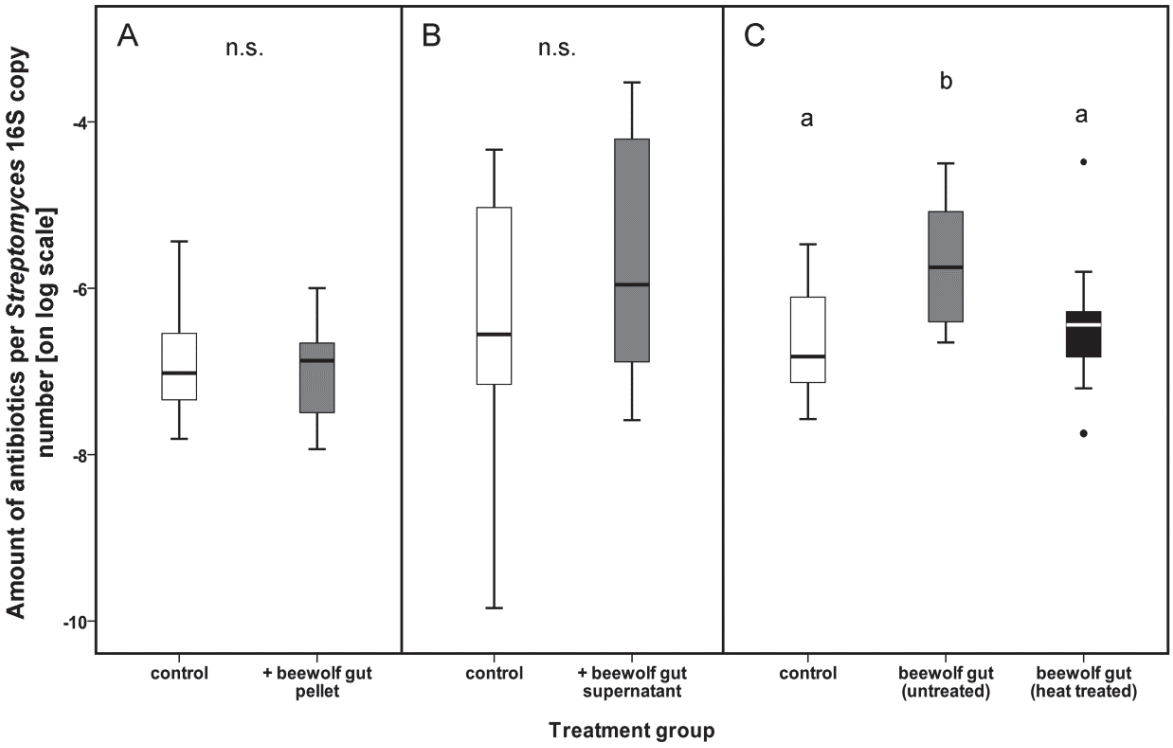

Fig. 4.3 Influence of the beewolf gut content on symbiont-mediated antibiotic production. The amount of antibiotics was normalized with the *Streptomyces* 16S copy numbers, data shown on log scale. **A)** Influence of the cellular fraction of the beewolf´s gut content (pellet) (t-test for dependent samples: t=-0.43, df=8, P=0.68), **B)** influence of the dissolved fraction of the beewolf´s gut content (supernatant) (t-test for dependent samples: t=2.17, df=8, P=0.06), **C)** influence of heat treated gut content on antibiotic production (Wilks´ λ=0.18, F=18.2, df=2, P<0.01). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers. Different letters above boxes denote significant differences (P<0.05).

4.4.3 Influence of the honey bee´s gut content on symbiont-mediated antibiotic production

To test for the specificity of the observed effect of beewolf larval gut content on symbiont-mediated antibiotic production, we added the gut content of adult honey bee workers (*Apis melifera*) to beewolf cocoons (Figure 4.4). For both the whole honey bee gut (Figure 4.4a, t-test for dependent samples: t=2.82, df=6, P=0.03) and for the supernatant of the gut content (Figure 4.4b, t-test for dependent samples: t=4.67, df=10, P<0.01), the amount of antibiotics was significantly higher on cocoons receiving treatment as compared to the water-treated controls.

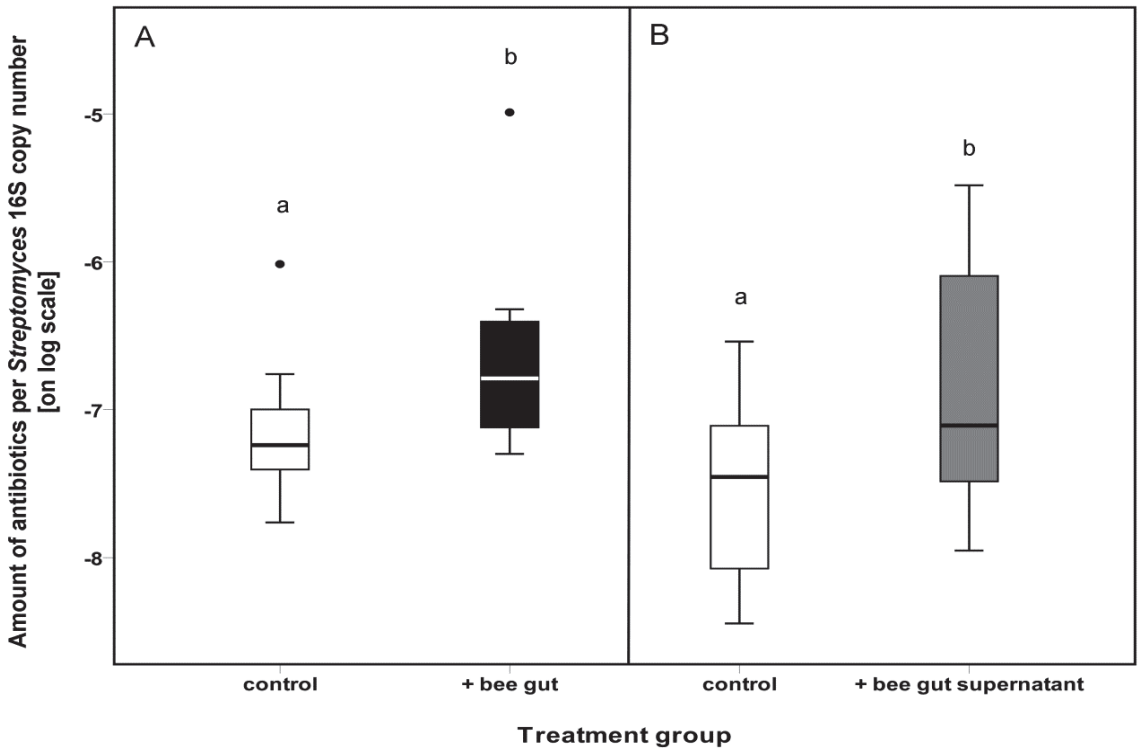

Fig. 4.4 Influence of the gut content of adult honey bee workers (*Apis melifera*) on symbiont-mediated antibiotic production on beewolf cocoons. The amount of antibiotics was normalized with the number of *Streptomyces* 16S copy numbers, data shown on log scale. **A)** Influence of whole honey bee gut (t-test for dependent samples: t=2.82, df=6, P=0.03), **B)** influence of the dissolved fraction of the honey bee´s gut content (supernatant) (t-test for dependent samples: t=4.67, df=10, P<0.01). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers. Different letters above boxes denote significant differences (P<0.05).

4.4.4 Influence of the *Streptomyces* culture medium on symbiont-mediated antibiotic production

Finally, to elucidate a potential nutritional effect responsible for the increase in the amount of antibiotics produced by the *Streptomyces* symbionts, we investigated the influence of a *Streptomyces* culture medium on the symbiont-mediated antibiotic production. Our results revealed that the amount of antibiotics was significantly higher on cocoon halves that received the medium as compared to the control (Figure 4.5a, t-test for dependent samples: t=3.43, df=11, P<0.01). However, the number of *Streptomyces* bacterial cells did not differ between the treatment and the control group (Figure 4.5b, t-test for dependent samples: t=-1.75, df=11, P=0.11), refuting the hypothesis that the increased amount of antibiotics originates from higher bacterial numbers on the cocoons.

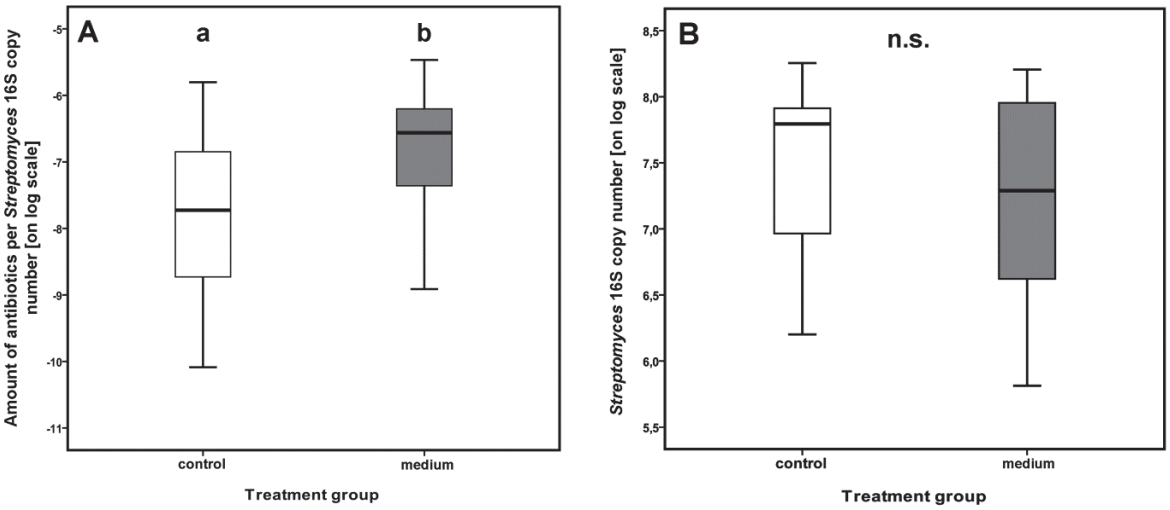

Fig. 4.5 Influence of adding *Streptomyces* culture medium to beewolf cocoons on symbiont-mediated antibiotic production and bacterial growth. **A)** Amount of antibiotics on beewolf cocoon halves normalized with the number of *Streptomyces* 16S copies, data shown on log scale (t-test for dependent samples: t=3.43, df=11, P<0.01), **B)** *Streptomyces* 16S copy number on beewolf cocoon halves (t-test for dependent samples: t=-1.75, df=11, P=0.11). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers. Different letters above boxes denote significant differences (P<0.05).

4.5 DISCUSSION

4.5.1 Influence of the beewolf larva on symbiont-mediated antibiotic production

Streptomyces produce a wide range of antibiotic compounds which have been shown to provide advantages over microbial competitors in nutrient-limited environments (Rasool and Wimpenny 1982; Turpin et al. 1992; Sanchez and Brana 1996; Slattery et al. 2001). However, in symbiotic associations, they can also provide a competitive advantage to macroorganisms that utilize the antimicrobial properties of their symbionts for means of protection (Currie et al. 1999a; Kaltenpoth et al. 2005; Scott et al. 2008; Haeder et al. 2009). Despite their importance as producers of defensive agents in symbioses, however, knowledge about the factors affecting symbiont-mediated antibiotic production *in vivo* is still scarce.

For many streptomycetes a complex program of regulation governs gene expression during morphological differentiation and the production of antibiotic substances (Champness and Chater 1994; Bibb 1996; Bibb 2005; van Wezel and McDowall 2011). Generally, the genes for the production of individual secondary metabolites are arranged in clusters that vary in size from a few to over 100 kb (Schwecke et al. 1995; Bentley et al. 2002; Ikeda et al. 2003). Most but not all of these clusters contain pathway-specific regulatory genes whose expression frequently depends on genes that are required for the production of several secondary metabolites made by the strain. Linking primary and secondary metabolism with growth and morphological development, many of these genes have been found to be nutritional regulators (van Wezel and McDowall 2011). The availability and source of carbon can have a substantial effect on the production of antibiotics and morphological development in *Streptomyces* (e.g. Ruiz et al. 2010; Sanchez et al. 2010; van Wezel and McDowall 2011). Additionally, many studies provided evidence that the nitrogen source can influence antibiotic production (Merrick and Edwards 1995; Sanchez and Demain 2002; Oskay 2011; van Wezel and McDowall 2011), and trace elements such as zinc and iron have as well been identified to affect the secondary metabolim of streptomycetes (see van Wezel and McDowall 2011). For defensive symbiotic associations, however, these regulatory check points may provide potential ways to control symbiont-produced substances, and thus, influence beneficial traits provided by the bacteria.

In the beewolf-*Streptomyces* symbiosis, the bacteria-produced antibiotics have been shown to protect the wasp offspring against pathogen attack during development (Kaltenpoth et al. 2005; Kroiss et al. 2010), and recent surveys revealed that the symbiont-mediated antibiotic production in the *Streptomyces* bacteria is not affected by environmental parameters such as temperature, humidity, or pathogen pressure (Koehler and Kaltenpoth 2013). However, our current analyses revealed a significant effect of the beewolf host on the production of antibiotic substances by the *Streptomyces* symbionts. In particular, we found that cocoons containing a beewolf larva showed significantly higher amounts of antibiotics as compared to control cocoons, from which the larva had been removed two days after cocoon spinning (Figure 4.1a). We hypothesized that an excretion from the beewolf larva, most likely from the larva´s gut, may trigger the antibiotic production in the symbiotic bacteria which are incorporated in the cocoon walls. Support for this hypothesis was provided by a subsequent experiment where we removed the larva from its cocoon only after the gut contents was excreted, which usually happens between day 4-5 after cocoon spinning. The late removal of the larva (at day 4 after cocoon spinning) abolished the positive effect on symbiont-mediated antibiotic production (Figure 4.1b). Moreover, we manually applied a crude larval gut suspension to cocoons

from which the larva had previously been removed, and the results show elevated amounts of antibiotics on these cocoons as compared to the controls (Figure 4.2).

There are several possibilities of how the beewolf´s gut excretion may influence the production of antibiotics in the *Streptomyces* cells. (i) The bacterial gut community of the beewolf larva´s gut triggers antibiotic production in the symbionts. (ii) Host-derived compounds in the beewolf larva´s gut trigger antibiotic production in the *Streptomyces* symbionts. (iii) Nutrients which are supplied to the cocoon with the gut content facilitate bacterial growth of the *Streptomyces* cells, resulting in an increased antibiotic amount.

To test these hypotheses, we first fractioned the larval gut content into a cellular fraction and a fraction containing dissolved components of the gut, separately added these fractions to beewolf cocoons, and subsequently, quantified the amount of antibiotics. The cellular fraction was used because prior studies on different *Streptomyces* spp. demonstrated *in vitro* that the amount of antibiotics produced by the bacteria can increase in the presence of competitors (Sanchez and Brana 1996; Wiener 1996; Slattery et al. 2001). However, our results revealed no significant effect of the cellular fraction of the larval gut suspension (Figure 4.3a), which is in accordance with previous studies finding no influence of competing fungi on symbiont-mediated antibiotic production in the beewolf symbiosis (Koehler and Kaltenpoth 2013).
Surprisingly, there was no significant effect of the larva´s gut supernatant on antibiotic production either. However, as demonstrated in this study, there is a strong effect of the whole beewolf larval gut content (Figure 4.2). Thus, the fractionation into pellet and supernatant by centrifugation should retain this effect in one of the fractions. Given that the pelleted fraction did not reveal a significant effect on symbiont-mediated antibiotic production (Figure 4.3a), the effect, observed by addition of the whole larval gut suspension, should be mediated by a dissolved component(s) in the supernatant. Indeed, cocoons treated with the larva´s gut supernatant showed a tendency towards higher amounts of antibiotics as compared to the control (Figure 4.3b). Possible explanations for the lack of significance may be the small sample size. However, the sample size was comparable across the different experiments, and significant effects have been detected for various treatments. Thus, it is more likely that due to the repeated centrifugation and washing procedure, we lost some of the activity, which may have impaired the effect of the supernatant on symbiont-mediated antibiotic production.
Moreover, heat treatment of the larval gut suspension abolished the effect on symbiont-mediated antibiotic production, indicating that heat-labile components are responsible for the observed effect (Figure 4.3c). Thus, the results already diminish potential factors that may stimulate the antibiotic production in the *Streptomyces* symbionts. As mentioned above, many studies have shown that the source of carbon or nitrogen can influence the production of antibiotics in *Streptomyces* (e.g. Merrick and Edwards 1995; Ruiz et al. 2010; Sanchez et al. 2010; Oskay 2011; van Wezel and McDowall 2011). Carbon sources such as glucose, however, appear to be stable at high temperatures (Robyt 1998), suggesting that they would still be available in the gut suspension even after heat treatment. Thus, heat labile substances such as proteins which are denatured when treated with high temperatures are more likely canditates for the stimulation of symbiont-mediated antibiotic production.

4.5.2 Influence of the honey bee´s gut content on symbiont-mediated antibiotic production

With regard to the positive influence of the beewolf larval gut on symbiont-mediated antibiotic production we examined whether this effect is specific to the beewolf host. Therefore, we used a gut suspension of honey bee workers (*Apis melifera*) and applied the suspension as well as the dissolved fraction of the honey bee gut (supernatant), respectively, to beewolf cocoons. Recent results indicated that the gut microbiota of beewolf larvae in different life stages is highly similar to the honey bee microbiota (Koehler and Rani et al, unpublished data), which seems plausible given that beewolves feed on paralyzed honey bees in the larval stage. However, it is not yet known whether these microbes are transient or constitute a resident "core" microbiota of the beewolf wasps (Koehler and Rani et al. unpublished data).
Surprisingly, our analyses revealed that antibiotic production is stimulated by the whole honey bee gut suspension as well as by the dissolved fraction of the honey bee gut (Figure 4.4), indicating that the effect on *Streptomyces*-produced antibiotics appears to be non-specific with regard to the beewolf host. Two possible hypotheses may explain this phenomenon. (i) Either the substance(s) which trigger(s) antibiotic production in the symbionts is/are produced by the host, yet not specific to beewolf wasp but rather generally produced throughout the hymenopteran order, or (ii) the compound(s) is/are produced by the insect´s gut microbiota.

4.5.3 Influence of the *Streptomyces* culture medium on symbiont-mediated antibiotic production

To test for a general growth effect of the larval excretions on the symbiotic bacteria we added *Streptomyces* culture medium to beewolf cocoons and quantified the amount of antibiotics as well as the *Streptomyces* population size. Previous studies already showed a positive correlation between the symbiont number and the amount of antibiotics produced (Koehler and Kaltenpoth 2013). Hence, an increased supply with nutrients may result in an enhancement of bacterial growth, which would be reflected in an increased production of antibiotics on beewolf cocoons.
Our analysis revealed a positive effect of the medium on symbiont-mediated antibiotic production (Figure 4.5a), however, there was no growth stimulation of the symbiotic bacteria with regard to the *Streptomyces* medium (Figure 4.5b). Likewise, the *Streptomyces* population size did not differ with addition of any of the aforementioned suspensions and solutions to beewolf cocoons (t-test for dependent samples: $p>0.05$ for all experiments, data not shown), despite the positive effect on antibiotic production. Given the fact that the bacteria undergo morphological differentiation on the cocoon shortly after their application by the larva (Koehler et al. 2013), our results suggest that the nutrients supplied cannot be used for growth in this phase, but may be directly channeled into antibiotic production instead (Mendez et al. 1985; Bibb 2005; Rigali et al. 2008; van Wezel and McDowall 2011).

Further experiments are necessary to identify underlying mechanisms and/or the key component(s) which is/are responsible for the enhancing effect on symbiont-mediated antibiotic production in *Streptomyces* symbionts. Assuming that the trigger is the same for the larval gut content and the medium, potential substances would already be limited to ~ 50 different compounds, distributed over four substance classes. Currently, our data suggests a heat labile, dissolvable substance which appears to be beewolf unspecific, and based on previous results, reasonable candidates may be proteins or vitamins, pointing to an involvement of the host gut microbiota in this effect. Moreover, recent experiments *in vitro*, yielded an increased amount of antibiotics after addition of single components of

the *Streptomyces* medium, e.g. biotin. While a solution of all vitamins seemed to inhibit antibiotic production in the streptomycetes, biotin alone lead to an increase in the symbiont-produced antibiotic substances (Nechitaylo et al. unpubl data). Interestingly, insects cannot produce biotin *de novo* and genomic analyses of the *Streptomyces* biosynthetic pathways revealed that, among others, the symbionts lack the pathway for biotin (Nechitaylo et al, unpubl data). Thus, the beewolf gut microbiota may take part in the production of a compound that triggers antibiotic production in the *Streptomyces* symbionts. However, the final candidate compound has not been identified yet, and further bioassay-guided experiments are necessary to disentangle the potential role of different players participating in the positive effect on symbiont-mediated antibiotic production. The first step for future experiments may be to eliminate groups of substances as well as single compounds in the medium or even in the larval gut using bioassay-guided fractionation, and evaluate the effect of these suspensions/solutions on the *Streptomyces*-mediated antibiotic production *in vitro*, before subsequently putting it into context with the natural conditions in the beewolf larva´s gut.

In summary, the data presented in this study provides strong evidence for host control over symbiont-mediated antibiotic production, either via compounds produced by the host itself or the host´s microbiota, which aims at increasing the efficiency of the symbiont-provided defense for the wasp offspring as well as successful acquisition of the bacterial symbionts by the next generation of beewolf wasps. Hence, the beewolf-*Streptomyces* symbiosis provides an ideal case study to address fundamental questions on host regulation and -control over symbiont-mediated antibiotic production in beneficial insect-bacteria associations.

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CHAPTER 5

LIFE CYCLE AND POPULATION DYNAMICS OF A PROTECTIVE INSECT SYMBIONT REVEAL SEVERE BOTTLENECKS DURING VERTICAL TRANSMISSION

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5.1 ABSTRACT

Insects engage in mutualistic relationships with a wide variety of microorganisms that are usually transmitted vertically to the next generation. During transmission, the symbiont populations often suffer significant bottlenecks that may entail major genetic and genomic consequences. Here we investigated the life-cycle and the severity of transmission bottlenecks in a symbiotic system with an unusual way of post-hatch vertical transmission by using quantitative PCRs and morphological 3D-reconstructions. European beewolves (*Philanthus triangulum*, Hymenoptera: Crabronidae) harbor symbiotic bacteria ('*Candidatus* Streptomyces philanthi') in specialized antennal gland reservoirs and secrete them into their subterranean brood cells. The symbionts are later taken up by the beewolf larva and incorporated into the cocoon material to provide protection against pathogenic microorganisms. Even after months of hibernation, the symbiont population on the cocoon is estimated to encompass around 1.4×10^5 cells. However, our results indicate that only few of these bacterial cells (about 9.7×10^2) are taken up from the cocoon by the emerging female. The symbiont population subsequently undergoes logistic growth within the antennal gland reservoirs and reaches a maximum of about 1.5×10^7 cells 3–4 days after emergence. The maximum specific growth rate is estimated to be 0.084–0.105 h^{-1}. With a total reduction in cell numbers of about 6.7×10^{-5} during vertical transmission, the symbiont population experiences one of the most severe 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.

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). Recently, however, several cases of defensive alliances have been described in which the symbionts confer protection to their hosts against pathogens or parasitoids (e.g. Currie et al. 1999; e.g. Oliver et al. 2003; Kaltenpoth et al. 2005; Scott et al. 2008). Although a case of environmental uptake of symbionts in each host generation has 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; Pais et al. 2008). Many Hemipteran 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 (Buchner 1965; Prado et al. 2006; Kikuchi et al. 2009), coprophagy (Buchner 1965), or the deposition of specialized symbiont-containing capsules (Fukatsu and Hosokawa 2002; Hosokawa et al. 2005). 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, with factors of the reduction in cell numbers ranging from 0.3 to about 5×10^{-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 grampositive 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 unique gland reservoirs that are located in five segments of the antennae of female beewolves (Goettler et al. 2007). The reservoirs constitute invaginations of the outer cuticle that are surrounded by numerous gland cell units and sealed by a membranous flap mechanism (for details see Goettler et al. 2007). Reservoirs are very flexible in volume and the filling status varies within a broad range depending on the number of bacteria (W. Goettler et al. unpubl. data). The

antennal symbionts are transmitted vertically by an unusual mechanism of post-hatch 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). During cocoon spinning, the larva takes the bacteria up and transfers them to the 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 emergence of adults (Strohm and Linsenmair 1995). Although the exact route of vertical transmission is not yet known, preliminary studies provide evidence that the symbionts are taken up by adult females from the cocoon during emergence (M. Kaltenpoth, unpubl. data). To estimate the degree of population reduction during transmission and to reconstruct the subsequent population dynamics, we quantified '*Ca.* Streptomyces philanthi' in the antennal glands of female European beewolves (*Philanthus triangulum*) before and after emergence from the cocoon, as well as in the antennal gland secretion and on the cocoon using quantitative real-time PCR and 3D-reconstructions based on histological sections. The results do not only allow us to reconstruct the route of transmission and assess the growth rate of this symbiotic actinomycete in comparison to free-living relatives, but they also have implications for the consequences 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 prepupae were obtained from a laboratory culture of the European beewolf (*P. triangulum*). After hibernation of 6–9 months at 10°C, they were transferred to room temperature to induce further development. Pupation occurred after 3–4 weeks. About 2–3 weeks later, wasps 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. For each individual, both antennae were removed and processed on two different days (the second one was always removed 2 days later than the first, respectively) to yield two data points for each female, since the number of female beewolves was limited. Antennae for genetic analyses were stored in Eppendorf tubes at -20°C. 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 (Figure S1). Thus, although the order of the pre-emergence specimens could be established with high certainty, the estimated absolute age represents an approximate value (±2–3 days). After removing the first antenna, the pre-emergence specimens were placed back into their cocoons until removal of the second antenna 2 days later. Morphology and 3D-reconstruction of antennal glands and symbiotic bacteria semithin sections of 28 beewolf antennae (7 pre-emergence and 21 post-emergence) 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® 812 (Polysciences, Eppelheim, Germany). Serial sections of 4 μm thickness were cut with a diamond knife on a Reichert 2040 Autocut microtome and stained with 1% toluidine blue. For each antenna, digital photos of the serial sections of the eighth antennomere were obtained with a Nikon DS-2Mv camera attached to a Zeiss Axioplan microscope. Due to technical difficulties with the

semithin sectioning of the hard antennomeres, complete sets of high-quality slices for the eighth antennomere were not available for all specimens. In these cases, the seventh or sixth antennomere was used. The image stack of the slices was automatically aligned with the 3D-visualization software Amira® (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.

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 antennomere was measured in the middle section, and the volume of the antennomere was approximated as a cylinder ($V_A=\pi r^2h$), using half of the antennal width as the radius and the number of sections of one antennomere multiplied by 4 µm (=thickness of the sections) as the height (in µm). The relative reservoir volume was calculated by dividing the reservoir volume by the volume of the whole antennomere. Likewise, the relative volume of the bacteria was calculated as the bacterial volume divided by the reservoir volume. 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 ± 9.3 µm (mean ± SD) when measured in the antennal gland secretion (Koehler and Kaltenpoth 2013), and a mean diameter of about 0.5 µm (Kaltenpoth et al. 2005). Assuming a cylindrical shape of the bacterial cells, the volume can be calculated as $V_C=\pi r^2h$. This yields a mean volume of about 2.69 x 10^{-6} nl for a single cell. Thus, the bacterial cell number in the antennal reservoirs could be approximated as

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

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.

5.3.2 DNA extraction and quantitative PCR

For the genetic analyses, 38 antennae of beewolf females of different age were placed individually in Eppendorf tubes, submerged in liquid nitrogen and ground with a sterile pestle. The DNA was extracted using the MasterPure™ DNA isolation kit (Epicentre®) according to the manufacturer's instructions and finally eluted in 50 µl LowTE (1 mM Tris, 0.1 mM EDTA, pH 7.5). Likewise, DNA samples were obtained from ten female cocoons (right after emergence of the females) and from the antennal gland secretions harvested from ten different beewolf brood cells. To quantitatively obtain antennal gland secretions from brood cells, they were covered with a plastic foil during the construction of a new cell by a beewolf female. Since beewolf females always apply the secretion to the ceiling of the brood cell, it could later be harvested by collecting the plastic foil and re-suspending the bacteria for DNA extraction (for information on the observation cages used and more detailed methods for manipulating the antennal gland secretion in the brood cells see Strohm and Linsenmair 1995). Quantitative PCRs were performed on an Eppendorf® Realplex cycler in final reaction volumes of 25 µl, containing 1 µl of template DNA, 2.5 µl of each primer (10 pM), and 12.5 µl of SYBR Green Mix (SensiMixPlus SYBR Kit, Quantace). Primers used were specific for '*Ca.* S. philanthi' and amplified 135 bp of 16S rDNA (S16S) and 115 bp of gyraseB (*gyrB*), respectively (Table 5.1). Standard curves were established by using 10^{-8}–10^{-2} ng of PCR product as templates for the

qPCR. A NanoDrop™1000 spectrophotometer (Peqlab) was used to measure DNA concentrations for the templates of the standard curve. PCR conditions were as follows: 95°C for 10 min, followed by 50 cycles of 60°C for 30 s, 72°C for 20 s, and 95°C for 15 s; then a melting curve analysis was performed by increasing the temperature from 60°C to 95°C within 20 min. Based on the standard curve, the amount of S16S or *gyrB* template could be calculated from the qPCR threshold values (C_t). The template copy numbers of S16S and *gyrB*, respectively, were then calculated by the absolute quantification method (Lee et al. 2006; 2008). For the estimation of symbiont cell numbers from the template copy numbers obtained by qPCR, bacterial cells were extracted from four different beewolf antennae. The number of bacterial cells was counted in 1 µl of bacterial suspension under a microscope after staining the cells with crystal violet. The total number of bacteria in the suspension was calculated, and dilution series (tenfold) of 10^6–10^2 cells were prepared for each antennal sample. The DNA was extracted and used for quantitative PCR as described above. From the resulting copy numbers of S16S and *gyrB*, respectively, and the known symbiont cell numbers in the dilutions, the median copy numbers of S16S and *gyrB* per symbiont cell were estimated to be 48.8 and 11.0, respectively. These values allowed for the calculation of the cell numbers in all other samples from the gene copy numbers obtained by qPCR.

Table 5.1 Primers used for the real-time qPCR assays

Gene	Primer name	Primer sequence (5´-> 3´)	Direction	Target group	Amplicon length
S16S	Strep_phil_fwd3mod[a]	TGGTTGGTGGTGGAAAGC	Fwd.	*Ca.* S. philanthi	135
	S16S_rev	GTGTCTCAGTCCCAGTGTG	Rev.	Many Eubacteria	
gyrB	S_gyrB_fwd	CGCCAACACGATCCACAC	Fwd.	*Ca.* S. philanthi	115
	S_gyrB_rev	GTCCTTCTCCCGCAGCAG	Rev.	*Ca.* S. philanthi	

[a]Modified from Kaltenpoth et al. (2006)

5.3.3 Statistical analysis and calculation of specific growth rate and generation time

For all analyses, symbiont cell numbers represent extrapolated total values for an individual in the respective life stage. Thus, estimated symbiont cell numbers obtained from the 3D-reconstructions of one antennal gland reservoir were multiplied by 10 (five antennal glands per antenna, two antennae per female), those obtained from qPCRs for one antenna were multiplied by 2. To analyze the bacterial growth in the antennae after emergence, four-parameter logistic regression curves were fit to the log-transformed data using SigmaPlot 8.0 with the following equation:

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

For the calculation of the maximum specific growth rate μ_{max} of bacterial cells, the first and second derivatives of function (2) were calculated using the software Maple 7.00. Since the specific growth

rate is equivalent to the slope of the logistic regression, the maximum specific growth rate can be calculated as the maximum of the first derivative (Text S2). The doubling time or generation time t_D was calculated as follows:

(3) $$t_D = \frac{ln2}{\mu_{max}}.$$

To analyze the population dynamics of '*Ca.* S. philanthi', the symbiont cell numbers estimated in the qPCRs were compared among the following beewolf life stages: in the antennae of adult females (4–16 days after emergence), in the antennal gland secretion from the brood cell, on female cocoons, and in the antennae of female beewolves on the day of emergence from the cocoon. Differences in symbiont numbers (log-transformed to meet the assumptions of normal distribution and equal variances) among life stages were analyzed in an ANOVA using SPSS 16.0 software, with the two genes treated as repeated measures.

5.4 RESULTS

5.4.1 Development of the antennal gland reservoir

The antennal gland reservoirs of 28 female *P. triangulum* antennae and their symbiotic bacteria were reconstructed (for some representative reconstructions see Figure 5.1). Seven antennae were obtained from specimens before emergence from the cocoon, three immediately after emergence (on the same day), and 18 one to nine days later. Up to about 1 day prior to emergence, the antennal sections clearly showed the pupal skin still surrounding the antennal cuticle. This probably inhibits any invasion of bacteria into the antennal gland reservoirs from outside of the insect body. The volume of the antennal gland reservoir increased significantly from about 2 nl at emergence to about 20 nl at day 3–4 (four-parameter logistic regression: R^2=0.689; $F_{3,25}$=18.7; P<0.001). By that time, the antennal gland reservoir accounted for about 25–30% of the total antennomere volume. The antennomere volume was independent of the age of the beewolf females (linear regression: F=1.34, P=0.258; four parameter logistic regression: $F_{3,25}$=0.298, P=0.827).

5.4.2 Uptake of symbiotic bacteria

Despite extensive search, no bacterial cells could be detected in any of the pre-emergence specimens. The first bacterial cells were visible in the antennal gland reservoirs of females on the day of their emergence, suggesting that the symbionts are taken up from the cocoon 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 prior to emergence (Movie S3). 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 10–100 cells are taken up by the females per antennomere (i.e. 100–1,000 cells per total female).

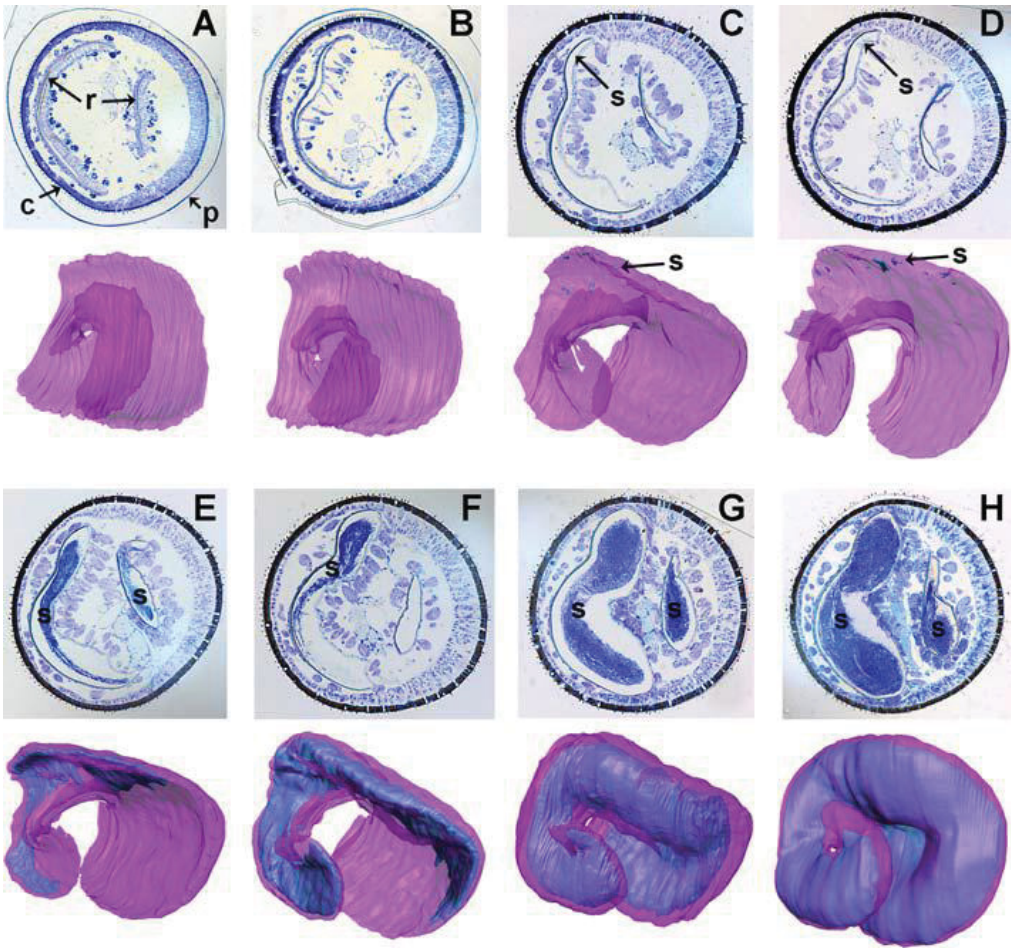

Fig. 5.1 Representative sections and 3D-reconstructions of antennal gland reservoirs and symbiotic bacteria from female European beewolves (*Philanthus triangulum*) of different age: A about 8 days prior to emergence (day -8), B about 5 days prior to emergence (day -5), C day of emergence (day 0), D 1 day after emergence (day 1), E 2 days after emergence (day 2), F 3 days after emergence (day 3), G 5 days after emergence (day 5), H 9 days after emergence (day 9). p pupal skin, c antennal cuticle, r antennal gland reservoir, s symbiont cells.

5.4.3 Growth of symbiont cells within the antennal gland reservoir

Based on the symbiont cell numbers estimated from the 3D-reconstructions of the antennal gland reservoirs and from the qPCR analyses of 16S rDNA and *gyrB* copy numbers, four-parameter logistic regressions could be used to model the growth of the symbionts in the antennal glands after emergence from the cocoon (Figure 5.2; Table 5.2). All regressions exhibited high regression coefficients and were highly significant (R^2 values range from 0.85 to 0.96; see Table 5.2). While the qPCR analyses of the two genes yielded very similar regression lines, cell numbers estimated by the morphological reconstructions were generally 3–14 times higher, which is likely due to interspaces between cells that are not taken into account by the morphological analysis, so the cell numbers tend to be overestimated. On the day of emergence, females harbored about 9.1×10^2–1.8×10^4 symbionts cells in their antennae (Table 5.2). This number increased to a maximum of about 1.3×10^7–4.1×10^7 cells within the following 3 days (Table 5.2). The maximum cell number corresponded to a volume of about 65% of the antennal gland reservoir volume. From the logistic regressions, the maximum specific growth rate (μ_{max}) of the symbionts was estimated to be between 0.069 and 0.105 h^{-1}, which corresponds to a doubling time of 6.6–10.0 h (Table 5.2).

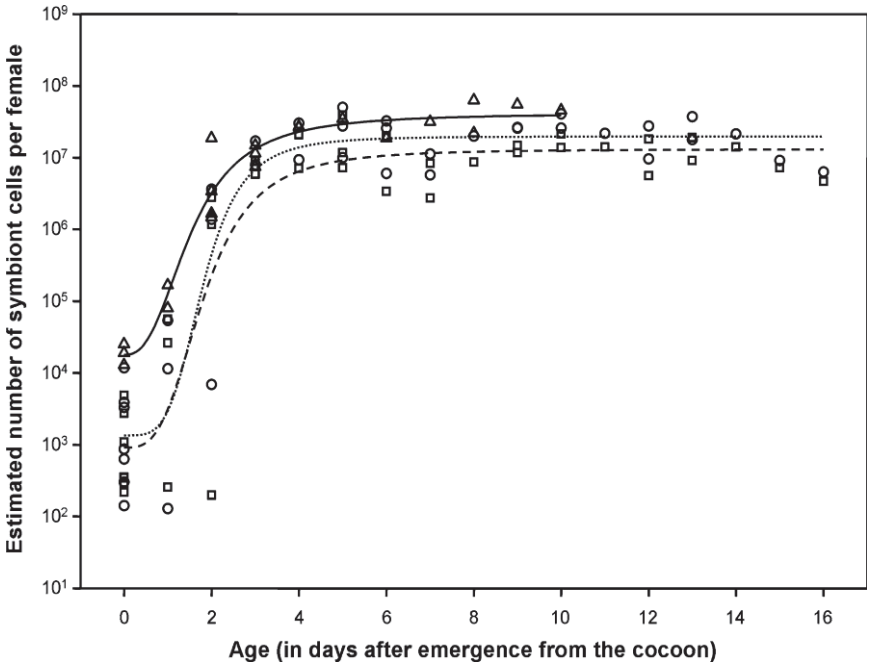

Fig. 5.2 Increase in symbiont cell number within the antennal gland reservoirs of female European beewolves after emergence from the cocoon. Circles (and dotted line) and squares (and dashed line) represent values obtained from qPCRs of 16S rDNA (R^2=0.900, $F_{3,34}$=101.7, P<0.0001) and gyrB genes (R^2=0.853, $F_{3,34}$=66.0, P<0.0001), respectively. Triangles and the solid line indicate cell number estimates from morphological 3D-reconstructions (R^2=0.962, $F_{3,17}$=143.2, P<0.0001).

Table 5.2 Analysis of symbiont growth within the antennal gland reservoirs of female beewolves after emergence from the cocoon

	3D- reconstruction	qPCR-S16S	qPCR-gyrB
Four-parameter logistic regression			
Sample size (n)	**21**	**38**	**38**
Regression coefficient (R^2)	**0.962**	**0.900**	**0.853**
F-value	**143.2**	**101.7**	**66.0**
P-value	**<0.0001**	**<0.0001**	**<0.0001**
Cell number and growth rate calculations			
Estimated symbiont cell number on day0	**1.8×10^4**	**1.3×10^3**	**9.7×10^2**
Estimated maximum cell number	**4.1×10^7**	**2.0×10^7**	**1.3×10^7**
Maximum specific growth rate (per hour)(μ_{max})	**0.069**	**0.105**	**0.084**
Doubling time (in hours)(t_D)	**10.00**	**6.63**	**8.24**

Given are the results of the four-parameter logistic regressions for the morphological 3D-reconstructions, the 16S rDNA and gyrB copy numbers versus the age of the females, respectively. The symbiont cell number on the day of emergence, the maximum cell number, the maximum specific growth rate and the corresponding doubling time were calculated from the regression parameters

5.4.4 Symbiont life cycle and population dynamics

The qPCR results for the 16S rDNA and the *gyrB* copy numbers were used to elucidate changes in the symbiont population size among different beewolf life stages (adult females, antennal gland secretion in the brood cell, cocoons, emerging females; see Figure 5.3). The ratios of 16S rDNA to

gyrB copy numbers calculated from all samples suggested an rrn operon number of 6–7 in the genome of '*Ca.* S. philanthi' (median ratio=6.68 copies). Adult females (4–16 days after emergence) harbored on average about 1.5 x 10^7 symbiont cells in their antennae, 1.9% of which (=2.7 x 10^5) were secreted into the brood cell (Figure 5.3). The number of symbiont cells that was present on the cocoon of a female at the time of emergence did not differ significantly from the number of symbionts secreted into the brood cell by the mother, although it was on average about 50% lower (Figure 5.3). The strongest reduction in symbiont population size occurred during the uptake of the bacteria from the cocoon by the emerging female, with more than a 100fold decrease in cell numbers (Figure 5.3). Only about 9.7 x 10^2 *Streptomyces* cells are taken up by the female, i.e. on average about 100 cells per antennal gland reservoir. The total bottleneck experienced by the symbiont population from the adult female via the antennal gland secretion and the cocoon to the emerging daughter is estimated to be 6.7 x 10^{-5}.

5.5 DISCUSSION

In the present study, we analyzed the population dynamics of the symbiotic bacteria of female European beewolves. The results provide evidence that the bacteria are taken up by female beewolves from the cocoon around the time of emergence and subsequently undergo logistic growth within the reservoirs. Furthermore, the symbiont population experiences severe bottlenecks during every vertical transmission event. The beewolf-*Streptomyces* symbiosis constitutes a specialized association with an unusual vertical transmission route of the symbionts via the brood cell and the cocoon. After secretion into the brood cell, the bacteria are taken up and applied to the cocoon by the beewolf larva (about 7–12 days after oviposition), where they grant protection against pathogenic fungi (Kaltenpoth et al. 2005). After hibernation in the cocoon for about 8–10 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 is 8–10 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. Possibly, the symbionts enter a metabolically inactive stage by forming spores (as other *Streptomyces* spp. do, see e.g. Holt et al. 1994) after an active phase of antibiotic production for the protection of the cocoon.

Within the antennal gland reservoirs of female beewolves, the endosymbiont growth can be approximated with a logistic regression. The maximum specific growth rate of 0.069–0.105 h^{-1} observed for '*Ca.* S. philanthi' lies within the lower range of growth rates found for non-symbiotic *Streptomyces* spp.: Depending on the species and the growth medium, specific growth rates between 0.024 and 1.13 h-1 have been reported (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' based on the 3D-reconstructions probably underestimates 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. Thus, the results obtained from the qPCR of the 16S rDNA and the *gyrB* gene probably provide more accurate estimates for the true maximum specific growth rate of '*Ca.* S. philanthi' (μ_{max}=0.084–0.105 h^{-1}, which corresponds to a doubling time of 6.6–8.2 h).

The results of our qPCR assays indicate high 16S rDNA and *gyrB* copy numbers per cell, and the ratio of 16S/*gyrB* copy numbers suggests that 6–7 rrn operons are present in the genome of '*Ca.* S. philanthi', which agrees with earlier studies on other *Streptomyces* species reporting 6 rrn operons (van Wezel et al. 1991; Kim et al. 1993; Yap and Wang 1999; Omura et al. 2001; Ohnishi et al. 2008). Based on the estimated number of rrn operons in the '*Ca.* S. philanthi' genome and the estimated

copy numbers of 16S rDNA and *gyrB* per symbiont cell (48.8 and 11.0, respectively), the number of genome copies per average '*Ca.* S. philanthi' cell is probably around 7–12. Multinucleate hyphae with several genome copies have been reported as a general feature of actinomycete bacteria (Chater 1993; Holt et al. 1994). It should be noted, however, that due to the likely variation in genome copy numbers among symbiont cells and possibly between different life stages of the host, symbiont numbers calculated from gene copy numbers are not always in agreement with direct methods

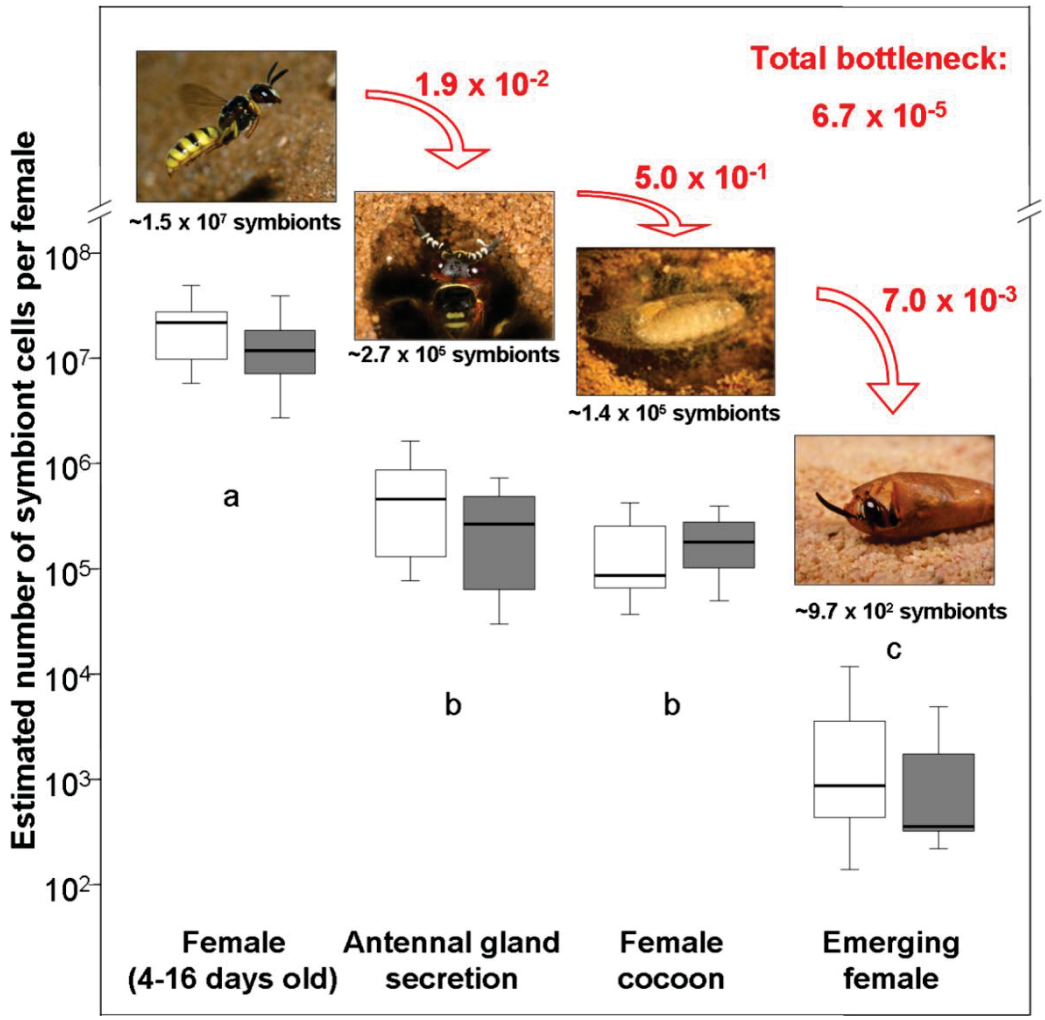

Fig. 5.3 Life cycle of '*Candidatus* Streptomyces philanthi' in European beewolves. Symbiont numbers represent mean estimated cell numbers calculated from S16S (white boxes) and *gyrB* (grey boxes) copy numbers obtained from qPCR assays. Bold lines represent medians, boxes comprise the interquartile range, and bars indicate minimum and maximum values. Red numbers by the arrows denote estimated reduction factors in cell numbers (in %). Different letters by the boxes indicate significant differences in symbiont cell numbers between different beewolf life stages (repeated-measures ANOVA, difference between beewolf life stages: $F_{3,46}$=246.2; P<0.001).

based on CFU counts (Komaki and Ishikawa 1999; Komaki and Ishikawa 2000; Ijichi et al. 2002). However, qPCR remains the most accurate and reliable quantification method for unculturable bacteria such as many insect endosymbionts. The qPCR results suggest that female beewolves secrete only 1.9% of the symbiont cells present in their antennae into a brood cell (i.e. 2.7 x 10^5 cells). However, direct counting of symbiont numbers secreted into the brood cell revealed much higher estimates for symbiont cell numbers (mean ± SD = 2.1 x 10^6 ± 8.4 x 10^5; (Koehler and Kaltenpoth 2013)). The following factors may contribute to the differences in observed and estimated symbiont numbers in the antennal gland secretion: (1) Due to the liquid nature of the antennal gland secretion,

it cannot be homogenized under liquid nitrogen as efficiently as beewolf antennae and cocoons, which may lead to a lower efficiency in the DNA extraction process; (2) in the brood cell the symbionts probably cannot be supplied with nutrients by the beewolf; therefore, they may enter a dormant stage (e.g. by forming spores) which may also impair DNA extraction; (3) due to the unfavorable conditions in the brood cell, symbiont cells may die or exhibit reduced effective genome copy numbers per cell (especially if they form spores), thus, leading to an underestimation in the number of cells by qPCR. Variation in genome copy numbers of symbiotic bacteria between different symbiont life stages has been reported in several earlier studies (Komaki and Ishikawa 1999; Komaki and Ishikawa 2000). Symbiont numbers on the cocoon were not significantly lower than in the antennal gland secretion in the brood cell, which may be due to one or several of the following reasons: Larvae may take up a large proportion of symbionts and completely apply them to the cocoon; there is a growth phase of the symbionts on the cocoon leading to an increase in cell and/or genome copy numbers; and/or the symbiont cell number in the brood cell may have been underestimated as mentioned above. The high number of symbiont cells on the cocoon at the time of emergence is insofar surprising as it indicates that the symbionts survive and possibly even thrive during the long time of hibernation on the cocoon.

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 6.7×10^{-5} from the mother (1.5×10^{7} cells) to the emerging offspring (9.7×10^{2} cells). The strongest reduction in symbiont numbers occurs with the uptake of the bacteria from the cocoon by the emerging female. The bottleneck experienced by '*Ca.* S. philanthi' during vertical transmission is significantly more severe than in most other symbiotic systems investigated so far: The symbiont populations of aphids (*Acyrthosiphon pisum*), weevils (*Sitophilus oryzae*), and stinkbugs (*Megacopta punctatissima*) suffer reductions of 1.6×10^{-2}–4.6×10^{-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 magnitude as in the beewolf-*Streptomyces* association (3×10^{-4}–5×10^{-5}) (Anbutsu and Fukatsu 2003). Additionally, the absolute number of '*Ca.* S. philanthi' cells transmitted from one beewolf generation to the next is extremely low (about 9.7×10^{2}), which can be expected to further augment the deleterious effects of the transmission bottleneck.

The severe transmission bottlenecks observed for the *Philanthus-Streptomyces* symbiosis 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 γ-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.

5.6 SUPPLEMENT

5.6.1 Supplementary figures

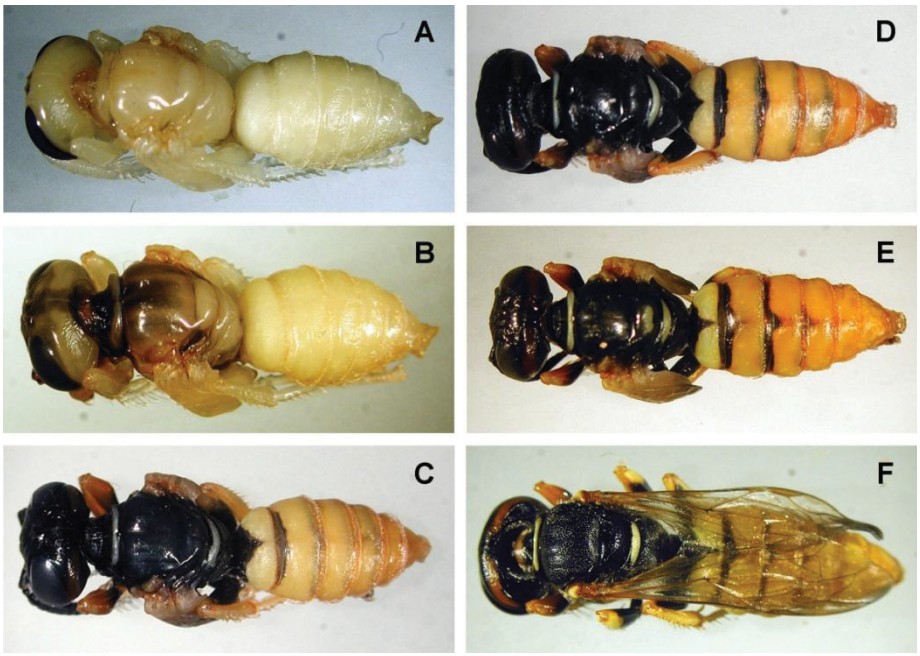

Fig. S1 Melanization of the cuticle in female European beewolves prior to emergence. Approximate time before emergence: **(A)** Eight days, **(B)** six days, **(C)** five days, **(D)** three days, **(E)** two days, **(F)** one day.

5.6.2 Supplementary methods

Text S2: Calculation of bacterial growth rate

Four-parameter logistic regression:

(2) $$f\ x\ = f\ x_0\ + \frac{a}{1+\frac{x}{x_o}\ b}$$

First derivative of the four-parameter logistic regression:

(4) $$f'\ x\ = \frac{a*b*\frac{x^{b-1}}{x_0^b}}{(1+(\frac{x}{x_0})^b)^2}$$

Second derivative of the four-parameter logistic regression:

(5) $$f''\ x\ = \frac{a*b*\ b+1\ *\frac{x^{b-2}}{x_0^b}}{(1+(\frac{x}{x_0})^b)^2} + \frac{2a*b^2*\frac{x^{2b-b}}{x_0^{2b}}}{(1+(\frac{x}{x_0})^b)^3}$$

Since the growth rate is equivalent to the slope of the logistic function (equation (2)), the maximum growth rate represents the maximum of the first derivative (equation (3)), which can be calculated by identifying the nulls of the second derivative (equation (5)) and calculating the respective values of the first derivative (equation (6)).

(6) $$f'' \, x_{max} = 0 \rightarrow x_{max} = x_0 * \sqrt[b]{\frac{b-1}{b+1}}$$

(7) $$\mu_{max} = f'(x_{max})$$

Movie S3: Putative symbiont uptake behavior by a female European beewolf in the cocoon prior to emergence (in real-time).
The antennae are bent backwards and rubbed against the inner wall of the cocoon, so symbiotic bacteria may be inoculated into the antennal gland reservoirs.
(for movie see: http://link.springer.com/article/10.1007/s10682-009-9319-z)

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CHAPTER 6

PARTNER CHOICE AND FIDELITY STABILIZE CO-EVOLUTION IN A CRETACEOUS-AGE DEFENSIVE SYMBIOSIS

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6.1 ABSTRACT

Background – Many insects rely on symbiotic microbes for survival, growth or reproduction. Over evolutionary timescales, intracellular symbioses are stabilized by partner fidelity through strictly vertical symbiont transmission. However, little is known about how symbioses with extracellular symbionts, representing the majority of insect-associated microorganisms, persist despite opportunities for horizontal exchange and *de novo* acquisition of symbionts from the environment. Here, we aimed to elucidate the evolutionary history of the defensive mutualism between solitary beewolf wasps and antibiotic-producing *Streptomyces* bacteria, and to assess the importance of partner fidelity and partner choice for the maintenance of the association.

Methodology and principal findings – Multi-gene co-phylogenies of 39 host species and their symbionts within the wasp genera *Philanthus*, *Trachypus*, and *Philanthinus* (Hymenoptera, Crabronidae) reveal that beewolves cultivate a distinct clade of *Streptomyces* for protection against pathogenic microorganisms. The symbionts evolved from a soil-dwelling ancestor at least 68 million years ago, and vertical transmission via the brood cell and the cocoon surface resulted in host-symbiont co-diversification. However, the external mode of transmission also provides opportunities for horizontal transfer and uptake of other bacteria from the environment. Concordantly, our results show that strains of symbiotic bacteria were indeed exchanged among host species, possibly through predation or nest reuse, and that other actinobacteria are occasionally taken up by beewolves. However, behavioral observations reveal that the transmission of these non-symbiotic bacteria is blocked, thereby ensuring specificity in the symbiotic association.

Conclusions and significance – Our results establish the beewolf-*Streptomyces* symbiosis as an ancient and co-evolved mutualism that has served to efficiently protect the wasp offspring against a broad range of opportunistic pathogens since the Cretaceous. Host control over symbiont transmission is pivotal for stabilizing the cooperative association, indicating that partner choice can reinforce partner fidelity and thereby play an important role even in vertically transmitted symbioses. As most insects maintain a mutualistic microbiota while simultaneously confronting pathogens, strategies to control symbiont identity during transmission and establishment must be common, and understanding their mechanistic basis will help explain the abundance and persistence of extracellular mutualisms in insects.

6.2 INTRODUCTION

Mutualistic associations are ubiquitous in nature, yet they present a conundrum to evolutionary biology, because acts that are beneficial to the receiver but costly to the actor should not be favored by natural selection (Axelrod and Hamilton 1981). The two most important models to explain the maintenance of interspecific cooperation are partner fidelity and partner choice (Bull and Rice 1991; Sachs et al. 2004). In partner-fidelity associations, host and symbiont interact repeatedly and reward cooperating individuals while punishing cheaters, thereby reinforcing mutually beneficial interactions (Bull and Rice 1991; Sachs et al. 2011). In partner-choice associations, individuals may interact only once, but one member can select its partner in advance of any possible exploitation (Bull and Rice 1991; Sachs et al. 2011). Partner choice appears to select for cooperative strains among environmentally acquired microbial symbionts, e.g. the bioluminescent *Vibrio fischeri* bacteria of squids (Nyholm and McFall-Ngai 2004), the nitrogen-fixing rhizobia of legumes (Kiers et al. 2003), and mycorrhizal fungi of plants (Kiers et al. 2011). By contrast, partner fidelity is generally assumed to be the major stabilizing force in the widespread and ecologically important vertically transmitted symbioses of insects (Sachs et al. 2011).

However, localization and transmission routes of mutualistic bacteria in insects are diverse, and the differences across symbiotic systems have important implications for the evolutionary trajectory of the associations. Symbionts with an obligate intracellular lifestyle are usually tightly integrated into the host's metabolism (e.g. McCutcheon and von Dohlen 2011) and development (Koga et al. 2012), and the mutual interdependence of both partners coincides with perfect vertical symbiont transmission. Over evolutionary timescales, the high degree of partner fidelity results in host-symbiont co-cladogenesis, and, concordantly, phylogenies of hosts and their intracellular symbionts are usually found to be congruent (Moran et al. 1993; Bandi et al. 1995; Baumann and Baumann 2005; Moran et al. 2008). While such a pattern is also observed for some extracellular symbioses with especially tight host-symbiont integration (Hosokawa et al. 2006; Kikuchi et al. 2009), the ability of many extracellularly transmitted symbionts to spend part of their life cycle outside of the host's body is often reflected in more or less extensive horizontal transmission or *de novo* acquisition of symbionts from the environment (Prado and Almeida 2009; Cafaro et al. 2011). In these cases, partner choice mechanisms are expected to ensure specificity in the establishment and maintenance of the association (Zhang et al. 2007). The nature of such control mechanisms, however, remains poorly understood.

While many of the well-studied mutualistic associations in insects have a nutritional basis (Douglas 2009; Feldhaar 2011), an increasing number of symbioses for the defense of the host against predators (Kellner 2002), parasitoids (Oliver et al. 2003), or pathogens (Teixeira et al. 2008; Brownlie and Johnson 2009; Kaltenpoth 2009) have recently been discovered. Among defensive symbionts, the high-GC gram-positive Actinobacteria are particularly prevalent, probably due to their ubiquity in the soil and their ability to produce secondary metabolites with antibiotic properties (Kaltenpoth 2009). Antibiotic-producing actinobacterial symbionts have been discovered on the cuticle of leaf-cutting ants (Currie et al. 1999), in the fungal galleries of a bark beetle (Scott et al. 2008), and in the antennae and on cocoons of beewolf wasps (Kaltenpoth et al. 2005). While in the former two cases, the symbionts have been implicated in the defense of the hosts' nutritional resources against competing fungi (Currie et al. 1999; Scott et al. 2008), the beewolves' bacteria protect the offspring in the cocoon against pathogenic microorganisms (Kaltenpoth et al. 2005; Kroiss et al. 2010).

Beewolves are solitary wasps in the genera *Philanthus*, *Trachypus* and *Philanthinus* (Hymenoptera, Crabronidae, Philanthini). They engage in a defensive alliance with the actinobacterium '*Candidatus* Streptomyces philanthi' (CaSP) (Kaltenpoth et al. 2005; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012), which is cultivated by female beewolves in specialized antennal gland reservoirs (Goettler et al. 2007). The uniqueness and complexity of the glands suggest a long history of host-symbiont co-evolution (Goettler et al. 2007). From the antennae, the streptomycetes are secreted into the brood cell, taken up by the larva and incorporated into its cocoon (Kaltenpoth et al. 2010a), where they provide protection against pathogenic fungi and bacteria (Kaltenpoth et al. 2005) by producing at least nine different antimicrobial compounds (Kroiss et al. 2010). Weeks or months later, eclosing adult females acquire the bacteria from the cocoon surface (Kaltenpoth et al. 2010a), thus completing the vertical transmission of CaSP. However, this mode of transmission provides opportunities for the horizontal transfer of symbionts among beewolf species or the *de novo* uptake of bacteria from the environment. Despite these opportunities, a monophyletic clade of CaSP strains has previously been found in 31 species of beewolves, suggesting an ancient and highly co-evolved relationship (Kaltenpoth et al. 2006; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012).

Here we combine co-phylogenetic analyses of beewolves and their vertically transmitted defensive symbionts with behavioral observations of symbiont transmission from female antennal gland reservoirs into the brood cell in order to (i) reconstruct the co-evolutionary history of the symbiosis, (ii) estimate the age of the symbiosis, (iii) elucidate the ancestral lifestyle of the symbionts, and (iv) assess the importance of partner fidelity and partner choice for the long-term stability of the association.

6.3 MATERIALS AND METHODS

6.3.1 Insect specimens

Specimens of 43 *Philanthus* species and subspecies from North America, Europe, India, and South Africa, 6 *Trachypus* species from South America, and one *Philanthinus* species from Turkey were collected or kindly supplied by colleagues (Table S1). Species were identified using published keys for the North American (Bohart and Grissell 1975; Ferguson 1983a; Ferguson 1983b) and South African *Philanthus* (Arnold 1925), and for the South American *Trachypus* (Rubio 1975), respectively. Indian specimens were identified by comparison to the original descriptions as well as the reference collection at the Natural History Museum in London. Fresh beewolf specimens were freeze-killed or placed directly into 70% or 95% ethanol and stored until DNA extraction. As outgroup taxa, crabronid species of the closely related genera *Aphilanthops, Clypeadon,* and *Cerceris* were collected*,* and additional sequences for the more distantly related *Bembix*, *Bicyrtes*, as well as *Apis mellifera* (Apidae) were obtained from the NCBI database (Table S1).

6.3.2 DNA extraction, PCR and sequencing of host genes

DNA was extracted either from insect thoraces or, to allow for later morphological determination of single specimens, from three legs. The MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) was used for DNA isolation according to the manufacturer's instructions. PCR amplifications were performed on a T-Gradient Thermocycler (Biometra, Göttingen, Germany), in final reaction volumes of 12.5 µl, composed of 1 µl genomic DNA extract, 1

µl of each primer (10 µM), 1.5 µl dNTP-Mix (2 mM; Fermentas, St. Leon-Rot, Germany), 1.25 µl Peqlab reaction buffer (200 mM Tris-HCl (pH 8.55 at 25 °C), 160 mM $(NH_4)_2SO_4$, 0.1% Tween 20, 20 mM $MgCl_2$) and 0.5 units SAWADY Taq DNA polymerase (Peqlab, Erlangen, Germany). Cycle parameters were as follows: 3 min initial denaturation at 94°C, followed by 35 cycles of 94°C for 40 s, the primer-specific annealing temperature for 40 s, 72°C for 40 s (or 90 s for longer fragments), and a final extension of 4 min at 72°C. Primer sequences and references are listed in Table S2, details on primer combinations, annealing temperatures and the corresponding fragment lengths are summarised in Table S3. Prior to sequencing, PCR products were purified with the peqGOLD MicroSpin Cycle-Pure Kit (Peqlab Biotechnologie GmbH, Erlangen, Germany) following the manufacturer's protocol. Sequencing was done commercially at Seqlab Sequence Laboratories (Göttingen, Germany).
Partial sequences of six different genes were obtained, all of which have previously been shown to be useful for phylogenetic analyses in Hymenoptera (Cameron and Mardulyn 2001; Kawakita et al. 2003; Danforth et al. 2004; Ramirez et al. 2010): A fragment of the subunit 1 of the mitochondrial cytochrome oxidase gene (*coxI*; 841 bp) was amplified and sequenced, as well as a fragment of the ribosomal 28S gene (*28S*; 865 bp). Additionally, the following four single-copy nuclear genes were used: Wingless (*wnt*, comprising of 378 bp cds), long-wavelength rhodopsin (*lwrh*, comprising of 608 bp of cds and 156 bp ncs), arginine kinase (*argK*, with 825 bp cds and 111 bp ncs), and elongation factor 1α (*ef1a*, including 1,041 bp cds and 696 bp ncs). The listed fragment lengths are those of the processed sequences used for the phylogenetic analyses. Primer sequences and PCR conditions for amplification of the host genes are given in Tables S2 and S3. Outgroup sequences for *Apis*, *Bembix,* and *Bicyrtes* could be obtained from the NCBI database. Accession numbers for all sequences are given in Table S1.

6.3.3 Reconstruction of the host phylogeny

Sequences were aligned using BioEdit 7.0.5.3 (Hall 1999) and SeaView 4.2.6 (Gouy et al. 2010). All alignments were checked and corrected manually. Open reading frames and intron / exon boundaries were identified by comparison with published coding sequences for *Apis mellifera* (*lwrh*: BK005514.1; *argK* AF023619.1; *ef1a*: NM_001014993.1) or via a blast search against non-redundant sequences in the Genbank database. As substitution rates and patterns can differ greatly between coding (cds) and non-coding sequences (ncs), we split the dataset into nine partitions: *28S*, *coxI*, *wnt*, *lwrh*-cds, *lwrh*-ncs, *argK*-cds, *argK*-ncs, *ef1a*-cds, and *ef1a*-ncs. Due to high substitution rates, the non-coding sequences could only be reliably aligned within the Philanthini species. Therefore, we coded the intron sequences of all outgroup taxa as missing data and thus excluded them from the analyses.
In a first step, we reconstructed nine separate gene trees using fast likelihood inferences with the software RAxML v7.0.4 (Stamatakis et al. 2005; Stamatakis 2006; Stamatakis et al. 2008) corresponding to the nine partitions determined above. Maximum likelihood (ML) searches were conducted with the rapid hill-climbing algorithm (Stamatakis et al. 2005) under the General Time-Reversible model with four gamma parameters GTR+G (Tavaré 1986; Yang 1993; Yang 1994). Support values (100 bootstrap steps) were calculated for each node and topologies were manually compared among the gene trees. Because none of the strongly supported nodes were different, we combined all loci in one supermatrix.
Additionally, searches for a saturation effect within one of the three codon positions were conducted for the genes *wnt*, *coxI*, *lwrh*, *argK*, and *ef1a* by calculating homoplasy indices (HI) for each codon position and gene separately. The software PAUP* 4.0 beta (Swofford 2003) was used for these analyses. The homoplasy index of the third codon position of the genes *coxI* and *lwrh* (HI(*coxI*)=0.66, HI(*lwrh*)=0.46) were higher compared to the first and second positions (HI: CO-1st=0.52, *coxI*-2nd=0.25, *lwrh*-1st=0.34, *lwrh*-2nd=0.18). Therefore, we excluded the third codon positions of the genes *coxI* and *lwrh* from further analyses, or we used the translated amino acid sequences (stated for each analysis).

In a next step, multiple independent analyses with different data partitioning strategies (1-4) were performed to test for the robustness of the phylogenetic reconstructions: (1) unpartitioned, (2) four partitions with combined nuclear introns, exons and mitochondrial sequences separately, plus *28S* sequences, (3) nine partitions with single genes separately and splitting coding and non-coding sequence parts, (4) complete random partitioning in 9 partitions; all analyses were conducted with excluded third codon positions of the genes *coxI* and *lwrh* and also with base sequences translated into amino acid sequences. The best fitting evolutionary model for the amino acid-translated sequences (*coxI*, *lwrh*) was inferred with ProtTest v1.4 (Abascal et al. 2005). The CPREV model showed the highest fit for *lwrh*, and the MTREV for *coxI*. From these different runs, we chose the tree with the highest likelihood for presentation. Bootstrap support values were obtained through a full non-parametric bootstrap inference with 10000 replicates, carried out separately with RAxML.
Bayesian inferences were run with the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003). The searches were also conducted under the GTR+G model with four rate categories. We ran each analysis for 10,000,000 generations and sampled trees every 1,000 generations. We checked if the standard deviation of split frequencies was consistently less than 0.01, and we used a "Burnin" of 20%, i.e. the first 20% of the sampled trees were discarded. We computed 50% majority rule consensus trees for each analysis with posterior probability values for every node. Different partition schemes (1-4) were analyzed as well (see above). However, mixed data sets consisting of DNA and protein sequences cannot be analyzed in MrBayes, so only nucleotide sequences were used, and third codon positions were excluded for *coxI* and *lwrh*.
Equal weighted maximum-parsimony (MP) analyses were performed using the program PAUP* 4.0 beta (Swofford 2003). We used a heuristic search and TBR (tree-bisection-reconnection) for branch swapping. Bootstrap supports were obtained from 1,000 independent replicates. The third codon positions of *coxI* and *lwrh* were excluded for all MP analysis as well. Further, MP analyses were only conducted for the partition schemes (1) and (3). Since all three analyses (ML, Bayesian, and MP) yielded very similar tree topologies, the results were combined for visualization (Figure 6.1).

6.3.4 Dating of the host phylogeny

Divergence time estimations were inferred using BEAST v1.7.5 (Drummond and Rambaut 2007). MCMC analyses with HKY and GTR nucleotide substitution models (empirical or estimated base frequencies, various site heterogeneity models [none, G, I+G]) were conducted under a strict clock (using a single rate of sequence evolution across the phylogeny) and an uncorrelated lognormal relaxed clock model (allowing variable substitution rates; Drummond et al. 2006). In each analysis, 25 million steps were performed, and trees were sampled every 2,500 steps. To estimate the influence of partitioning, analyses were conducted with the partitioned (9 gene partitions, codon partitioning (1+2, 3) for *argK, ef1a* and *wnt*) as well as with the unpartitioned dataset (3rd codon positions excluded for *coxI* and *lwrh* in both datasets due to saturation). The phylogenetic tree from the ML analysis (see previous section) was used as starting tree in all analyses. In some of the analyses, the input tree was fixed by removing the tree priors from the BEAST input file.
Four calibration points were included in the initial dating analysis: (A) The age of the Bembicinae with oldest fossils known from Florissant beds in Colorado (*Psammaecius sepultus,* originally described as *Hoplisus sepultus* by Cockerell (Cockerell 1906), reviewed by Pulawski and Rasnitsyn (Pulawski and Rasnitsyn 1980) and transferred to the extant bembicin genus *Psammaecius*), which date back to the latest Eocene (~34.1 Mya) (Evanoff et al. 2001), (B) the age of the oldest *Cerceris* fossil from late Stampian (*Cerceris berlandi*, ~30 Mya) shales in France (Timon-David 1944), (C) the age of the oldest *Philanthus* fossils from Colorado (*Philanthus saxigenus* and *Prophilanthus destructus,* ~34.1 Mya) (Rohwer

1909; Theobald 1937), and (D) the root age was calibrated based on earlier phylogenetic analyses (Grimaldi and Engel 2005; Cardinal and Danforth 2013). Minimum age constraints for the Bembicinae and the *P. saxigenus* fossil were modelled with lognormal distributions (mean±SD=34.1±0.5, offset=20.0 for both fossils). The age of the *Cerceris* fossil was used to place a hard lower boundary on the age of the Cercerini+Aphilanthopini clade (uniform distribution, minimum=30.0, maximum=1000.0; or lognormal distribution with mean±SD=30.0±0.75, offset=20.0). As the phylogenetic relationship of Crabronidae subfamilies and bees ("Apidae" *sensu lato*) is still controversial (Melo 1999; Debevec et al. 2012; Cardinal and Danforth 2013), we did not enforce monophyly of the Crabronidae (Philanthinae+Bembicinae).

Five compression fossils described from 1906 to 1944 have been assigned to the Philanthinae by earlier authors: *Prophilanthus destructus* (Cockerell 1906), *Philanthus saxigenus* (Rohwer 1909), *Philoponites clarus* (Cockerell 1915), *Philanthus annulatus* (Theobald 1937), and *Cerceris berlandi* (Timon-David 1944). No recent publication has reviewed the systematic affinities of these specimens. In our view, only the *Cerceris* specimen is clearly assignable to Philanthinae. Timon-David's (Timon-David 1944) description and illustration leave no doubt that the specimen belongs to the philanthine tribe Cercerini. When it comes to the other four specimens, however, no structures are described or illustrated that would convincingly associate them with the Philanthinae, much less the tribe Philanthini. Therefore, the dating analyses were also repeated excluding the *Philanthus* fossil calibration point.

The root of the tree was modelled with a normal distribution with mean±SD=140.0±10.0, since both the divergence of Sphecidae from the other Apoidea and that of Crabronidae and bees have been estimated to the period of 130-150 mya (Grimaldi and Engel 2005; Cardinal and Danforth 2013). This time period coincides with the estimated rise of the angiosperms. Due to their tight association with angiosperms, bees and crabronid wasps have likely evolved with or after the origin of angiosperms (Brady et al. 2009; Cardinal and Danforth 2013). However, to assess the effect of root age on the divergence estimates, we additionally performed analyses without a root age prior.

Evaluation and comparison of the models was performed using Tracer v1.5 (Rambaut and Drummond 2007). Bayes factors (BF) were computed for comparison of marginal likelihood values, and $\log_{10}$BF>100 were interpreted as decisive evidence for differences in model performance. For visualization of the results, the maximum clade credibility tree was inferred with TreeAnnotator (Drummond and Rambaut 2007), using a burnin of 1,000 and a posterior probability limit of 0.5. The consensus tree was visualized with FigTree v1.3.1 (Rambaut 2010), including highest posterior density (HPD) intervals (Figure 6.2). Due to the unclear systematic position of the putative Philanthini fossils, the analyses excluding this calibration point were displayed (Figure 6.2). It should be noted, however, that the analyses including the Philanthini fossils yielded identical tree topologies and very similar age estimates.

6.3.5 DNA extraction, PCR and sequencing of CaSP genes

Genomic DNA was extracted from whole beewolf antennae according to a standard phenol-chloroform extraction protocol (Sambrook et al. 1989) or with the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre Biotechnologies) according to the manufacturer's instructions. The presence of '*Candidatus* Streptomyces philanthi' in the antennae was confirmed by diagnostic PCR using the specific 16S rDNA primer Strep_phil_fwd3 in combination with the general actinomycete primer Act-A19 as described earlier (Kaltenpoth et al. 2006). Almost complete 16S rDNA sequences of many '*Ca.* S. philanthi' ecotypes had already been sequenced earlier (Kaltenpoth et al. 2006; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). The 16S rDNA of additional specimens was amplified with the primers fD1 and Spa-2R, and sequenced bi-directionally with fD1 and rP2 (Tables S2 and S3). PCR amplifications were performed on a Biometra® T-Gradient Thermocycler

or on a VWR Gradient Thermocycler in a total reaction volume of 25 µl containing 2 µl of template, 1x PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.08% Nonidet P40), 2.5 mM $MgCl_2$, 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 s, 65°C for 1 min, and 72°C for 1 min, and a final extension time of 4 min at 72°C.

Parts of the elongation factor Tu and the elongation factor G as well as the intergenic spacer region (collectively referred to as *fus-tuf* in the following) of '*Ca.* S. philanthi' were amplified by using the primer pairs EF-Tu-1F/EF-Tu-2R and EF-Tu-3F/EF-Tu-3R, respectively, and sequenced using the same primers (Tables S2 and S3). The primer pairs gyrB-F1/gyrB-R3 and gyrB-F3/gyrB-R10 amplified overlapping fragments of the gyrase B gene (*gyrB*) of the endosymbionts that could be sequenced by using the same primers. Additionally, a 627 bp fragment of gyrase A (*gyrA*) was amplified using primers gyrA-5F/gyrA-5R and sequenced unidirectionally using primer gyrA-5F (Tables S2 and S3). PCR reaction mixtures were the same as described for the amplification of the 16S rDNA. Cycle parameters were as follows: 3 min at 94°C, followed by 35 cycles of 94°C for 40 s, 65°C (*fus-tuf* primers) or 62°C (*gyrB* primers) or 60°C (*gyrA* primers) for 40 s, and 72°C for 40 s, and a final extension time of 4 min at 72°C. Sequencing was done in the Department of Entomology at the Max Planck Institute for Chemical Ecology (Jena, Germany) or commercially by SEQLAB Sequence Laboratories (Göttingen, Germany).

6.3.6 Symbiont phylogenetic analysis

For the phylogenetic analysis, 16S rRNA, *gyrA*, *gyrB*, and *fus-tuf* sequences of all *Streptomyces* species for which fully sequenced or good draft genomes were available were retrieved from the NCBI database. Additionally, cultures of three closely related strains (based on 16S rRNA, *Streptomyces ramulosus* DSM 40100*, S. abikoensis* DSM 40831, and *S. mutabilis* DSM 40169) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), and the four gene fragments were sequenced as described above.

All protein-coding sequences were assembled and aligned based on their translated amino acid sequences using Geneious Pro 5.4 (Drummond et al. 2011). The 16S rRNA gene sequences were imported into ARB and aligned against closely related *Streptomyces* sequences based on the secondary structure prediction (Ludwig et al. 2004). The alignments were concatenated in BioEdit 7.0.5.3 (Hall 1999). The concatenated alignment consisted of a total of 4653 bp (1391 bp of 16S rDNA, 639 bp of *fus*, 930 bp of *tuf*, 249 bp of *fus-tuf* intergenic spacer, 765 bp of *gyrB*, and 549 bp of *gyrA*).

Approximately-maximum-likelihood trees were reconstructed with FastTree 2.1 using the GTR model (Price et al. 2010). Local support values were estimated with the Shimodaira-Hasegawa test based on 1,000 resamples without reoptimizing the branch lengths for the resampled alignments (Price et al. 2010). Additionally, a maximum likelihood tree was reconstructed using PHYML (Guindon and Gascuel 2003) as implemented in Geneious Pro 5.4 (Drummond et al. 2011). The GTR+I+G model was chosen, the transition/transversion ratio was set to 4 (fixed), and both the proportion of invariable sites and the gamma distribution parameter were estimated. Bootstrap values were obtained from a search with 1,000 replicates.

Bayesian inferences were run with the program MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001; Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003), with the concatenated alignment split into six partitions: 16S rRNA, *gyrA, gyrB*, *fus, tuf*, and the *fus-tuf* intergenic spacer. The searches were conducted under the GTR+I+G model. We ran each analysis for 20,000,000 generations and sampled trees every 1000 generations. A "burnin" of 25% was used, i.e. the first 25% of the sampled trees were discarded. We checked if the standard deviation of split frequencies was consistently lower than 0.01. We computed a 50% majority rule consensus tree with posterior probability values for every node.

Since the phylogenetic trees reconstructed with the three different methods were topologically very similar, the results were combined into a single figure.
To gain more comprehensive insights into within-species patterns of symbiont phylogenetic relationships, we sequenced *gyrA* for the symbionts of 109 beewolf individuals across 41 species. We aligned the sequences as described above and used FastTree 2.1 for phylogenetic reconstruction, with the same settings as for the concatenated alignment (Figure 6.5).

6.3.7 Host-symbiont co-phylogenetic analysis

To test for co-diversification between hosts and symbionts, three different methods were used: (i) Host and symbiont trees were imported into TreeMap 1.0 (Page 1995). Both trees were randomized (1,000 replicates), and the number of observed cospeciation events (21) was compared to the resulting distribution of cospeciation events in the randomized dataset. (ii) Host and symbiont distance matrices were computed in BioEdit 7.0.5.3 (Hall 1999) based on the concatenated alignments, and permutation tests (1,000 replicates) were run as implemented in ParaFit (Legendre et al. 2002). (iii) Host and symbiont trees were imported into Jane 3 (Conow et al. 2010) and tested for congruence by using both edge- and node-based cost models. In addition to an analysis using the default cost parameters, a second analysis with the cost for symbiont loss reduced to 1 was performed. The number of generations was set to 30 and the population size to 500 for both analyses, as neither parameter appeared to influence the results (several combinations tested). Statistical assessment of the observed cost of the optimal trees was achieved by randomizing the symbiont tree (beta=-1) or permuting host-symbiont associations (100 resamplings, respectively).
For visualization, a tanglegram was reconstructed and optimized in Dendroscope V3.0.13beta (Huson et al. 2007) and used as a template for visualization of the comparative phylogenies in Microsoft PowerPoint, including both branch lengths (both trees) and divergence time estimates (host tree only) (Figure 6.2) In the symbiont tree, a lower number of free-living *Streptomyces* strains was included for better visualization of the relationships among CaSP isolates. The monophyly of the symbiont clade and the within-clade relationships were identical to the full bacterial tree.

6.3.8 Detection of other bacteria in *Philanthus* antennae

In a few cases, bacteria other than CaSP could be found in the antennae of female beewolves. To assess the incidence of CaSP across beewolf species, 338 specimens of 34 different Philanthini species were screened for the presence of CaSP by diagnostic PCR using the specific 16S rDNA primer Strep_phil_fwd3 in combination with the general actinomycete primer Act-A19 as described earlier (Kaltenpoth et al. 2006). Additionally, since bacteria of the genus *Amycolatopsis* were detected repeatedly, and notably in the only two specimens of *P.* cf. *basalis* investigated, the same specimens were screened for the presence of *Amycolatopsis* by using the specific primer Amy_16S_1F in combination with the actinobacterial primer Act-A19 (Table S3). Antennal specimens that were negative for both CaSP and *Amycolatopsis* were tested with the general actinobacterial primer pair Act-S20/Act-A19 (Table S3) and subsequently with the general eubacterial primers EUB933F-GC (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCAC-GGGGGGGCACAAGCGGTGGA GCATGTGG-3') and EUB1387R (5'-GCCCGGGAACGTAT-TCACCG-3') (Iwamoto et al. 2000; Kawai et al. 2002). Amplification products of the actinobacterial PCR were sequenced directly, whereas those of the eubacterial PCR were separated by temperature-gradient gel electrophoresis (TGGE) prior to sequencing as described earlier (Stoll et al. 2007). Briefly, TGGE gels (50 ml) were prepared with a final concentration of 8% polyacrylamide (60:1), 8 M urea, 0.1X TBE buffer and 2%

glycerol, and polymerized on polybond films (Biometra) by adding 110 µl TEMED (N,N,N',N'-tetramethylethan-1,2-diamine) and 40 µl ammoniumpersulfate (50%). After electrophoresis for 18 hours at 150V with a temperature gradient from 40°C to 50°C on a TGGE Maxi System (Biometra), gels were stained with silver nitrate as described previously (Stoll et al. 2007). Bands were excised using a sterile scalpel, and the DNA was re-eluted overnight at 4°C in 50 µl LowTE buffer (1 mM Tris, 0.1 mM EDTA). Excised bands as well as amplicons from the *Amycolatopsis*- and actionobacterial PCRs were sequenced and compared against the NCBI database using BLASTn.
Diagnostic PCRs for *Trachypus boharti* antennae consistently yielded positive results for both CaSP and *Amycolatopsis*. *Amycolatopsis* PCR products were sequenced and turned out to stem from CaSP, indicating that the Amy_16S_1F primer successfully amplified the *T. boharti* CaSP strain despite two mismatches in the primer binding site (as opposed to 3-5 mismatches for all other CaSP strains, 1-2 of which are located towards the 3'-end of the primer). Hence, *T. boharti* specimens that yielded positive PCRs for both CaSP and *Amycolatopsis* were assumed to harbor pure cultures of CaSP, and only CaSP-negative specimens were subsequently screened with *Amycolatopsis*, general actinobacterial, and general eubacterial primers.
Sequences of actinobacterial 16S rRNA were aligned to the SILVA-ARB SSU database (Pruesse et al. 2007) using the SINA aligner (Pruesse et al. 2012) and imported into ARB (Ludwig et al. 2004). The most closely related strains for each beewolf isolate as well as representative strains of the actinobacterial genera containing isolates were selected for phylogenetic analysis. Furthermore, CaSP strains were included as a reference. The alignment was exported from ARB, and an approximately-maximum-likelihood tree was reconstructed with FastTree 2.1 using the GTR model (Price et al. 2010). Local support values were estimated with the Shimodaira-Hasegawa test based on 1,000 resamples without reoptimizing the branch lengths for the resampled alignments (Price et al. 2010).

6.3.9 Localization of *Amycolatopsis* in the antennal gland reservoirs

To exclude the possibility of contamination and confirm that the *Amycolatopsis* sequences originated from bacteria within the antennal gland reservoirs, we performed morphological analyses and fluorescence in-situ hybridization (FISH) on an antenna of *P.* cf. *basalis* that was positive for *Amycolatopsis*. Half of the antenna was fixated in 95% ethanol, and semithin sections and 3D-reconstructions of the antennal gland reservoirs and the bacteria inside were obtained as described earlier (Goettler et al. 2007; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012) (Figure 6.3). The second half of the *P.* cf. *basalis* antenna containing *Amycolatopsis* was embedded in cold-polymerizing resin (Technovit 8100, Heraeus Kulzer) and used for FISH as described earlier (Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). The specific fluorescent probes Cy3-SPT177 (specific for '*Ca.* S. philanthi', see Kaltenpoth et al. 2006) and Cy3-Amy_16S (specific to *Amycolatopsis*; complementary to primer Amy_16S_1F) as well as the general eubacterial probe Cy3-EUB338 (Amann et al. 1990) were used to stain the bacteria within the antennal gland reservoirs (Table S3, Figure 6.4). To confirm the specificity of the Cy3-Amy_16S probe, an antenna of a female *P. triangulum* specimen was prepared for FISH in the same way and stained with the same probes (data not shown).

6.3.10 Detection of CaSP in sand surrounding beewolf nests

In order to assess the possibility for horizontal uptake of CaSP from nest material, we screened sand from observation cages which had previously been occupied by beewolves for the presence of CaSP using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) of bacterial 16S rRNA genes. Total DNA was extracted from microorganisms separated by Nicodenz® gradient centrifugation as

described previously (Berry et al. 2003). Briefly, six sand samples (30 g each) were filled up to 50 ml with disruption buffer (0.2 M NaCl, 50 mM Tris-HCl pH 8.0) and thoroughly mixed. Large sand particles were sedimented by centrifugation at 100 × g for five minutes at room temperature. The supernatant was transferred into the tubes with Nicodenz® and cells were separated from sand particles at 10,000 × g for 20 min at 4°C. Cells were collected from the surface of Nicodenz®, washed three times with PBS and finally, total DNA was extracted with the SoilMaster™ DNA Extraction Kit (Epicentre). The quality of extracted DNA was checked by 1% agarose gel electrophoresis and PCR with the general eubacterial 16S rRNA primers fD1 and rP2. The DNA extracts from the six samples were pooled for bTEFAP.
BTEFAP was done commercially by Research and Testing Laboratory (Lubbock, TX, USA). In total, 8665 reads were generated using primers Gray28F (5'-GAGTTTGATCNTGGCTCAG-3') and Gray519r (5'-GTNTTACNGCGGCKGCTG-3') (Ishak et al. 2011; Sun et al. 2011). Generation of the sequencing library was established through one-step PCR with 30 cycles, using a mixture of Hot Start and HotStar high-fidelity Taq polymerases (Qiagen). Sequencing extended from Gray28F, using a Roche 454 FLX instrument with Titanium reagents and procedures at Research and Testing Laboratory (RTL), based upon RTL protocols (http://www.researchandtesting.com). All low-quality reads (quality cut-off = 25) and sequences <200 bp or >600 bp were removed following sequencing, which left 7,123 sequences for subsequent analysis. Processing of the high-quality reads was performed using QIIME (Caporaso et al. 2010). The sequences were denoised using the denoiser algorithm (Reeder and Knight 2010) and subsequently clustered into operational taxonomic units (OTUs) using multiple OTU picking with cdhit (Li and Godzik 2006) and uclust (Edgar 2010) with 97% similarity cut-offs. For each OTU, one representative sequence was extracted (the most abundant) and aligned to the Greengenes core set (available from http://greengenes.lbl.gov/) using PyNast (Caporaso et al. 2010), with the minimum sequence identity per cent set to 75. Taxonomy was assigned using RDP classifier (Wang et al. 2007), with a minimum confidence to record assignment set to 0.80. For visualization of the results, OTUs were combined based on phylum-level taxonomic affiliation. To assess the number of CaSP reads within the sample, all high-quality sequences were compared to the '*Ca.* S. philanthi triangulum' 16S reference sequence (GenBank accession number DQ375802) by BLAST, and identical sequences were counted using a custom-made Perl script (Figure 6.6). This provided a conservative estimate for the number of CaSP sequences in the samples, as it excluded highly similar sequences containing even low numbers of sequencing errors.

6.3.11 Behavioral assays

Female beewolves were reared in observation cages as described previously (Strohm and Linsenmair 1995) and provided with honey and bees *ad libitum*. Freshly constructed brood cells were checked for the presence of the white antennal gland secretion (AGS) containing the symbiotic bacteria. In the observation cages, the AGS is usually visible with the unaided eye after secretion by the female beewolf to the ceiling of the brood cell (Strohm and Linsenmair 1995). Six females did not apply AGS to any of their brood cells (AGS-), whereas the AGS was regularly found in brood cells of all other females (AGS+ females). The AGS- and seven randomly selected AGS+ females were sacrificed, and RNA and DNA were extracted from the antennae using the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. DNA extracts were screened with CaSP- (Strep_phil_fwd3/Act-A19) and Actinobacteria-specific (Act-S20/Act-A19) primers as described above, and products from Act-PCRs were sequenced bidirectionally. In case of double signals obtained from the sequencing reaction, PCR products were cloned into *E. coli* using the TOPO TA cloning kit (Invitrogen), and cloned products were sequenced bidirectionally. Sequences were aligned with the '*Ca.* S. philanthi triangulum' 16S

rRNA sequence to check for similarity and compared with the NCBI database using BLASTn. Sequences that were distinct from CaSP were included in the phylogenetic analyses described above.

6.4 RESULTS AND DISCUSSION

6.4.1 Dated phylogeny of beewolves

To reconstruct the phylogenetic relationships across beewolves and closely related wasps, we determined sequences of five nuclear (*28S*, *lwrh*, *argK*, *wnt*, *ef1a*) and one mitochondrial gene (*coxI*) for 50 Philanthini (*Philanthus, Trachypus, Philanthinus*) that engage in a defensive symbiosis with CaSP, as well as several outgroup taxa that lack antennal symbionts (Kaltenpoth et al. 2006) (Tables S1-S3). Based on the concatenated alignment of 5,521 bp, phylogenetic analyses strongly supported monophyly of the three genera with antennal symbionts (Figure 6.1). As previously hypothesized (Alexander 1992), our results indicate that *Trachypus* renders *Philanthus* paraphyletic. Since we included representatives of all genera in the subfamily Philanthinae (sensu Alexander 1992) except for the very rare *Pseudoscolia* (which is probably most closely related to Cerceris and Eucerceris, see Alexander 1992), we conclude that the symbiosis with CaSP in antennal gland reservoirs had a single origin in the ancestor of the tribe Philanthini.

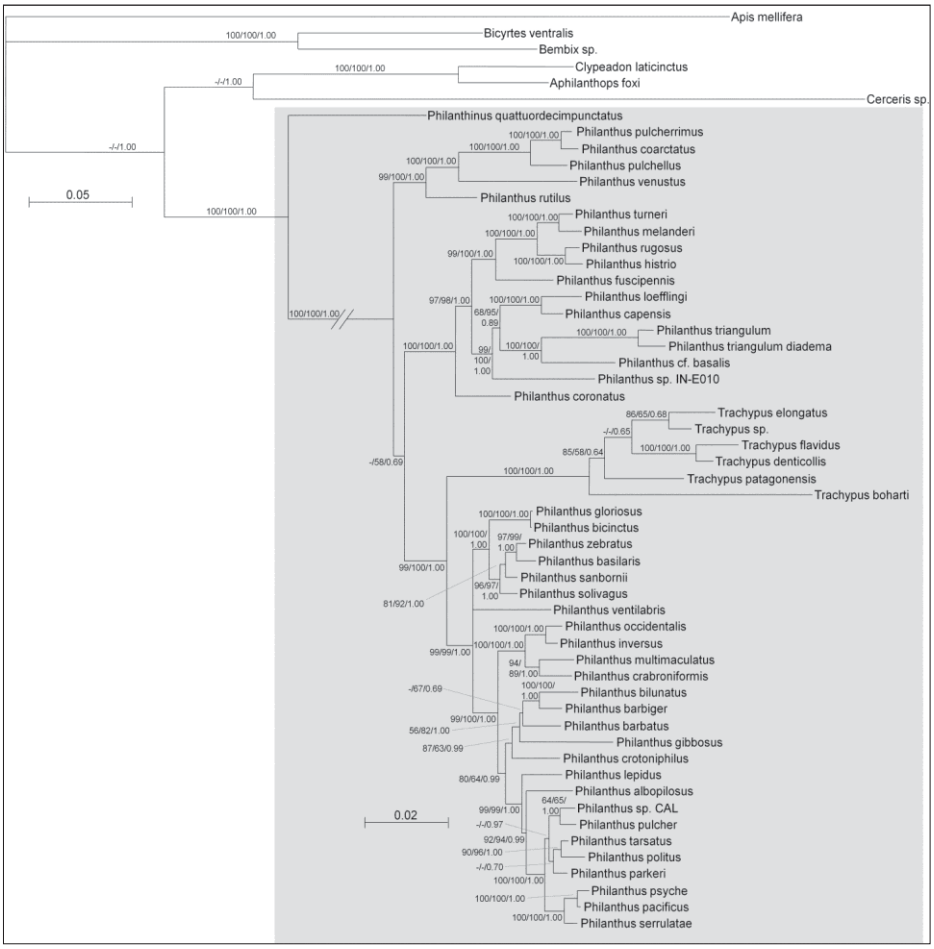

Fig. 6.1 Reconstruction of phylogenetic relationships among Philanthini digger wasps, based on the concatenated alignment of 5521 bp of *28S, lwrh*, *argK*, *wnt*, *ef1a*, and *coxI*. Bootstrap values (>50%) from maximum-parsimony (MP, 1000 replicates) and maximum likelihood (ML, 10000 replicates) analyses as well as Bayesian posterior probabilities (>0.5) are provided at the nodes. Taxa with antennal *Streptomyces* symbionts are highlighted with grey background. Scale bars represent substitutions per site.

Three fossil calibration points were used to infer minimum ages of divergence within the beewolf phylogeny : (A) *Psammaecius sepultus* (Bembecinae) from Florissant beds in Colorado (Cockerell 1906; Pulawski and Rasnitsyn 1980), which date back to the latest Eocene (~34.1 Mya) (Evanoff et al. 2001), (B) *Cerceris berlandi* from late Stampian shales (~30 Mya) in France (Timon-David 1944), and (C) two Philanthini fossils from Colorado (*Philanthus saxigenus* and *Prophilanthus destructus,* ~34.1 Mya) (Rohwer 1909; Theobald 1937) and one from France (*Philanthus annulatus*, ~30 mya) (Theobald 1937). Due to the doubtful systematic affiliation of the *Philanthus* and *Prophilanthus* fossils, the analyses were also repeated excluding these fossil calibration points, which did not significantly affect the age estimation for the origin of the symbiosis. The age for the root was set to 140±10 mya (mean±SD), since both the divergence of Sphecidae from other Apoidea and that of Crabronidae and bees have been estimated to the period of 130-150 mya (Grimaldi and Engel 2005; Cardinal and Danforth 2013), coincident with the rise of the angiosperms.

Among the tested evolutionary models (GTR, GTR+I+G, HKY, HKY+G, HKY+I+G), assessment of convergence and Tracer v1.5 (Rambaut and Drummond 2007) evaluation of Bayes factors revealed the HKY+G and HKY+I+G as the best models. Across all models, the partitioned dataset (nine gene partitions, and codon partitioning [1+2, 3] for *argK, ef1a* and *wnt*) consistently yielded better likelihood scores than the non-partitioned dataset, and the uncorrelated lognormal relaxed clock model outperformed the strict clock model. Despite some minor topological discrepancies within the Philanthini (i.e. the placement of *Trachypus boharti, Philanthus albopilosus*, and *P. ventilabris*), both HKY+G and HKY+I+G models consistently yielded age estimates of 64.7 to 68.7 mya (lower boundary) to 102.0 to 107.5 mya (upper boundary) for the age of the beewolf-*Streptomyces* symbiosis, regardless of whether the input tree was fixed to the ML input tree or not. Furthermore, estimates for the symbiosis age changed only slightly when a uniform distribution was used to model the ancestral age of the Cercerini+Aphilanthopini instead of a lognormal distribution, or when the putative *Philanthus* and *Prophilanthus* fossils or the root calibration was omitted (60.1 to 68.3 mya for the lower and 92.3 to 110.5 for the upper boundary). Omitting both calibration points, however, resulted in low performance of the HKY+G model and yielded considerably lower age estimates for the symbiosis (39.4 to 56.3 mya for the lower and 62.8 to 86.4 mya for the upper boundary).

Based on the model evaluations, the HKY+G substitution model with fixed input tree, relaxed uncorrelated lognormal clock model, and the inclusion of the *Cerceris, Psammaecius*, and root calibration points yielded the most reliable age estimate of 68.3 mya (95% HPD: 44.8-92.8 mya) to 110.0 mya (95% HPD: 80.9-140.4 mya) for the origin of the association with *Streptomyces* (Figure 6.2). Thus, the beewolf-*Streptomyces* symbiosis evolved more recently than many of the intimate nutritional mutualisms in insects, e.g. the aphid-*Buchnera* (160 - 280 mya, see Moran et al. 1993), cockroach-*Blattabacterium* (135 - 250 mya, see Bandi et al. 1995), planthopper-*Vidania* (>130 mya, see Urban and Cryan 2012), and Auchenorrhyncha-*Sulcia* (260-280 mya, see Moran et al. 2005) associations. However, it is probably more ancient than the functionally similar defensive association between leaf-cutter ants and antibiotic-producing *Pseudonocardia* bacteria, because fungus-farming did not evolve in ants before around 50 mya (Schultz and Brady 2008). Therefore, to our knowledge, the beewolf-*Streptomyces* mutualism represents the oldest known defensive symbiosis in insects to date.

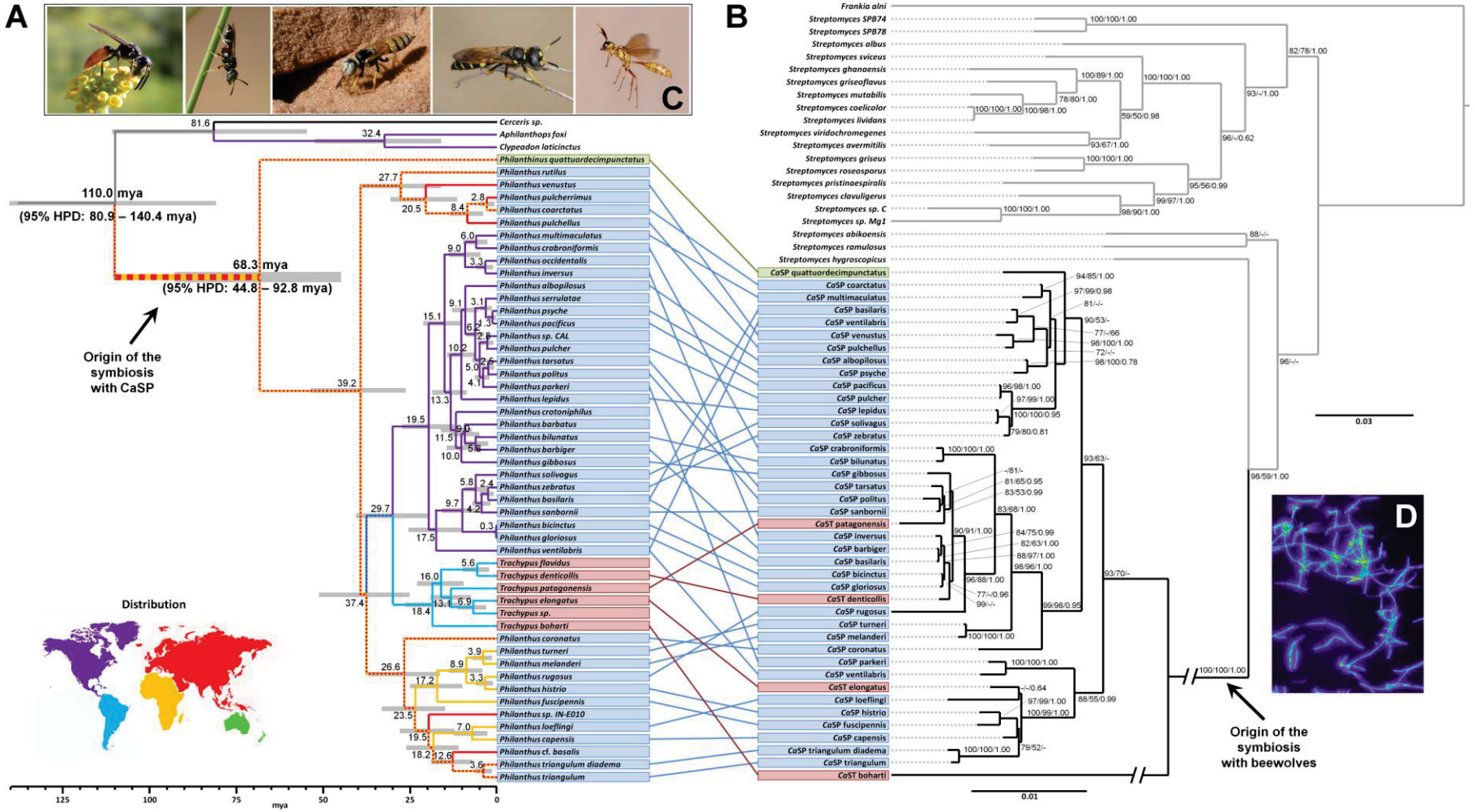

Fig. 6.2 Co-phylogenetic analysis of beewolves (**A**) and their defensive antennal symbionts (**B**). Node ages in the host phylogeny are shown in million years ago (mya) with 95% highest posterior density (HPD) interval bars. Branches are color-coded according to the geographic distribution of the host species (see world map). Colored boxes around host and symbiont names denote host genera (green=*Philanthinus*, blue=*Philanthus*, red=*Trachypus*). Host-symbiont associations are shown by connecting lines. Values at the nodes of the symbiont phylogeny are local support values from the FastTree analysis (GTR model), bootstrap values from PHYML, and Bayesian posteriors, respectively. The origin of the symbiosis is highlighted in both phylogenies by arrows. (**C**) Photographs of selected Philanthini host species: *Philanthus loefflingi* male, *P. pulcherrimus* male, *P. basilaris* female at its nest entrance, *P. coronatus* male, and *Trachypus boharti* female (from left to right). (**D**) Fluorescence micrograph of CaSP from the antennal gland secretion of a female *Philanthus triangulum* (in pseudocolors).

6.4.2 Biogeography of beewolves

The molecular phylogeny of the Philanthinae revealed Eurasia or Africa as the likely geographic origin of beewolves, since *Philanthinus* and all the basal *Philanthus* species show a palearctic or paleotropic distribution (Figure 6.2). The adaptive radiation of beewolves was likely promoted by the evolution of angiosperms and their most important pollinators, the bees, as adult beewolves feed on the nectar of angiosperms and predominantly use bees as larval provisions (Evans and O'Neill 1988). Bees originated during the early to mid-Cretaceous in the southern hemisphere (Gondwana), most likely in Africa (Danforth et al. 2006). Considering this and the high diversity of extant *Philanthus* species in Africa (77 out of 137 species), it seems likely that the Philanthini evolved on this continent as well. Subsequently, they spread across the paleotropics and palearctic and subsequently colonized the Americas about 31-38 mya (95% CI: 23-48 mya), possibly via the Thulean bridge (Tiffney 1985) or the northern land bridge Beringia (about 55–35 mya, Wolfe 1972). The exchange between North and South America may have occurred via the Aves ridge in the Eocene-Oligocene boundary, corresponding to the time of the estimated split between *Trachypus* and the north American *Philanthus* clade (about 31 mya, see Figure 6.2) (Iturralde-Vincent and MacPhee 1999). Interestingly, the South Indian *Philanthus* species are interspersed among Asian and African taxa in our phylogeny, suggesting that the Indian species were recruited from both Africa and Asia.

6.4.3 Prevalence of antennal *Streptomyces* symbionts across beewolves

To assess the prevalence of antennal symbionts across beewolf host species, we screened 338 females from 34 species and subspecies for the presence of CaSP using diagnostic 16S rRNA gene primers (Kaltenpoth et al. 2006). We detected CaSP in 93% of all individuals, and prevalence ranged from 67-100% within species, with the exception of *Philanthus* cf. *basalis* (Table 6.1). We tested apparently symbiont-free individuals for other eubacterial taxa and occasionally found actinobacteria other than CaSP, proteobacteria or mollicutes, in or on female beewolf antennae. *Amycolatopsis* was found in the antennae of both available individuals of *P.* cf. *basalis* and in two *P. triangulum* individuals (out of 68) from Germany. For *P.* cf. *basalis*, we verified the replacement of CaSP by *Amycolatopsis* and its growth in the antennal gland reservoirs by specific fluorescence *in-situ* hybridization (Figures 6.3-6.4). Whether these symbiont replacements represent rare individual cases or a complete lineage replacement in *P.* cf. *basalis* cannot be determined because of the small sample size (N=2). The occurrence of proteobacteria (*Wolbachia, Serratia*) and mollicutes (*Spiroplasma*) probably represent systemic infections of the hosts, including the antennal hemolymph, rather than specialized colonization of the antennal gland reservoirs.

Table 6.1 Infection prevalence of CaSP across 34 different species of beewolves, as revealed by diagnostic PCRs for CaSP. Diagnostic PCRs for *Amycolatopsis* and general PCRs for Actinobacteria and Eubacteria were used to detect other bacterial symbionts in beewolf antennae.

			Antennal symbionts				CaSP infection rate	
Species	**Number of species (total)**	**CaSP**	**CaSP+ others (co-infection)**	**other Actino-bacteria**	**other bacteria**	**no bacteria**	**of all (%)**	**of colonized antennae (%)[1]**
Philanthus albopilosus	3	2		1			67	67
Philanthus barbiger	28	27				1	96	100
Philanthus cf. *basalis*	2	0		2			0	0
Philanthus basilaris	25	25					100	100
Philanthus bicinctus	3	3					100	100
Philanthus capensis	1	1					100	100
Philanthus coarctatus	1	1					100	100
Philanthus coronatus	1	1					100	100
Philanthus crabroniformis	2	2					100	100
Philanthus fuscipennis	5	5					100	100
Philanthus gibbosus	2	2					100	100
Philanthus gloriosus	6	6					100	100
Philanthus histrio	1	1					100	100
Philanthus inversus	1	1					100	100
Philanthus lepidus	1	1					100	100
Philanthus loefflingi	6	4	1	1		1	83	100
Philanthus melanderi	3	2				1	67	100
Philanthus multimaculatus	19	17				2	89	100
Philanthus pacificus	3	3					100	100
Philanthus parkeri	36	36					100	100
Philanthus psyche	15	15					100	100
Philanthus pulchellus	2	2					100	100
Philanthus pulcher	4	4					100	100
Philanthus rugosus	4	4					100	100
Philanthus triangulum	68	53	2	6	2	7	81	93
Philanthus triangulum diadema	7	6			1		86	100
Philanthus turneri	1	1					100	100
Philanthus ventilabris	6	6					100	100
Philanthus venustus	2	2					100	100
Philanthus zebratus	2	2					100	100
Trachypus bohartí	68	66		1	1		97	99
Trachypus elongatus	5	5					100	100
Trachypus patagonensis	2	2					100	100
Philanthinus quattuordecimpunctatus	3	3					100	100
Total	**338**	**311**	**3**	**11**	**4**	**12**	**93**	98

[1]Excluding all antennae without Actinobacteria ("no bacteria" and "other bacteria"), as the latter probably represent systematic infections with *Wolbachia, Spiroplasma*, or *Serratia*

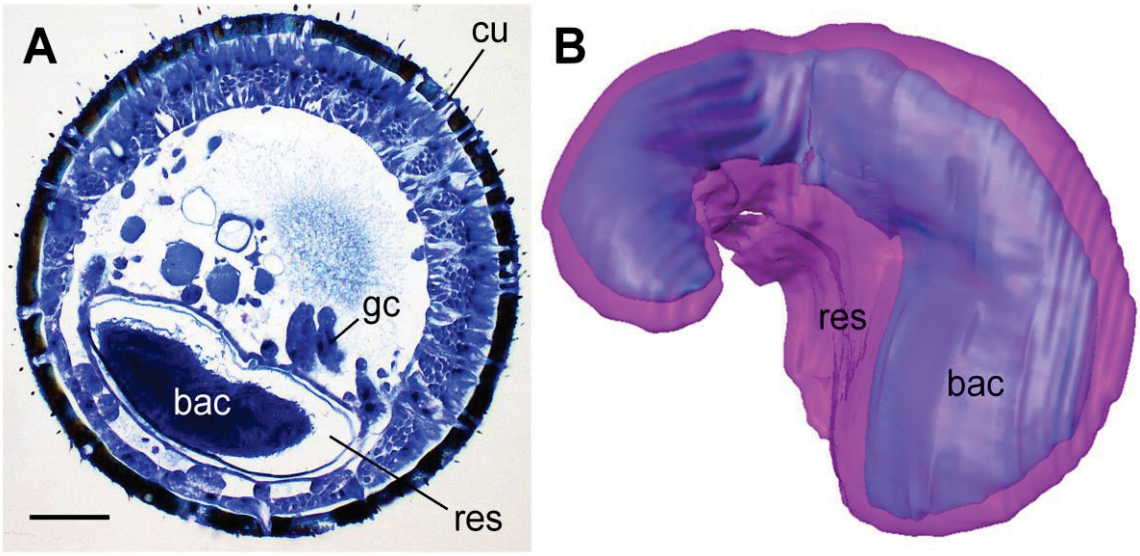

Fig. 6.3 Antennal gland reservoir of a female *P.* cf. *basalis* with non-symbiotic *Amycolatopsis* bacteria. (A) Cross section through the antenna, with the reservoir and the bacteria within clearly visible. Scale bar: 50µm. (B) Three-dimensional reconstruction of the same antennal gland reservoir (pink) containing bacteria (blue). Note that the same antenna was used for FISH to confirm the identity of *Amycolatopsis* (see Figure 6.4). res=reservoir; bac=bacteria; gc=gland cells; cu=antennal cuticle.

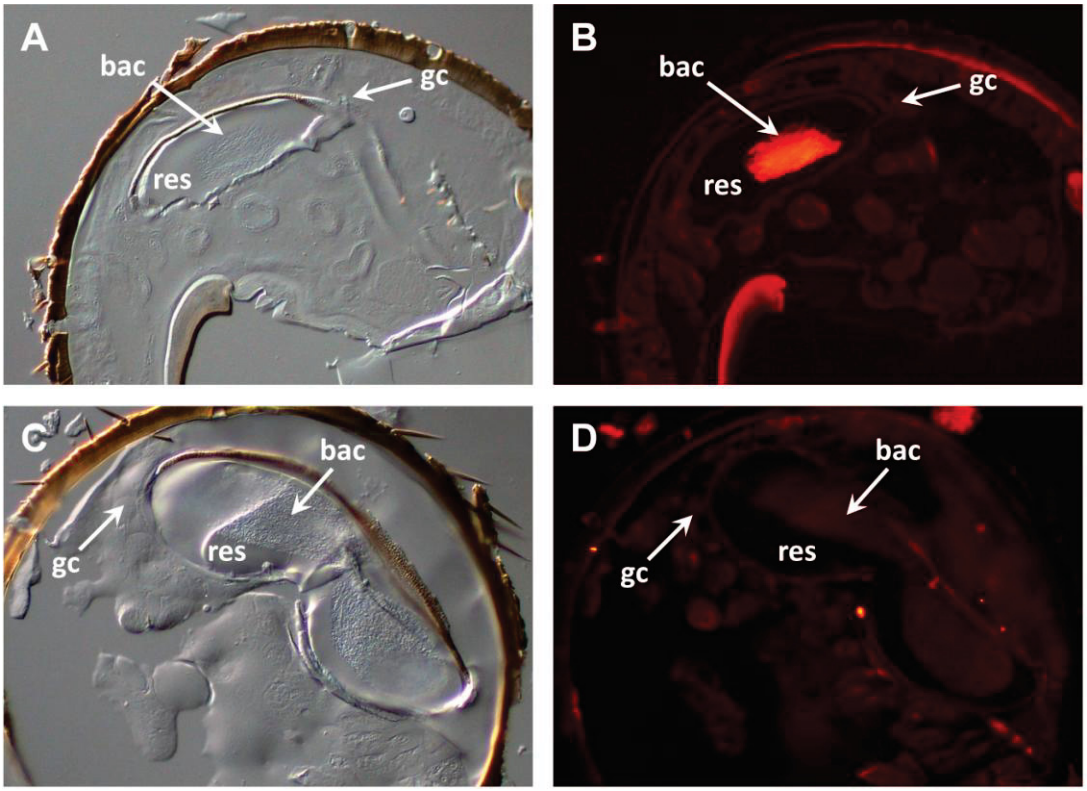

Fig. 6.4 Replacement of CaSP symbionts by *Amycolatopsis* in antennae of a female *Philanthus* cf. *basalis*. (**A**) and (**C**) Differential interference contrast micrographs of antennal cross-sections. (**B**) and (**D**) Fluorescent micrographs of the same areas, after staining with the *Amycolatopsis*-specific probe Amy_16S-Cy3 (**B**) or the CaSP-specific probe SPT177-Cy3 (**D**). bac=bacteria, res=antennal gland reservoir, gc=gland cells.

6.4.4 Host-symbiont co-evolutionary history

We reconstructed the phylogeny of CaSP symbionts from 34 *Philanthus*, four *Trachypus* and one *Philanthinus* host species, using partial sequences of 16S rRNA, elongation factor-G and -Tu (*fus-tuf*), gyrase B (*gyrB*) and gyrase A (*gyrA*). Both BI and ML analyses provided strong support for the monophyly of the symbiont clade within *Streptomyces* (Figure 6.2), implying a single origin of the association. Randomization tests yielded evidence for overall co-cladogenesis of beewolves and CaSP (Parafit: P=0.001; TreeMap: P=0.003, Jane3: P<0.05). However, a comparison of the phylogenies also revealed numerous discrepancies between host and symbiont trees, indicating horizontal transmission of symbionts among host species (Figure 6.2).

To explore the prevalence of symbiont exchange within and across beewolf populations, we sequenced *gyrA* from the symbionts of 109 beewolf individuals in 41 species. The topology of the *gyrA* tree was very similar to the multi-gene phylogeny, and symbiont sequences of individuals from the same host species were identical or clustered together for all but three species (Figure 6.5). While for *P. gibbosus*, CaSP strains were closely related, this was not the case for *P. ventilabris* and *P. basilaris*. These species occur sympatrically with other beewolves, and interspecific predation among *Philanthus* has occasionally been observed (Evans and O'Neill 1988), so it is conceivable that some lineages have recently acquired symbionts horizontally from congeneric beewolf females that served as larval provisions (specifically, *P. ventilabris* and *P. basilaris* may have acquired symbionts from the two smaller sympatric species *P. parkeri* and *P. barbiger*, respectively, see Figure 6.2). A second possible explanation for horizontal transfer of symbionts is reuse of nests and brood cells that occurs in some beewolf species (Evans and O'Neill 1988). A third alternative is that a reservoir of CaSP spores might subsist in beewolf habitats and thereby facilitate diffuse horizontal exchange (Koehler et al. 2013). Consistent with the latter two hypotheses, we detected CaSP DNA in sand from used beewolf observation cages by pyrosequencing bacterial 16S amplicons (385 out of 7123 total sequences=5.4%, Figure 6.6). Although Actinobacteria are subject to intensive search efforts due to their pharmacological potential, screenings for novel strains are based on the cultivation of environmental isolates and would therefore miss CaSP due to its refractoriness to cultivation (Kaltenpoth et al. 2006).

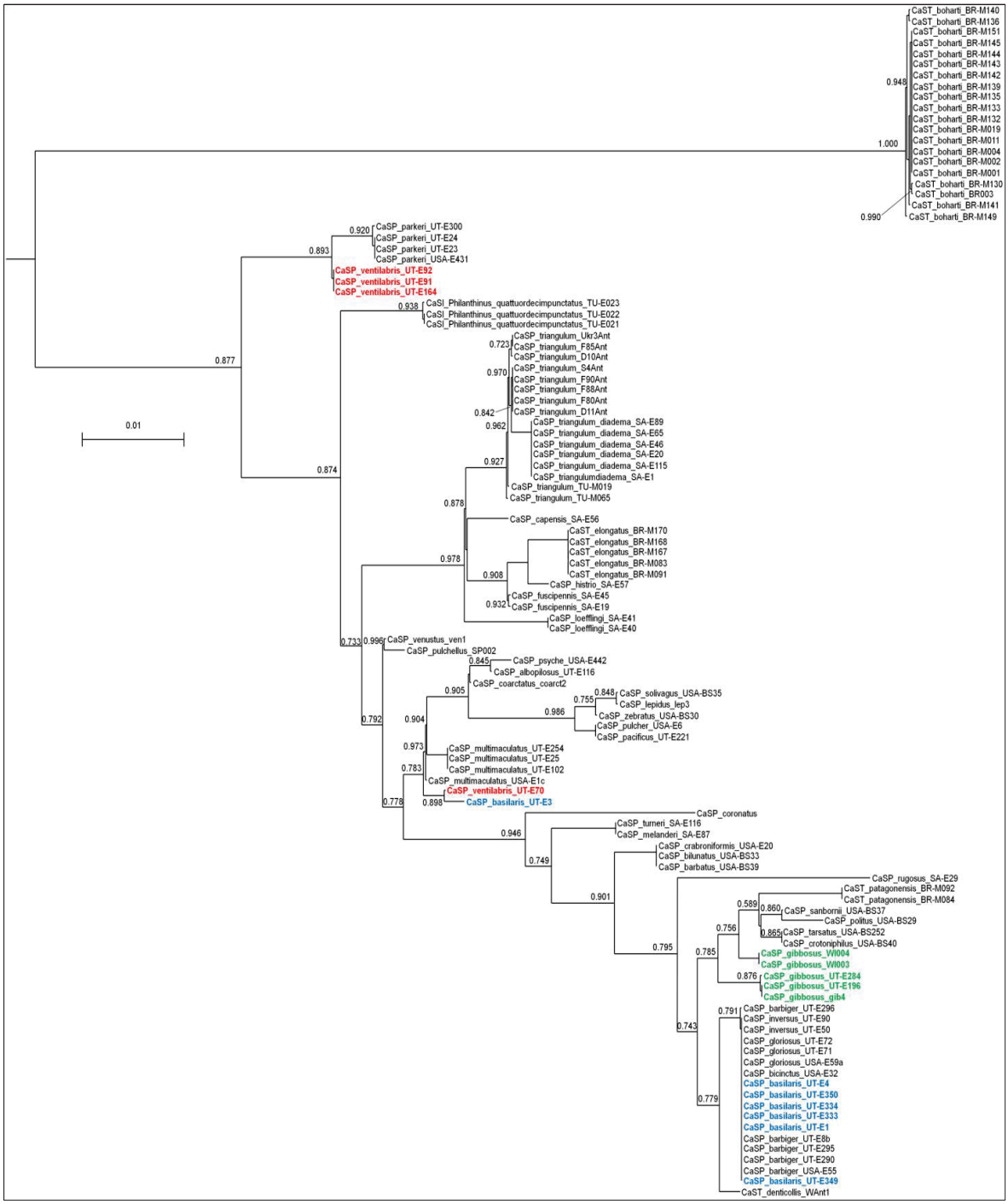

Fig. 6.5: Phylogenetic relationships among symbionts of 109 beewolf individuals across 41 species, based on partial gyrase A (gyrA) sequences. The phylogeny was reconstructed using FastTree (GTR model), and numbers at the nodes represent local support values. Host species with individuals carrying symbionts in different clades are highlighted in different colors. Scale bar represents substitutions per site.

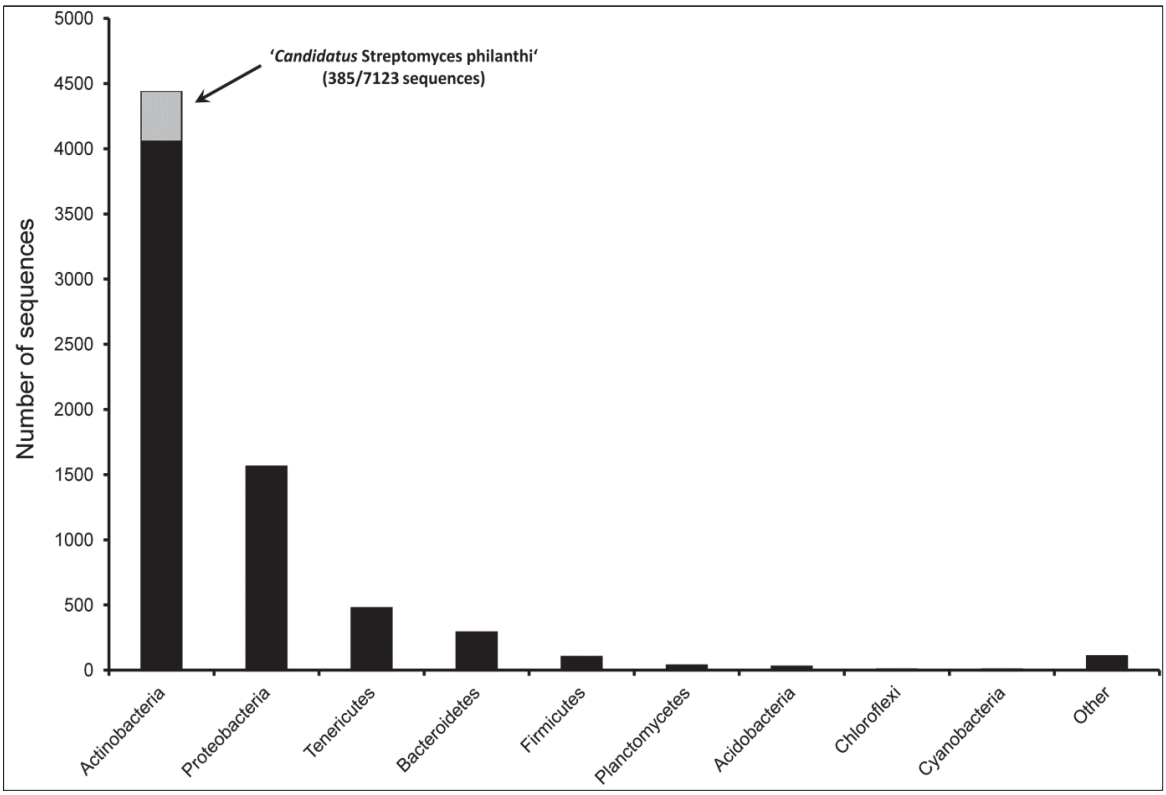

Fig. 6.6: Detection of CaSP in sand from beewolf rearing cages. The microbial community composition was determined by bacterial tag-encoded FLX amplicon sequencing (bTEFAP) of bacterial 16S rRNA. After quality control, denoising and OTU picking (cdhit and uclust), OTUs were combined based on phylum-level taxonomic affiliation. The proportion of '*Candidatus* Streptomyces philanthi triangulum' 16S reads is highlighted in grey.

6.4.5 Maintenance of specificity in the symbiotic association

Considering the ample opportunities for non-symbiotic actinobacteria to be taken up by beewolf females, how is specificity maintained in the beewolf-CaSP symbiosis? Behavioral observations indicate that females harboring non-symbiotic actinobacteria in their antennal gland reservoirs do not secrete these bacteria into their brood cells (homogeneity test with Yates's correction, χ^2=5.49, P=0.019, Table 6.2). Thus, the lack of transmission apparently mediates partner choice and thereby contributes to the long-term stability of the beewolf-*Streptomyces* mutualism. Two alternative hypotheses may provide mechanistic explanations for these observations: either beewolves are able to recognize beneficial symbionts and sanction non-cooperating microbes, or the conditions in the antennal reservoirs are such that only CaSP can grow and/or be successfully transmitted.

Specific recognition of mutualistic partners by the interaction of microbial oligo- or polysaccharides with membrane-bound sugar-binding proteins (lectins) and subsequent host sanctions have been demonstrated for several environmentally acquired symbionts, notably mycorrhizal fungi and nitrogen-fixing rhizobia of plants (Denarie et al. 1996; Kiers et al. 2003; Kiers et al. 2011; Maillet et al. 2011), and bioluminescent bacteria of squid (Nyholm and McFall-Ngai 2004; Nyholm and Graf 2012). The alternative hypothesis of a selective host environment that favors establishment of certain antibiotic-producing symbionts has recently been proposed to shape the association between leaf-cutter ants and their actinobacterial community (Scheuring and Yu 2012). Although certain bacteria other than CaSP can apparently grow in beewolf antennae (Figures 6.3-6.4), it is possible that their long-term subsistence and/or secretion into the brood cell are prevented by the selective conditions provided by

the host. Both mechanisms require the transport of specific host factors into the antennal gland reservoirs, which is likely to occur from the hemolymph (Kaltenpoth et al. 2009) or the associated gland cells (Goettler et al. 2007). The adaptive significance of blocking transmission during application of the antennal gland secretion to the brood cell rather than at symbiont uptake from the cocoon remains to be investigated.

Table 6.2 Antennal symbionts of beewolf females (*Philanthus triangulum*) applying (AGS+) or not applying (AGS-) visible amounts of antennal gland secretion to their brood cells.

Species number	Age (d)	AGS visible	Number of brood cells	Brood cells with visible AGS		Antennal symbionts (diagnostic PCRs and sequencing)	BLAST identity
				number	proportion (%)		
10b	51	+	39	29	74,4	CaSP	
12a	56	+	48	42	87,5	CaSP	
15d	57	+	36	30	83,3	CaSP	
24c	24	+	9	7	77,8	CaSP	
25c	46	+	12	12	100	CaSP	
29c	36	+	32	26	81,3	CaSP	
04c	53	+	18	11	61,1	Rhodococcus baikonurensis,	100%
						Nocardioides simplex	100%
08a	18	-	2	0	0	none detected	
29b	60	-	35	0	0	Streptomyces pluricolorescens	99%
04b	63	-	17	0	0	Streptomyces flavofuscus	100%
19a	31	-	5	0	0	Streptomyces ramulosus	99%
10c	44	-	23	0	0	Streptomyces rochei	99%
15c	34	-	20	0	0	Streptomyces phaeochromogenes	99%

6.5 CONCLUSIONS

The observed pattern of diffuse co-evolution between beewolves and defensive *Streptomyces* symbionts indicates that, despite the fact that they are localized in specialized antennal gland reservoirs, their extracellular lifestyle and external route of transmission allow for horizontal symbiont replacement and uptake of non-symbiotic Actinobacteria. However, in contrast to other insect symbioses which rely on partner choice rather than fidelity (Zhang et al. 2007; Cafaro et al. 2011; Kikuchi et al. 2011), only a distinct monophyletic clade of symbionts appears to be able to successfully establish a long-term association with the host. Thus, the beewolf-*Streptomyces* mutualism presents an interesting intermediate case between strictly vertically transmitted primary symbionts and more loosely associated secondary symbionts. Partner choice at the point of symbiont transmission may have reinforced host-symbiont fidelity and thereby promoted the long-term stability of the mutualistic association with a specific clade of symbionts since origin of the association in the Cretaceous.

6.6 SUPPLEMENT

6.6.1 Supplementary tables

Table S1 Collection localities and GenBank accession numbers for beewolf specimens used to reconstruct the host phylogeny.

			Collection	NCBI accession number					
Species	Specimen no.	Sex	locality	Wingless	LWRh	EF1a	28s	ArgK	COI
P. fuscipennis	SA-E69	Male	South Africa	JN74210	N/A	N/A	JN674263	JQ083453	JQ040272
P. gibbosus	UT-E186	Male	USA	JN74211	N/A	N/A	JN674264	JQ083454	-
P. gloriosus	USA-E60f	Male	USA	JN74212	N/A	N/A	JN674265	JQ083455	JQ040273
P. histrio	SA-E58	Male	South Africa	JN74213	N/A	N/A	JN674266	JQ083456	JQ040274
P. inversus	USA-E53b	Male	USA	JN74214	N/A	N/A	JN674267	JQ083457	-
P. lepidus	CAN-E1	Male	Canada	JN74215	N/A	N/A	JN674268	JQ083458	-
P. loeffllingi	SA-E13	Male	South Africa	JN74216	N/A	N/A	JN674269	JQ083459	JQ040275
P. melanderi	SA-E79	Male	South Africa	JN74217	N/A	N/A	JN674270	JQ083460	JQ040276
P. multimaculatus	UT-E76	Male	USA	JN74218	N/A	N/A	JN674271	JQ083461	JQ040277
P. occidentalis	CAL-Eth4	Male	USA	JN74219	N/A	N/A	JN674272	JQ083462	JQ040278
P. pacificus	USA-E19	Male	USA	JN74220	N/A	N/A	JN674273	JQ083463	JQ040279
P. parkeri	UT-E45	Male	USA	JN74221	N/A	N/A	JN674274	JQ083464	JQ040280
P. politus	JS-32a		USA	JN74222	N/A	N/A	JN674275	JQ083465	-
P. psyche	UT-E154/ *JS-A	Male	USA	JN74223	N/A	N/A *	JN674276	JQ083466	JQ040281
P. pulchellus	SP-001	Male	Spain	JN74224	N/A	N/A	JN674277	JQ083467	JQ040282
P. pulcher	USA-E8b	Female	USA	JN74225	N/A	N/A	JN674278	JQ083468	JQ040283
P. pulcherrimus	IN-E064	Male	India	JN74226	N/A	N/A	JN674279	JQ083469	JQ040284
P. rugosus	SA-E23	Male	South Africa	JN74227	N/A	N/A	JN674280	JQ083470	JQ040285
P. rutilus	JS-32			JN74228	N/A	N/A	JN674281	JQ083471	-
P. sanbornii	m28	Male	USA	JN74229	N/A	N/A	JN674282	JQ083472	JQ040286
P. serrulatae	JS-63	Female	USA	JN74230	N/A	N/A	JN674283	-	-
P. solivagus	USA-BS36	Male	USA	JN74231	N/A	N/A	JN674284	JQ083473	-
P. sp. IN-E010	IN-E010	Male	India	JN74232	N/A	N/A	JN674285	JQ083474	JQ040287
P. sp. CAL	CAL-Eth14	Male	USA	JN74233	N/A	N/A	JN674286	JQ083475	-
P. tarsatus	JS-44	Male	USA	JN74234	N/A	N/A	JN674287	JQ083476	-
P. triangulum	N14/ *JS-B	Male	Germany	JN74235	N/A	N/A *	JN674288	JQ083477	JQ040288
P. triangulum diadema	SA-E8	Male	South Africa	JN74236	N/A	N/A	JN674289	JQ083478	JQ040289
P. turneri	SA-E116	Female	South Africa	JN74237	N/A	N/A	JN674290	JQ083479	JQ040290
P. ventilabris	USA-E30	Male	USA	JN74238	N/A	N/A	JN674291	JQ083480	JQ040291
P. venustus	Ph02	Male	Greece	JN74239	N/A	N/A	JN674292	JQ083481	-
P. zebratus	USA-E25	Male	USA	JN74240	N/A	N/A	JN674293	JQ083482	JQ040292
Trachypus bohartı	BR-002	Female	Brasil	JN74250	N/A	N/A	JN674294	JQ083483	JQ040293
Trachypus denticollis	JS-11		Chile	JN74241	N/A	N/A	JN674295	JQ083484	-
Trachypus elongatus	BR-E032	Male	Brasil	JN74242	N/A	N/A	JN674296	JQ083485	JQ040294
Trachypus flavidus	BR-E067	Male	Brasil	JN74243	N/A	N/A	JN674297	JQ083486	JQ040295
Trachypus patagonensis	BR-E092	Female	Brasil	JN74244	N/A	N/A	JN674298	JQ083487	JQ040296
Trachypus spec.	JS-52		Chile	JN74245	N/A	N/A	JN674299	JQ083488	-
Philanthinus quattuordecimpunctatus	TU-EY-E027	Male	Turkey	JN74246	N/A	N/A	JN674300	JQ083489	JQ040297
Aphilanthops foxi	CAL-Eth10	Male	USA	JN74247	N/A	N/A	JN674301	JQ083490	JQ040298
Bembix amoena/ *B. troglodytes	-		-	EU367331.1	-	EU367212.1	EU367154.1	-	EF203767.1*
Bicyrtes ventralis	-		-	-	DQ116701.1	AY585161	AY654458.1	-	-
Cerceris sybiensis/Eucerceris	*Cerc1/**Cerc2/***JS-C	Female	Germany/USA	* JN74248	N/A **	N/A ***	AY654460.1	JQ083491**	-
Clypeadon laticinctus	UT-E177/ *BS32a/ **JS-1	Female	USA	JN74249	N/A *	N/A **	JN674302	JQ083492	JQ040299
Apis mellifera	-		-	AY703618.1	U26026.1	NM_001014993.1	AY703551.1	NM_001011603.1	AF214668.1

Note:
N/A: accession numbers will be provided upon acceptance of the manuscript

Table S2 Primers used for the amplification and sequencing of host and symbiont genes, and probes for the fluorescence in-situ hybridization to detect CaSP and *Amycolatopsis* in beewolf antennae.

Target organism	Target sequence	Primer/probe name	5'-3' Sequence	Fwd/ rev	5'-mod.	Target taxon	Reference
Host primers	Wingless	beewgfor	TGCACNGTSAAGACCTGYTGGATG	fwd	-	Apoidea	Danforth et al. 2004
		Lepwg2a	ACTICGCARCACCARTGGAATGTRC	rev	-	Apoidea	Brower & DeSalle 1998, Danforth et al. 2004
	LWRh	LWRH_Rev1744	GCDGCTCGRTAYTTHGGATG	rev	-	Philanthinae	this study
		LWRhFor4_N	GAGAARAAYATGCGNGARCAAGC	fwd	-	Philanthinae	this study (modified from Danforth et al. 2004)
		LWRhFor1	AATTGCTATTAYGARACNTGGGT	fwd	-	Apoidea	Mardulyn & Cameron 1999, Danforth et al. 2004
		LWRhRev1	ATATGGAGTCCANGCCATRAACCA	rev	-	Apoidea	Mardulyn & Cameron 1999, Danforth et al. 2004
	EF1a	For1deg	GYATCGACAARCGTACSATYG	fwd	-	Apoidea	Danforth et al. 2003
		F2Rev1	AATCAGCAGCACCTTTAGGTGG	rev	-	Apoidea	Danforth et al. 2003
		HaF2for	GGGYAAAGGWTCCTTCAARTATGC	fwd	-	Apoidea	Danforth et al. 1999
		Cho10	ACRGCVACKGTYTGHCKCATGTC	rev	-	Apoidea	Danforth et al. 2003
	ArgK	ArgK_Loretta	TGATCGATGATCACTTCCTTTTCAA	fwd	-	Philanthinae	this study
		ArgK_fwd2	GACAGCAARTCTCTGCTGAAGAA	fwd	-	Apoidea	Kawakita et al. 2003
		ArgK_KLTrev2	GATKCCATCRTDCATYTCCTTSACRC	rev	-	Apoidea	www.danforthlab.entomology.cornell.edu/resources.html
	COI / COII	CO_fwd1	TGGAGCHTCWTTYAGATTAATAATY	fwd	-	Philanthinae	this study
		CO_rev2	TCCWCCAATWGTRAATAATAARAYA	rev	-	Philanthinae	this study
		CO_LCO	GGTCAACAAATCATAAAGATATTGC	fwd	-	insects	Folmer et al. 1994
		CO_Ben	GCWACWACRTAATAKGTATCATG	rev	-	insects	Kronauer et al. 2004
	28s rRNA	28s_3665F	AGAGAGAGTTCAAGAGTACGTG	fwd	-	Apoidea	Cameron & Mardulyn 2001
		28s_4749R	GTTACACACTCCTTAGCGGA	rev	-	Apoidea	Danforth et al. 2006
Symbiont primers	16S rRNA	fD1	AGAGTTTGATCCTGGCTCAG	fwd	-	Eubacteria	Weisburg et al. 1991
		rP2	ACGGCTACCTTGTTACGACTT	rev	-	Eubacteria	Weisburg et al. 1991
		Spa-2R	KTTCGCTCGCCRCTAC	rev	-	Eubacteria	Hain et al. 1997
		Strep_phil_fwd1	TACCGATCGCATGGTTGGTG	fwd	-	*Ca*. S. philanthi	Kaltenpoth et al. 2006
		Strep_phil_fwd2	TATGACTACYGAYCGCATGG	fwd	-	*Ca*. S. philanthi	Kaltenpoth et al. 2006
		Strep_phil_fwd3	CATGGTTRGTGGTGGAAAGC	fwd	-	*Ca*. S. philanthi	Kaltenpoth et al. 2006
		Strep_phil_fwd4	GTGGTGGAAAGCTCCGGC	fwd	-	*Ca*. S. philanthi	Kaltenpoth et al. 2006
		Amy_16S_1F	CCTGTACTTTGGGATAAGCCT	fwd	-	*Amycolatopsis*	this study
	EF-Tu	EF-Tu-1F	ATYACCAAGGTGCTGCACG	fwd	-	*Ca*. S. philanthi	this study
		EF-Tu-3F	TTCAAGGTCGAGGCCAACG	fwd	-	*Ca*. S. philanthi	this study
		EF-Tu-2R	GCCACCCTCGTCCTTSGAS	rev	-	*Ca*. S. philanthi	this study
		EF-Tu-3R	GCACCGGTGATCATGTTCTT	rev	-	*Ca*. S. philanthi	this study
	gyrB	gyrB-F1	GAGGTCGTGCTGACCGTGCTGCA	fwd	-	*Ca*. S. philanthi	Hatano et al. 2003
		gyrB-F3	TTCGTGAAGTACCTGAACTCG	fwd	-	*Ca*. S. philanthi	this study
		gyrB-R3	SAGCTTGACCGAGATGATCG	rev	-	*Ca*. S. philanthi	this study
		gyrB-R10	CGACTTGCGGATGATGTCC	rev	-	*Ca*. S. philanthi	this study
	gyrA	gyrA-5F	AACCTGCTGGCCTTCCAG	fwd	-	*Ca*. S. philanthi	this study
		gyrA-5R	AACGCCCATGGTGTCACG	rev	-	*Ca*. S. philanthi	this study
Symbiont probes	16S rRNA	SPT177	CACCAACCATGCGATCGGTA	rev	Cy3	*Ca*. S. philanthi	Kaltenpoth et al. 2005
		Amy_16S	AGGCTTATCCCAAAGTACAGG	rev	Cy3	*Amycolatopsis*	this study
		EUB338	GCTGCCTCCCGTAGGAGT	rev	Cy3	Eubacteria	Amann et al. 1990

Table S3 Primer combinations and PCR conditions used for amplification and sequencing of host and symbiont genes.

Target organism	Target sequence	Forward primer	Reverse primer	PCR cycle number	Annealing temp. (°C)	Fragment length (bp)	Sequencing primers Forward	Reverse
Host	28s	28s_3665F	28s_4749R	35	62,9	1080	28s_3665F	28s_4749R
	Opsin	LWRhFor1	LWRhRev1	35	58,5	650	LWRhFor1	-
		LWRhFor4_N	LWRH_Rev1744	35	53,8	800	-	Rev1744
		LWRhFor1	LWRH_Rev1744	35	53,8	1200	LWRhFor1	LWRH_Rev1744
	wingless	beewgFor	Lepwg2a	35	65,6	450	beewgFor	-
	ArgK	ArgK_fwd2	ArgK_KLTrev2	35	50,5	1200	ArgK_fwd2	ArgK_KLTrev2
		ArgK_Loretta	ArgK_KLTrev2	35	53,0	700	ArgK_Loretta	-
	COI / COII	CO_LCO	CO_Ben	35	49,0	1100	CO_LCO	CO_Ben
		CO_fwd1	CO_rev2	35	52,8	1000	CO_fwd1	CO_rev2
	EF1a	For1deg	F2Rev1	35	56,8	1300	For1deg	F2Rev1
		HaF2for	Cho10	35	58,0	1700	-	Cho10
Symbiont	16S rDNA	fD1	Spa-2R	32	65,0	2090	fD1	rP2
	EF-Tu	EF-Tu-1F	EF-Tu-2R	35	65,0	870	EF-Tu-1F	-
		EF-Tu-3F	EF-Tu-3R	35	65,0	1220	EF-Tu-3F	EF-Tu-3R
	gyrB	gyrB-F1	gyrB-R3	35	62,0	740	gyrB-F1	-
		gyrB-F3	gyrB-R10	35	62,0	440	-	gyrB-R10
	gyrA	gyrA-5F	gyrA-5R	35	60,0	630	gyrA-5F	-

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CHAPTER 7

EXPERIMENTAL SYMBIONT EXCHANGE PROVIDES EVIDENCE FOR PARTNER SPECIFICITY IN THE DEFENSIVE BEEWOLF-*STREPTOMYCES* SYMBIOSIS

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7.1 ABSTRACT

For many obligate mutualisms, successful symbiont transmission from one host generation to the next constitutes an essential process, and one would expect that various adaptations would arise in both the insect host as well as the bacterial symbiont to facilitate the maintenance and specificity of these associations. Here we demonstrate that, in the beewolf-*Streptomyces* symbiosis, partner choice during symbiont transmission reinforces host-symbiont fidelity, thereby stabilizing the cooperative association over long evolutionary timescales.

Using a controlled re-infection approach we were able to experimentally re-introduce either con- or heterospecific bacterial symbionts into the antennal gland reservoirs of aposymbiotic beewolf females (*Philanthus triangulum*, Hymenopter: Crabronidae). Our results revealed that both conspecific *´Ca.* Streptomyces philanthi` as well as heterospecific *Amycolatopsis* bacteria are able to grow in the antennal reservoirs, once the aposymbiotic females have been re-infected with a bacterial culture suspension. However, behavioral observations as well as molecular and chemical analyses suggest that the transmission of heterospecific symbionts may be selectively hindered, most likely via complex mechanisms that involve host-symbiont recognition and communication. Moreover, we could demonstrate that the survival rate for offspring from re-infected females differs significantly with respect to the symbiont identity. Offspring from females re-infected with heterospecific *Amycolatopsis* experienced a significantly higher survival rate as compared to offspring from females with the native symbiont. Thus, the beewolf-*Streptomyces* association provides an optimal model system to address evolutionary as well as mechanistic questions on the maintenance and specificity of insect-bacteria symbioses.

7.2 INTRODUCTION

Symbiotic relationships between insects and microorganisms are common in nature and have been intensively studied in diverse taxa (Buchner 1965). More often than not, the biochemical abilities of the microbes exceed those of their host, thereby enabling the insect host to exploit novel ecological niches through balancing nutrient deficiencies, promoting growth and reproduction of the host, and contributing to defensive strategies for means of protection of the host from predators, pathogens and parasites (Douglas 1998; Currie et al. 1999; Kellner 2002; Oliver et al. 2003; Kaltenpoth et al. 2005; Scarborough et al. 2005; Oh et al. 2009; Kroiss et al. 2010).

Despite the prevalence of mutualistic interactions in almost all environments, the evolution of such inter-specific partnerships still remains a conundrum in evolutionary biology (Szathmary and Smith 1995). One conceivable explanation suggests that symbioses evolve from a parasitic ancestor, including successive reduction of virulence factors of the microbial partner as well as adaptations of the insect´s innate immune system, favoring the colonization by microorganisms (Ewald 1987). An alternative hypothesis, however, states that microbial mutualists may evolve from environmental commensals that provide beneficial traits to the host immediately upon contact (Ewald 1987). Regardless of the origin, most associations evolve towards highly specific interactions between the different organisms associated in such partnerships, raising the questions why symbiotic organisms selectively exclude partners, how this selection process is maintained and who chooses? Even though it seems plausible that discrimination of suitable partners versus incompatible or detrimental organisms may be beneficial, reducing conflict in the establishment of symbioses, little is known about factors promoting the formation of cooperative associations.

Intuitively, the partner who loses the most when associating with an ineffective partner should accomplish the choosing, thus, in symbiotic interactions between insects and bacteria, most often the host is likely to be the one choosing. The most prominent models that may explain the maintenance of such interspecific cooperations are partner fidelity and partner choice (Bull and Rice 1991; Sachs et al. 2004). In partner-fidelity associations, host and symbiont interact repeatedly and reward cooperating individuals while sanction cheaters, thus reinforcing beneficial interactions (Bull and Rice 1991; Sachs et al. 2011). Partner fidelity is generally assumed to be the major stabilizing force in vertically transmitted symbioses of insects (Sachs et al. 2011). By contrast, partner choice has been proposed to particularly select for cooperative strains among environmentally acquired symbionts, such as the nitrogen-fixing rhizobia of legumes (Denison 2000; Kiers et al. 2003), mycorrhizal fungi of plants (Kiers et al. 2011), and the bioluminescent *Vibrio fischeri* bacteria of squids (Nyholm and McFall-Ngai 2004). In partner-choice associations, the individuals may interact only once, however, one member can select the partner in advance of any possible exploitation (Bull and Rice 1991; Sachs et al. 2011).

To efficiently discriminate between beneficial and detrimental bacterial strains, the host must either (i) be able to recognize beneficial bacterial symbionts and sanction non-cooperating microbes, or (ii) provide selective conditions which favor the growth of beneficial strains.
The first hypothesis, emphasizing the importance of host-microbe recognition, suggests a high level of communication between the insect host and its microbial symbiont via signals and cues. In the legume-rhizobia symbiosis, a three component signal, initiated by the secretion of plant flavonoids from the roots, triggers a signaling cascade and, subsequently, changes the plant´s gene expression, leading to morphological changes in the roots which are essential for a successful association with rhizobia (Denarie et al. 1996; Denison 2000; Simms and Taylor 2002; Kiers et al. 2003; van de Velde

et al. 2010). Likewise, signaling has been demonstrated to be important in the formation of symbiotic associations between plants and arbuscular mycorrhiza (Gherbi et al. 2008; Maillet et al. 2011) as well as between plants and the nitrogen-fixing Actinobacterium *Frankia* (Gherbi et al. 2008).
Moreover, chemical cues are used by the squid, *Euprymna scolopes*, to locate its symbiotic partner *Vibrio fischeri*. By releasing fragments of cell wall peptidoglycan, *Vibrio* bacteria initiate the formation of the light emitting organ in their squid host, subsequently being assimilated (Davidson et al. 2004; Nyholm and McFall-Ngai 2004). A similar example is depicted by the jellyfish *Cassiopeia* spp. and symbiotic *Symbiodinum* dinoflagellates (Colley and Trench 1983; Fitt 1985). In the juvenile state, the jellyfishes are deprived of their symbiotic algae, which are essential nutritional symbionts, providing photosynthesis products to their host. To attract the symbiotic dinoflagellates, the jellyfish releases ammonia, and the algae actively follow the ammonia gradient, promoting contact with their potential host (Colley and Trench 1983; Fitt 1985).

As an alternative to specialized mechanisms for host-microbe recognition and communication, the host can provide selective conditions that favor its bacterial symbiont. As any environment, the host constitutes an ecological niche, which can only be colonized by organisms tolerating the predominant conditions. A selective environment favoring the colonization by beneficial, instead of detrimental, microorganisms is likely to be stabilized by natural selection because it enhances host fitness by associating with the most effective symbiont. Such mechanisms of partner choice have recently been proposed for gut microbiota (e. g. Schluter and Foster 2012) and the symbiosis between fungus-farming ants and their actinobacterial symbionts (Scheuring and Yu 2012). Over evolutionary timescales, the newly emerged host traits can result in changes in the colonizing microbes, subsequently leading to new adaptations in the host again, and this may finally result in the co-evolution of two species and specificity of the host-symbiont association.

The right level of specificity, however, usually represents a trade-off between risking association with an inefficient or detrimental partner through low specificity and the risk of failing to find a partner through high specificity. For vertically transmitted symbionts, the specificity is generally believed to be very high, which arises from a limited availability of symbionts to the host and, therefore, reduces the selection pressure on the host to discriminate between different putative symbionts.
In the solitary digger wasp, *Philanthus triangulum*, the symbiotic bacteria are vertically transmitted from mother to offspring via the brood cell and the wasp´s cocoon. The female wasps cultivate the symbionts of the genus *Streptomyces* (´*Candidatus* Streptomyces philanthi`) in specialized antennal gland reservoirs (Kaltenpoth et al. 2006; Goettler et al. 2007) and secret them into subterranean brood cells prior to oviposition. Later, the bacteria are incorporated into the silken cocoon walls by the larva during cocoon-spinning, where they provide antimicrobial protection against soil-dwelling fungal and bacterial pathogens (Kroiss et al. 2010), which significantly augments the progeny's conditional probability of survival (Kaltenpoth et al. 2005). Despite the vertical transfer of the symbionts from mother to offspring, however, the external mode of transmission via the cocoon surface provides the opportunity for horizontal uptake of different bacterial strains.

In beewolves, there are several possible routes for horizontal transmission or *de novo* uptake of heterospecific symbionts: (i) The symbionts could be acquired via spores from the soil surrounding the nest. *Streptomyces* species are known to produce spores to subsist on inhospitable conditions (Chater 1993; Flärdh and Buttner 2009), and recently, the symbiont of European beewolf wasps, ´*Candidatus* Streptomyces philanthi`, has also been shown to undergo morphological differentiation (Kaltenpoth et al. 2010; Koehler et al. 2013). Moreover, Kaltenpoth et al (in revision) detected DNA of ´*Candidatus* Streptomyces philanthi` in sand samples originating from used beewolf cages in the laboratory, which supports the hypothesis that spores of symbiotic *Streptomyces* may remain in the soil

of deserted nests, representing a potential source for cross infection and horizontal transfer. (ii) Evans and O'Neill (1988) observed interspecific predation among different *Philanthus* species, which share the same habitat. Thus, predation of smaller species as food for their larvae may constitute a second route of horizontal symbiont exchange between different beewolf wasps (Kaltenpoth et al., in revision). (iii) Similarly, symbiotic microorganisms may be horizontally acquired via other prey species of beewolves. Although nothing is known about Actinobacteria in European bees of the genus *Apis*, Promnuan et al. (2009) detected *Streptomyces* in beehives of bees of the genera *Apis* and *Trigona* in Thailand and *Streptomyces* sp. were also frequently found in pollen, provisions and alimentary canals of alfalfa leafcutter bees, *Megachile rotundata* (Inglis et al. 1993). (iv) A last path of transmission could be via parasitoids, visiting the nest of female beewolves. It has been shown before that the female´s nest can be highly parasitized by cuckoo wasps such as *Hedychrum rutilans* (Kimsey and Bohart 1990; Strohm et al. 2008). Given the ubiquitous distribution of Actinobacteria in the environment, which makes it easy for macroorganisms to encounter, it may be possible that parasitoids, visiting nests of different beewolves, transmit bacteria and/or their spores which, subsequently, could infect the wasp´s antennal reservoirs. Since parasitoids by definition usually kill the host, however, this path of horizontal symbiont transfer is very unlikely.

The present study demonstrates that European beewolves can indeed be colonized by different strains of symbiotic bacteria, other than their native symbiont. Using controlled re-infection experiments of aposymbiotic female beewolves (*Philanthus triangulum*) with a con- and heterospecific actionbacterial strain, we could validate that different Actinobacteria can not only be taken up into the female´s antennal reservoirs, but can also grow inside the beewolf´s bacteria cultivation organ. Behavioral observations as well as molecular and chemical analyses, however, revealed that the transmission of heterospecific bacteria seems to be selectively hindered, which may result in a discontinuous vertical transmission of the bacteria to the next generation, thus, facilitating the stabilization of this unique co-operation mediated by partner choice and fidelity.

7.3 MATERIALS AND METHODS

7.3.1 Symbiont manipulation

Female European beewolves, *Philanthus triangulum*, collected from natural populations in Berlin (Germany), were individually housed in breeding cages (Fig. 7.1) in a climate chamber with 25°C (+/- 3°C). The cages consisted of a "flight compartment" of 18 cm x 18 cm x 15 cm (Height x Width x Depth) in which the beewolf female was provided with honey bees (*Apis melifera*) as prey and honey *ad libitum,* and an additional "sand compartment" that measured 18 cm x 18 cm x 55 cm (H x W x D) where the female could establish a nest and construct brood cells containing offspring. The moisture of the sand inside the nesting cage was regulated via a tube, which span the whole cage area except the flying compartment. Additionally, the cage was equipped with a lid to open the nesting area.

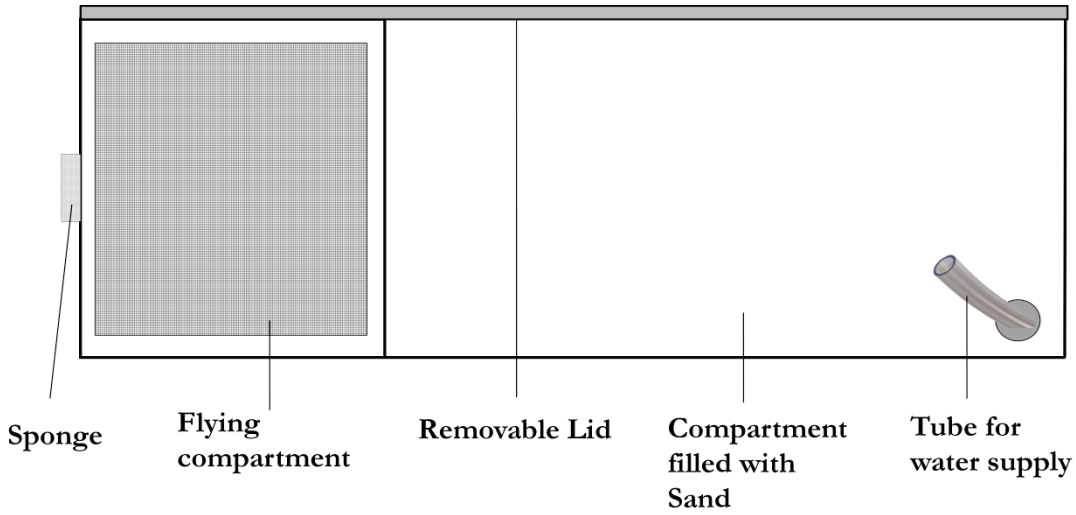

Fig. 7.1 Beewolf breeding cage as used for laboratory culture of female European beewolves.

In the fall, the beewolf´s offspring was excavated from the cages, placed in 1.5 ml Eppendorf cups inside a box with moist sand, and stored in the fridge at 6°C for hibernation. After approximately 6-8 months, cocoons were transferred to an incubator at 25°C to induce pupation.
Subsequently, aposymbiotic females were created by removing the developing beewolf from the cocoon prior to emergence. It has been demonstrated that, by bending and rubbing the antennae to the cocoon´s inner surface during eclosion, the symbionts are taken up into the spezialized antennal reservoirs of female beewolves (Kaltenpoth et al. 2010). The pupal skin, covering the female during its pupal development inside the cocoon, prevents earlier infection of the beewolf´s antennal reservoirs with the symbiontic bacteria. 1-2 days prior to emergence, the pupal-skin is shed off, allowing infection of the antennal gland reservoirs (Kaltenpoth et al. 2010).
We therefore monitored the progress of the wasp´s pupation through the semitransparent cocoon, which can be observed as an intensifying melanization of the pupa´s cuticle. Approximately 3-5 days prior to emergence (the pupa´s thorax is completely black and the abdomen starts to display black stripes), the beewolf pupa was dissected out of the cocoon to prevent natural uptake of the bacterial symbionts, thereby effectively creating aposymbiotic individuals. The sample dissection was conducted as follows: The cocoons were longitudinally cut to create an opening through which the pupa could carefully be removed using forceps. Subsequently, the pupa was placed in a sterile 1.5 ml

cup for further development. The cups were individually placed in a low-germ environment consisting of a small jar with autoclaved sand, which was moistened with autoclaved millipore water. Finally, the jars were closed with autoclaved sponges and placed in the incubator at 25°C until emergence. After successful emergence (pupal skin remained in the Eppendorf cup), the females were randomly assigned to two different treatment groups: i) re-infected with the native symbionts (´*Candidatus* Streptomyces philanthi`, 23Af2, **CaSP**), and ii) re-infection with a heterospecific actinobacterial strain that was previously isolated from the antenna of a female *Philanthus albopilosus* beewolf (*Amycolatopsis* sp., alb538-1, **Amy**).

Re-infection of aposymbiotic females was accomplished by spreading a bacterial culture suspension of either con- or heterospecific bacterial strains over the antennae of previously paralyzed (with CO_2) female beewolves using a 10 µl pipette tip. While applying the bacteria, the antennae were carefully bent with forceps (Fig. 7.2) to expose the reservoir openings (for detailed morphology of beewolf antennae see: Goettler et al. 2007), allowing bacterial cells to contaminate these bacterial cultivation organs.

After recovery from paralysis, the re-infected female beewolves were individually moved to observation cages according to Strohm and Linsenmair (1995). The cages were situated in a greenhouse (14:10 h, L/D, 23°C (+/- 3°C fluctuation)) at the Max Planck Institute for Chemical Ecology in Jena, Germany, where the female beewolves were provided with honey bee workers *(Apis melifera)* and honey *ad libitum*.

Fig. 7.2 Re-infection of aposymbiotic female European beewolves with a bacterial culture suspensions. The antennae were bent with forceps to expose the reservoir opening. The suspension has been applied using a 10µl pipette tip carefully moving back and forth over the antenna.

During their whole life, the reproductive output of the experimental females was monitored. For each re-infected female we counted the number of brood cells as well as the number of bees per brood cell, and documented the absence or presence of the white substance in the brood cells. All offspring of re-infected females were kept in the observation cages for 8-10 days after cocoon spinning, and the survival was monitored during this time of development. Finally, the offspring cocoons were removed from the nest and frozen at -80°C for subsequent analyses.

7.3.2 Detection of actinobacterial symbionts in female beewolf antennae

A single antenna from every re-infected beewolf female (CaSP: N=13, Amy: N=9) was subjected to DNA extraction using the Epicentre MasterPure™ DNA extraction kit (Epicentre Technologies, Madison, USA) with one minor modification: all centrifugation steps were done at room temperature. The DNA pellet was re-suspended in 50 µl nuclease-free low-TE buffer (1 mM Tris/HCl, 0.01 mM EDTA) and, subsequently, stored at −20°C. To validate the success of the re-infection procedure, primers specific for ´*Ca.* Streptomyces philanthi` (Kaltenpoth et al. 2010) and *Amycolatopsis* sp. (Tan et

al. 2006; Kyselkova et al. 2008) were utilized to screen for the con- and heterospecific symbionts, respectively, using diagnostic qPCR (quantitative polymerase chain reaction) (Table 7.1).
QPCR amplification was performed on a Rotor-Gene Q Cycler (Qiagen, Hilden, Germany) in a total reaction volume of 25 µl, including: 6.5µl RNase-free water and 12.5 µl SYBR-Mix (Rotor-Gene SYBR Green RT-PCR kit, Qiagen, Hilden, Germany); 2.5 µl of each primer (10 µM), 1 µl template. Cycle parameters were as follows: 95°C for 10 min, 35 cycles of 95°C for 10 s, 65°C for 20 s. Finally, a melting curve analysis was conducted by increasing the temperature from 65°C to 99°C with 1°C gain.
PCR success was additionally verified by gel electrophoresis using a GelRed™ (Biotium, Hayward, USA) stained 1.5% TBE agarose gel (150V, 30 min) and documented using GeneSnap image acquisition software (GeneSnap 7.09.06, Syngene, Cambridge, United Kingdom).

Table 7.1 Primers used for diagnostic PCR amplification of '*Ca.* S. philanthi' and *Amycolatopsis* sp.

Primer	Sequence (5'-3')	Direction	Ampl. length	References
Strep_phil_fwd3mod	TGGTTGGTGGTGGAAAGC	Fwd	135	Kaltenpoth et al., 2010
Strep_16S_rt_rev	GTGTCTCAGTCCCAGTGTG	Rev		Kaltenpoth et al., 2010
Amytop6_fwd	GCGCCAGACATCTCCAGAGA	Fwd	222	Kyselková et al., 2008
ATOP_rev	GTATCGCAGCCCTCTGTACCAGC	Rev		Tan et al., 2006

7.3.3 FISH of actionbacterial symbionts in beewolf antennae

To visualize the re-infected symbionts in the antennal gland reservoirs of *Philanthus triangulum,* fluorescence in situ hybridization (FISH) was performed using cross sections of the remaining antenna that had not been used for nucleic acid extraction. Only antenna of re-infected females that were reproductively active have been subjected to the analysis (CaSP: N=5, Amy: N=6). Each female´s antenna was individually fixed in 70% ethanol, dehydrated in acetone and then embedded in cold polymerizing resin (Technovit 8100, Germany) according to the manufacturer's instructions. Sections of 10 µm thickness were prepared with a diamond knife on a Microm HM 355 S microtome (Thermo Scientific, Germany). FISH on the embedded beewolf antennae was carried out using specific probes for ´*Ca.* Strep. philanthi` and *Amycolatopsis* sp. (Table 7.2), in combination with DAPI. Hybridization was achieved as described previously (Kaltenpoth et al. 2005), and localization of the bacterial symbionts was recorded using an Axioimager Z1 fluorescence microscope (Carl Zeiss, Germany).

Table 7.2 Specific FISH probes used for visualization of actinobacterial symbionts in beewolf antennae

Probe	Sequence (5'-3')	Direction	5´-mod.	Target	References
CaSP-SPT177	CACCAACCATGCGACGGTA	Rev	Cy5	CaSP	Kaltenpoth et al., 2005
Amy-16S	AGGCTTATCCCAAAGTACAGG	Rev	Cy3	Amy	This study

7.3.4 Quantification of antibiotics on beewolf cocoons using GC-MS

For the gas chromatography-mass spectrometry (GC-MS) analyses of the symbiont-produced antibiotics, all cocoons originating from females that had been re-infected with con- or heterospecific symbiont strains were placed individually in glass vials and extracted with 500 µl of methanol for 1 h. After extraction, cocoons were taken out of the GC-MS vials, placed on a tissue to drain methanol, transferred to clean 1.5 ml cups (Eppendorf), and finally frozen at -20°C for subsequent nucleic acid extraction. The methanol extracts were evaporated to dryness under a gentle stream of Argon and subsequently re-suspended in 50 µl of methanol. An aliquot of 1 µl was injected into the GC-MS using a split/splitless injector at 250°C, with the purge valve opened after 60 s. Analysis of the cocoon samples was performed using a Varian 450GC gas chromatograph coupled to a Varian 240MS mass spectrometer (Agilent Technologies, Böblingen, Germany). The GC was equipped with a DB5-MS capillary column (30 m x 0.25 mm diameter, film thickness: 0.25 µm, Agilent Technologies), programmed from 150 to 300°C at 5°C/min with a 1 min. initial and a 5 min. final isothermal hold. Helium was used as a carrier gas with a constant flow rate of 1 ml/min. Mass spectra were recorded using electron ionization (EI-MS), and data acquisition as well as quantification were done using MS Workstation Version 6.9.3 Software (Agilent Technologies). Absolute quantification of the three major antibiotic substances on beewolf cocoons, i.e. streptochlorin (SC), piericidin A1 (PA1), piericidin B1 (PB1), was achieved as described before (Kroiss et al. 2010; Koehler et al. 2013). The peaks were identified based on their mass spectra (Kroiss et al. 2010), and peak areas were integrated automatically using the MS Workstation Software. The success and accuracy of this integration was controlled manually for every peak.

7.3.5 Quantification of actinobacterial symbionts on beewolf cocoons

To assess the vertical transmission success of the artificially (re-)introduced symbionts to the next beewolf generation, we quantified the number of CaSP and Amy bacterial cells on con- and heterospecific cocoons that originated from re-infected females, respectively (**CaSP**: re-infected females: N=5, offspring cocoons: N=82; **Amy**: re-infected females: N=6, offspring cocoons: N=84). The beewolf cocoons were homogenized individually in liquid nitrogen followed by DNA extraction using the Epicentre MasterPure™ DNA extraction kit (Epicentre Technologies, Madison, USA), with the following adjustments: the lysozyme step was omitted, and all centrifugation steps were accomplished at room temperature. Finally, the DNA pellet was re-suspended in 100 µl nuclease free low-TE buffer (1 mM Tris/HCl, 0.01 mM EDTA) for subsequent qPCR analyses. Amplification was accomplished using the same primer pairs and qPCR conditions as described above (see passage 7.3.2 and Table 7.1). For all cocoon samples from CaSP as well as Amy re-infected females we used qPCR amplification with both primer pairs.

7.3.6 Statistical analysis

The proportion of beewolf brood cells with and without white substance secretion and the life-history parameter of the re-infected female beewolves were compared across treatments using the t-test for independent samples, since the samples followed a normal distribution.
Molecular data obtained from qPCR was checked for the presence/absence of bacterial DNA using a threshold of 10^{-7} ng/µl obtained from the standard curve. Samples showing equal or less amplification than the threshold value were assigned negative with regard to the presence of symbionts.

The GC-MS data was checked for presence/absence of CaSP-produced antibiotic substances. Subsequently, the molecular data obtained from qPCR analyses as well as the chemical data obtained from GC-MS runs were tested using the Yates´ chi-squared-test.
Finally, to investigate the effect of symbiont-mediated protection on the survival probability of the wasp progeny we performed a survival analysis for different life stages using the Gehan-Wilcoxon-test, and the Kaplan-Meier-Schätzer diagram for display.
Statistical analyses were performed using SPSS 17.0 Software (IBM, New York, USA) and BiAS 8.2.

7.4 RESULTS

7.4.1 Detection of actinobacterial symbionts in female beewolf antennae using qPCR

To validate the success of the re-infection procedure, the presence of CaSP and Amy was evaluated for one antenna per female using qPCR with specific primers (Figure 7.3, Table 7.1). Our results revealed a success rate of the re-infection procedure of at least 59-73% (13-16 antennae out of 22 in total), which constitutes a conservative estimate given the fact that sometimes only one beewolf antenna may be infected. The re-infection frequency for CaSP and Amy were 54% and 67%, respectively. The bands for W18, W19 and W26 show only weak amplification (Figure 7.3b), indicating that the antennae had either been surface contaminated with Amy or not all 5 antennal reservoirs were successfully re-infected with bacterial symbionts.

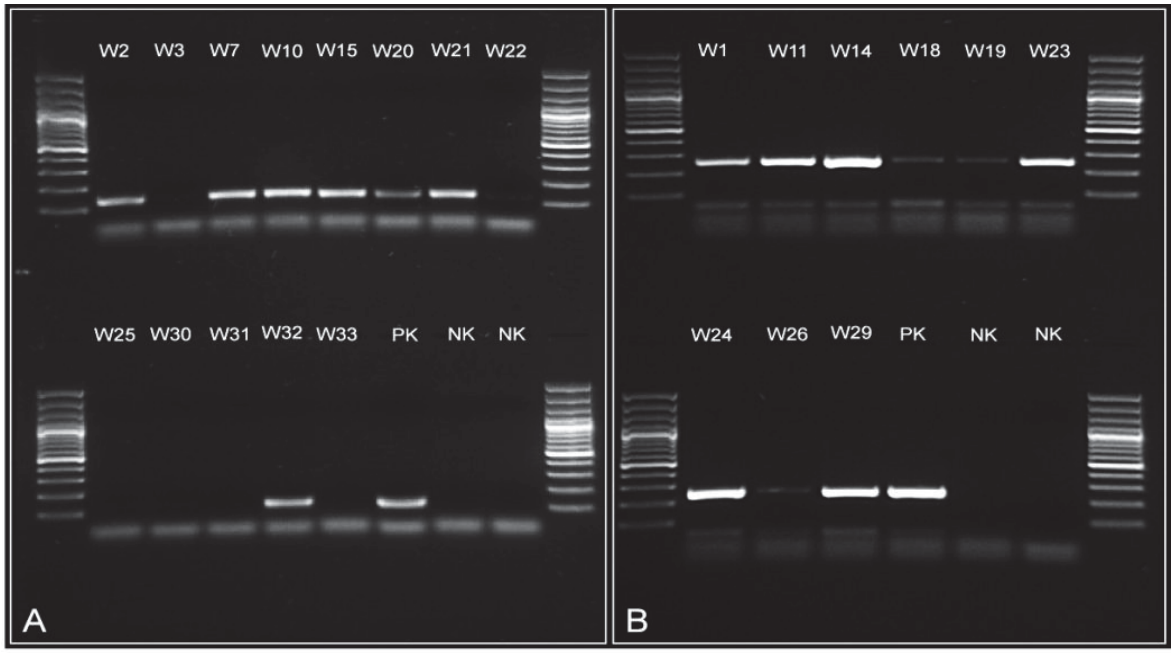

Fig. 7.3 Gel picture of the antennal qPCR from re-infected female beewolves with specific primer pairs. **A)** Total number of females re-infected with *´Ca*. Streptomyces philanthus`, **B)** total number of females re-infected with *Amycolatopsis* sp.

7.4.2 FISH of actinobacterial symbionts in female beewolf antennae

To verify that the actionbacterial symbionts *de facto* grow inside the antennal reservoirs we used FISH with specific probes for CaSP and Amy and DAPI (see Table 7.2). The pictures clearly show large amounts of *Streptomyces* (Figure 7.4a,b) as well as *Amycolatopsis* (Figure 7.4c,d) bacterial cells inside the beewolf´s antennal reservoirs. The absence of other specific structures, e.g gland cells, inside the antennae is due to artifacts that likely occurred during fixation, embedding, and sectioning.

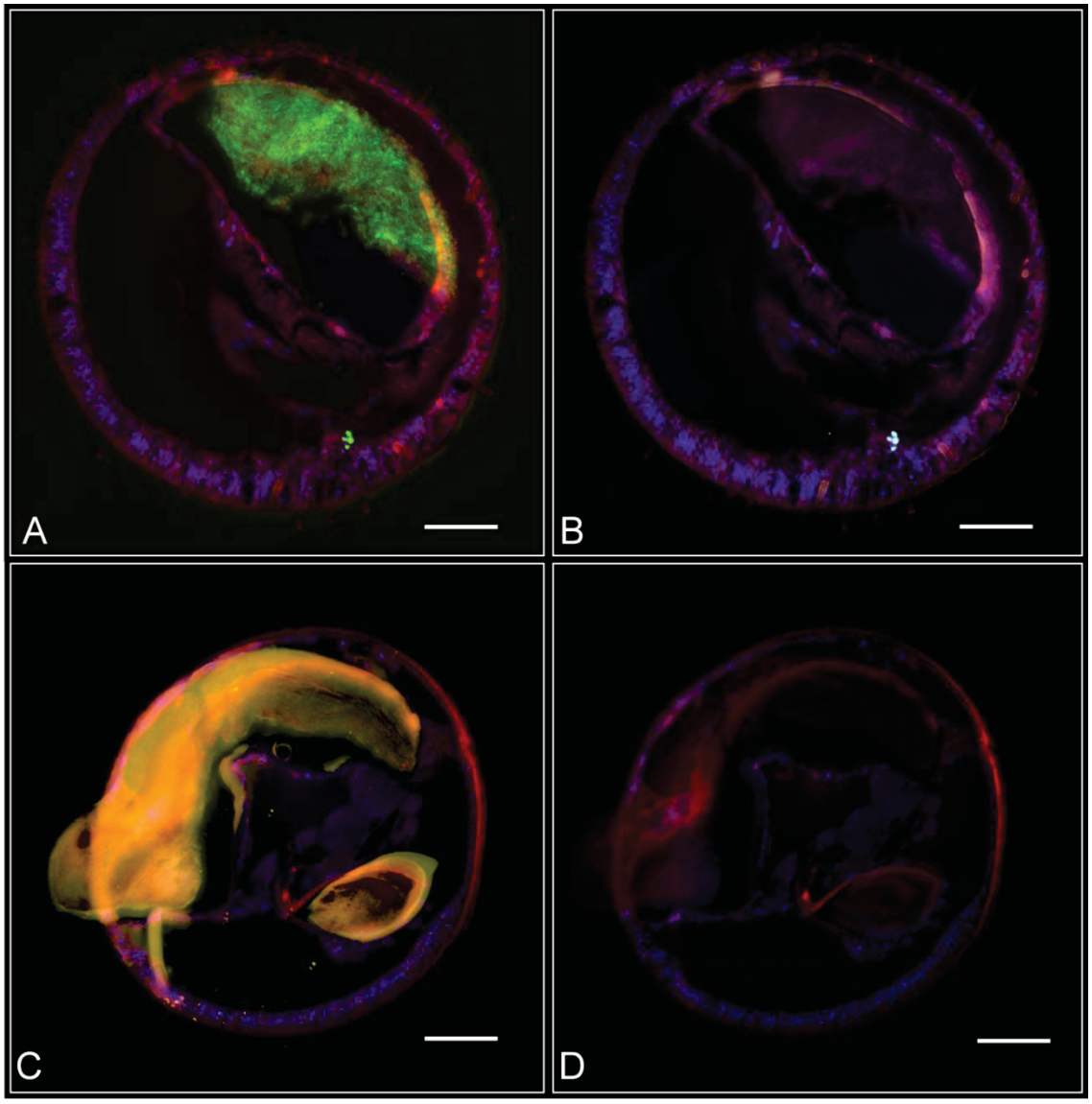

Fig. 7.4 FISH of antennal cross sections from re-infected female beewolves stained with a *´Ca.* Streptomyces philanthi` specific probe (CaSP-SPT177-Cy5, green) and an *Amycolatopsis* specific probe (Amy-16S-Cy3, yellow), counterstained with DAPI (blue). **A)** Antenna re-infected with con-specific *´Ca.* Streptomyces philanthi` (W10), the bacteria are clearly visible in the reservoir using the *Streptomyces* specific probe, **B)** Antenna re-infected with con-specific *´Ca.* Streptomyces philanthi` (W10), the bacteria are not visible with the *Amycolatopsis* specific probe, **C)** Antenna re-infected with hetero-specific *Amycolatopsis* sp. (W1), the bacteria are clearly visible in the reservoir using the *Amycolatopsis* specific probe, and **D)** Antenna re-infected with hetero-specific *Amycolatopsis* sp. (W1), the bacteria are not visible with the *Streptomyces* specific probe. Scale bars 50 μm.

7.4.3 Detection of white substance in beewolf brood cells

To survey the transmission of the bacterial symbionts into the brood cell we daily checked for new brood chambers and monitored the secretion of white substance at the ceiling of each brood cavity (Figure 7.5) for females with progeny (CaSP: N=5, Amy: N=6). The percentage of brood cells with white substance for CaSP re-infected females ranged between 57 to 80% (Figure 7.6). By contrast, there was no white substance visible in any of the brood cells of females that were re-infected with Amy (Figure 7.6). The statistical analysis showed a significant difference for the secretion of white substance between CaSP and Amy re-infected females (Figure 7.6, t-test: t=-17.6, df=9, P<0.001)

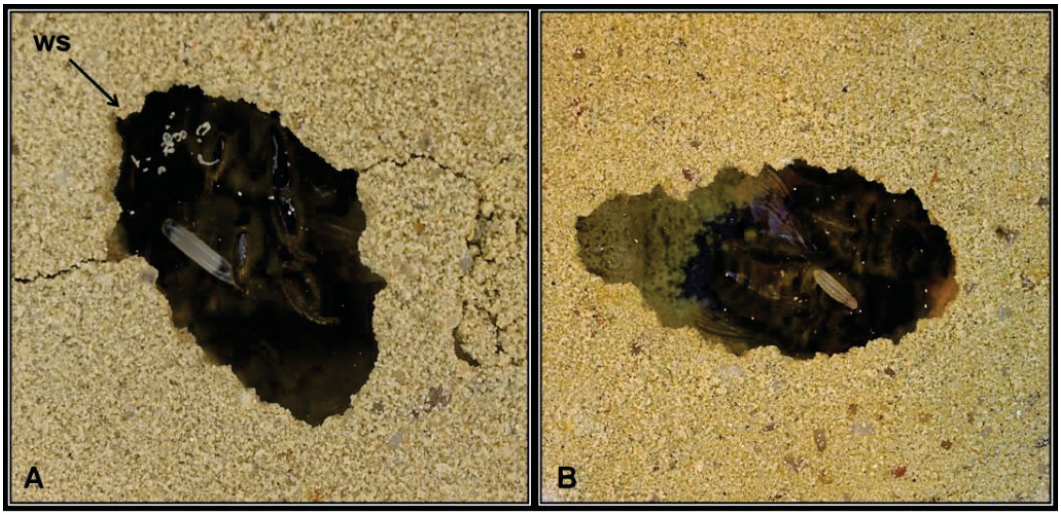

Fig. 7.5 Beewolf brood cells with honey bees and offspring, **A)** Brood cell of a female re-infected with *´Ca.* Streptomyces philanthi` (W10) containing a beewolf egg on paralyzed honey bees, **B)** Brood cell of a female re-infected with *Amycolatopsis* (W14), no white substance visible. **ws** white substance

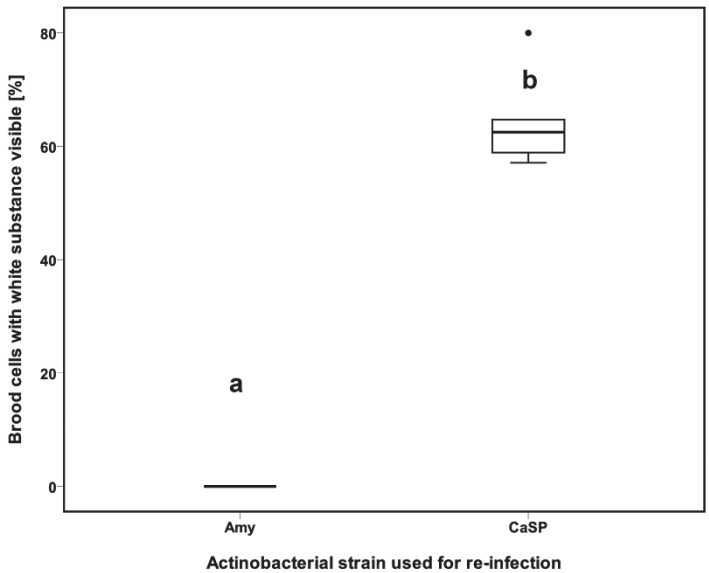

Fig. 7.6 Percentage of beewolf brood cells positive for white substance. Brood chambers originating from females re-infected with *Amycolatopsis* did not contain white substance secretion as compared to brood cells from females re-infected with *´Ca.* Streptomyces philanthi`(t-test: t=-17.6, df=9, P<0.001). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers. Different letters above boxes denote significant differences (P<0.05).

7.4.4 Detection of symbiont cells and symbiont-produced antibiotics on beewolf cocoons

To further characterize whether the transmission of symbiotic vs. non-symbiotic bacteria to the next generation depends on the symbiont identity, we checked for presence and absence of bacterial symbionts as well as the CaSP-produced substances on beewolf cocoon. Offspring of both CaSP- and Amy re-infected females were analyzed using both primer pairs. For all offspring (cocoons from CaSP and Amy re-infected females, N=166) tested with the CaSP primer pair the analysis revealed a higher number of positive cocoons originating from CaSP re-infected females (82.9%) as compared to cocoons from Amy re-infected females (44.1%) (homogeneity test with Yates´s correction: χ^2=26.1, P<0.001). By contrast, there was no significant difference for both treatment groups tested with the Amy primer pair (homogeneity test with Yates´s correction χ^2=1.67, P=0.19). The proportion of symbiont positive offspring cocoons was significantly higher for progeny from CaSP re-infected females (82.9%) as compared to offspring from Amy re-infected females (40.5%) when tested with the respective primer pair (Figure 7.7, t-test: t=-3.58, P=0.006).
The chemical analysis of symbiont-produced antibiotics revealed a significant difference in the presence or absence of antibiotics on cocoons from females re-infected with CaSP as compared to cocoons from females re-infected with Amy. We tested 79% of the cocoons from females re-infected with CaSP positive for antibiotics. By contrast 5.9% of the cocoons (5 out of 84) from females re-infected with Amy were positively tested for CaSP-produced antibiotics (homogeneity test with Yates´s correction: χ^2= 89.3, P<0.001).

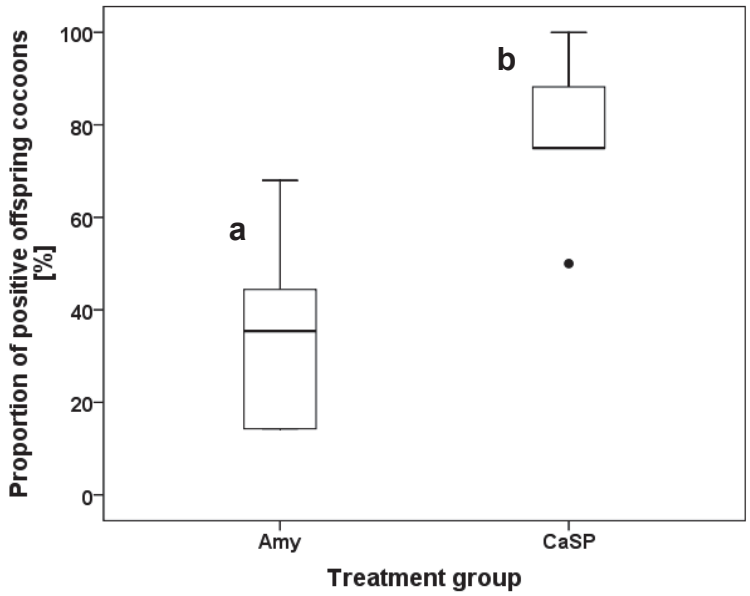

Fig. 7.7 Percentage of beewolf cocoons positive for *Amycolatopsis* and *´Ca.* Streptomyces philanthi` symbionts tested with the respective primer pair. Females re-infected with *´Ca.* Streptomyces philanthi` show a significantly higher proportion of symbiont-containing cocoons as compared to *Amycolatopsis* (t-test: t=-3.58, P=0.006). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers. Different letters above boxes denote significant differences (P<0.05).

Table 7.3 Success of symbiont transmission for reproductively active females of European beewolves used for re-infection with *´Ca.* Streptomyces philanthi` and *Amycolatopsis* bacteria.

Sample	Treat-ment group	Age (d)	Number of brood cells	WS pos.	Brood cells with WS visible (%)	Antennal symbionts		Cocoons total	qPCR primers -		Cocoons tested for CaSP antibiotics pos.
						qPCR	FISH		Amy pos.	CaSP pos.	
W01	Amy	41	6	no	0	+	+	6	2	2	0
W11	Amy	24	19	no	0	+	+	18	8	9	0
W14	Amy	45	34	no	0	+	- *	25	17	10	5
W18	Amy	13	15	no	0	+	+	14	2	6	0
W23	Amy	11	20	no	0	+	+	14	2	7	0
W29	Amy	22	8	no	0	+	-	7	3	3	0
W07	CaSP	21	7	yes	57,1	+	+	6	0	3	3
W10	CaSP	38	20	yes	80,0	+	+	20	4	15	14
W15	CaSP	38	39	yes	58,9	+	-	34	18	30	30
W21	CaSP	31	8	yes	62,5	+	+	8	0	6	6
W32	CaSP	42	17	yes	64,7	+	+	14	2	14	12

*negative for Amy, but positive for CaSP

7.4.5 Fitness of beewolf females re-infected with CaSP and Amy

To examine whether experimental symbiont exchange influences the survival and/or reproductive output of female beewolves, we analyzed the female´s longevity as well as the number of constructed brood cells for the two different treatment groups (Figure 7.8). On average females re-infected with CaSP and Amy survived 34 and 26 days and constructed 18 and 17 brood cells, respectively. The statistical analysis revealed that females re-infected with CaSP did not live significantly longer than females re-infected with Amy (t-test for independent samples: t=-1.11, df=9, P=0.29). Moreover, females re-infected with CaSP and Amy constructed a comparable number of brood cells (t-test for independent samples: t=-0.17, df=9, P=0.87).

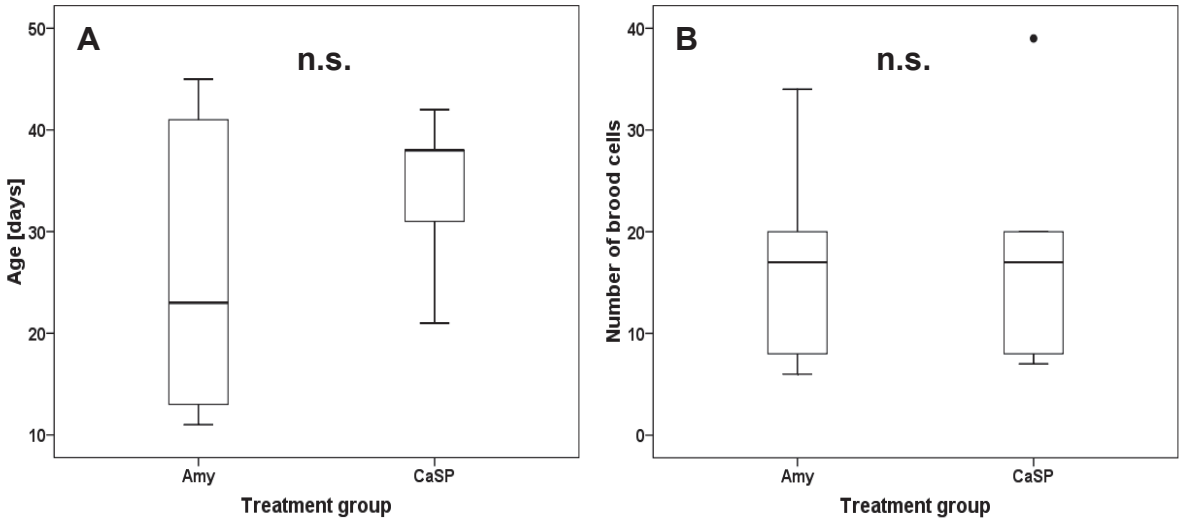

Fig 7.8 Life-history and fitness parameters of females re-infected with *Amycolatopsis* sp. and ´*Ca.* Streptomyces philanthi` bacteria. **A)** Longevity (in days) of females re-infected with Amy and CaSP. There is no significant difference for survival between re-infected females from both treatment groups (t-test for independent samples: t=-1.11, df=9, P=0.29). **B)** Number of brood cells from females re-infected with Amy and CaSP. The brood cell number did not differ significantly with regard to the treatments of the female wasps (t-test for independent samples: t=-0.17, df=9, P=0.87). Bold lines represent medians, boxes comprise the interquartile range, bars indicate the minimum and maximum values and dots represent statistical outliers.

7.4.6 Survival of beewolf progeny obtained from re-infected females

A survival analysis was performed for all offspring from five females re-infected with CaSP and six females re-infected with Amy (Figure 7.9). To discriminate between the effect of symbiont-mediated protection during development inside the cocoon and other threats which peril the larval survival in the early stages of development, a survival analysis was performed additionally for two separate life stages: (i) from egg to cocoon spinning, and (ii) from cocoon spinning to day 8 after cocoon spinning. Since cocoons of the same age were required for the chemical analysis, we froze the cocoons at day 8 after cocoon spinning. If, at this time point, the cocoons were not infested with fungi and the larva was alive (could be detected during dissection), we assumed that these offspring would have survived to reach adulthood and set the survival to an approximate value of 30 days. This period correlates with the developmental time from cocoon spinning to emergence under natural conditions.
Our results revealed that offspring that originated from females re-infected with the native symbiont (CaSP) has a significantly higher survival probability as compared to offspring originating from females that have been re-infected with Amy (Figure 7.9, Gehan-Wilcoxon-test: Z=2.84, P=0.005).

For the period from beewolf egg to larval cocoon spinning, we did not detect a significant effect on the offspring from differently treated females (Gehan-Wilcoxon-test: Z=1.04, P=0.29). However, for the phase of larval development inside the cocoon, we found a significant effect for the offspring of differently treated females, with progeny from CaSP re-infected females showing a significantly higher survival probability (Gehan-Wilcoxon-test: Z=3.36, P<0.001). Thus, larvae from Amy re-infected females had equal chances of reaching the cocoon-spinning stage as compared to offspring from CaSP females, but their cocoons were more often infested with fungus and, subsequently, the larva died within the first eight days after cocoon spinning.

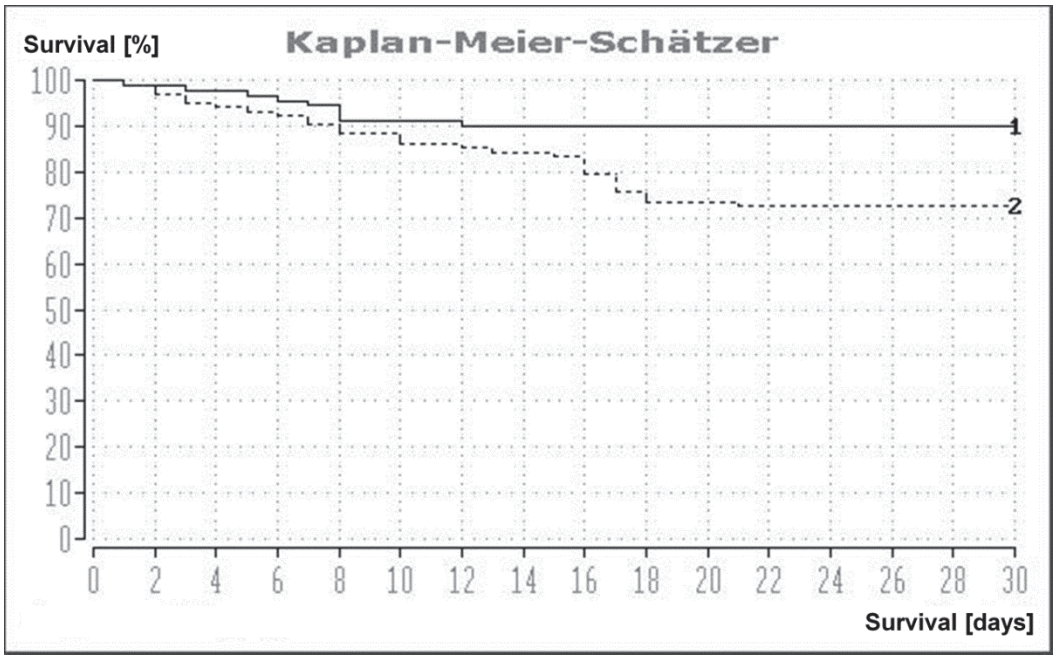

Fig 7.9 Survival of beewolf offspring originating from females re-infected with ´*Ca*. Streptomyces philanthi` (1) vs. *Amycolatopsis* sp. (2) bacteria. Gehan-Wilcoxon-test: Z=2.84, P=0.005.

7.5 DISCUSSION

7.5.1 Symbiont transmission

The establishment and persistence of symbiotic associations often depends on the ability of the participating organisms to discriminate between effective partners and a vast majority of incompatible or detrimental candidates. By choosing "wisely", the host reduces conflict with its partner, and therefore partner choice can select for cooperative strains among many available microorganisms.
In the beewolf-*Streptomyces* association, the bacterial symbionts have been shown to be vertically transmitted from the mother to the offspring via the cocoon surface (Kaltenpoth et al. 2010). As a result of this strict vertical transmission, beewolf wasps share a long co-evolutionary history with their symbiotic *Streptomyces*. Recently, phylogenetic analyses of different beewolf species and their bacterial symbionts provided evidence for some degree of co-cladogenesis of this unique symbiosis since its origin ~68 mya ago (Kaltenpoth et al, in revision). However, the mode of transmission, including an environmental persistence of the bacteria on the cocoon surface, provide opportunities for horizontal transmission of bacterial strains across beewolf species and *de novo* uptake of bacteria other than the wasp´s symbiont from the environment. Indeed, Kaltenpoth and colleagues (in revision) also provided evidence that other bacteria, among them *Amycolatopsis,* are occasionally taken up and grow in beewolf antennae (Kaltenpoth et al, in revision). Based on these results, we tested the specificity of the beewolf-*Streptomyces* association with focus on the potential for horizontal transmission of bacterial strains. Using a controlled re-infection approach we were able to experimentally re-introduce bacterial symbionts to aposymbiotic female beewolves by either re-infecting them with their native symbiont (´*Ca.* Streptomyces philanthi`) or a heterospecific bacterial strain (*Amycolatopsis* sp.).
The obtained results demonstrate that conspecific CaSP as well as heterospecific Amy bacteria are able to grow in the antennal reservoirs, once the aposymbiotic females have been re-infected (Figures 7.3 and 7.4). However, regularly observing the female beewolves' nests during their whole life, our observations revealed clear secretions of white substance only in brood cells from females re-infected with CasP (Figures 7.5 and 7.6, Table 7.3), suggesting that heterospecific symbionts may not be transmitted to the brood cell as white substance.

The molecular data of beewolf offspring cocoons obtained from qPCR analyses aiming at elucidanting the success of conspecific and heterospecific symbiont transmission, however, yielded inconclusive results. Offspring tested with the CaSP primer pair revealed a higher number of positive cocoons from CaSP re-infected females as compared to cocoons from Amy re-infected females (homogeneity test with Yates´s correction: $\chi^2=26.1$, $P<0.001$). However, repeated detection of CaSP on cocoons from females re-infected with Amy suggests unspecificity of the assay. By contrast, there was no significant difference for both treatment groups tested with the Amy primer pair (homogeneity test with Yates´s correction $\chi^2=1.67$, $P=0.19$), suggesting that Amy might occur on the cocoons as a result of contamination. The proportion of cocoons which are positive for symbionts, however, was significantly higher for progeny from CaSP re-infected females as compared to Amy re-infected females when tested with the respective primer pair (Figure 7.7, Table 7.3).

Two hypotheses may explain the observed pattern in our data. (i) It is possible that the heterospecific Amy bacteria are indeed transmitted from the wasp´s antennal reservoirs to the brood cell, but can´t be detected with the naked eye. The white substance usually secreted by beewolf females contains not only symbiotic CaSP but other compounds, e.g. hydrocarbons (Kaltenpoth et al. 2009). Thus, the composition of the antennal gland secretion may change in the presence of a foreign symbiont, rendering the secretion to be transparent. Moreover, *Streptomyces* have been shown to produce

pigments (e.g. Hobbs et al. 1990; Bystrykh et al. 1996) which may be responsible for the conspicuity of the white substance and may be lacking in *Amycolatopsis* sp. (ii) Heterospecific Amy is not transmitted to the wasp offspring but has been detected on the cocoon as a result of environmental contamination. Actinobacteria are common and widespread in soils (Goodfellow and Williams 1983; Holt et al. 1994), and the sand used for the beewolf cages had been untreated, i.e. possibly contaminated with environmental bacteria. Using specific Amy primers, we also detected positive cocoons for offspring from CaSP re-infected females, suggesting contamination from the soil. Thus, assuming transmission of symbionts for CaSP, but not for Amy, cocoons from females re-infected with CaSP would contain the symbiotic *Streptomyces* already which may hinder the colonization. By contrast, cocoons from females re-infected with Amy may be depleted of symbionts, which would facilitate the colonization.

The antibiotic profiling of cocoons of the females´ offspring revealed antibiotic substances almost exclusively on cocoons of CaSP re-infected females (Table 7.3). However, we detected piericidin antibiotics on five cocoons of one female re-infected with Amy. Since piericidins are so far known to be produced only by very few *Streptomyces* species, among them CaSP, the presence of these antibiotics on the cocoons most likely originates from a contamination with CaSP rather than environmental bacteria. Moreover, one of these cocoons was positively tested for streptochlorin. This further supports our hypothesis, since CaSP is the only strain known to produce both these classes of antibiotics (Kroiss et al. 2010). A possible scenario explaining these results is that the cocoons have been either contaminated with CaSP from the environment, as it was previously detected in the sand surrounding beewolf nests (Kaltenpoth et al, in revision), or other streptomycetes contaminating the sand. An alternative explanation is that the female has not been aposymbiotic in the first place and, thus, some CaSP cells might have invaded the antennal reservoirs. Indeed, the FISH results confirmed that the antenna of this specific female (W11) was positive when tested with the CaSP-specific probe and negative for the Amy-specific probe (Table 7.3). However, the positive results for the antennal qPCR with specific Amy primers, at the same time confirmed the presence of Amy bacteria in the female antenna. Also, if the females would have been exclusively infected with CaSP, instead of Amy, we might have expected a higher number of cocoons with antibiotics in general. Nevertheless, it should be kept in mind that each antenna contains five antennal reservoirs, and it is possible that not all of them had been successfully re-infected with Amy. One or few of them may have been contaminated with CaSP cells prior to re-infection with Amy, resulting in a low efficiency for the transmission of CaSP to the cocoon, which is supported by our results.

7.5.2 Survival of progeny from re-infected beewolf females

In the beewolf-*Streptomyces* symbiosis, the bacteria have been shown to provide efficient protection of the wasp progeny during their long and vulnerable phase of development (Kaltenpoth et al. 2005; Koehler et al. 2013). Moreover, it has been demonstrated that the beewolf offspring experiences a dramatic reduction in the survival rate without the symbiotic bacteria (Kaltenpoth et al. 2005). Thus, we examined the survival probability for beewolf offspring from females that had been re-infected with the native symbiont, CaSP, and females that had been re-infected with Amy. Our results revealed that the survival probability for the beewolf progeny is significantly higher in the presence of the native symbiont (CaSP) (Figure 7.9). Moreover, we divided the larval development in two time periods, (1) from egg to cocoon spinning and (2) from cocoon spinning to day 8 after cocoon spinning, since the symbionts have previously been shown to only affect the survival probability in the cocoon phase (Kaltenpoth et al. 2005). The symbionts are incorporated into the cocoon walls by the larva while spinning the cocoon, thus, there should be no difference in the survival rate between

progeny from CaSP and Amy re-infected females for the first period of larval development. Indeed, there was no significant difference in survival between offspring of CaSP- and Amy re-infected females for the larval development within the first days until the larva starts spinning the cocoon. However, the survival analysis performed for the larval development inside the cocoon (period 2) revealed that, for offspring from females re-infected with the native CaSP symbiont, the survival probability is significantly higher as compared to offspring from females re-infected with Amy, which is in agreement with the results of Kaltenpoth et al. (2005). Admittedly, the effect on the survival rate between groups in this study appeared to be much less dramatic than in the survey by Kaltenpoth et al. (2005). However, it is likely that the survival rate in the present study is overestimated, due to the termination of the experiment at day 8 after cocoon spinning. Thus, our analysis would have missed offspring that may have died throughout later developmental stages.

Considering that the transmission of Amy from the antennal gland reservoirs could not unambiguously be disproven nor verified in our qPCR assays (see above), there are two hypotheses that may explain the reduced survival rate of progeny from Amy re-infected females. (i) The Amy bacteria are present on the cocoon, but provide no or reduced protective benefits to the larva as compared to the native CaSP symbiont. The likely inhospitable environment on the cocoon may hamper the colonization for the bacteria, and although the genus Amy has been demonstrated to be a potent producer of antibiotic substances (e.g. Nadkarni et al. 1994; Wink et al. 2003), these conditions may as well hinder the production of antimicrobial substances. Moreover, the substances that may be produced by Amy might not be as efficient as compared to the CaSP produced compounds in warding off soil microbes that threaten the beewolf offspring during development. For nutritional symbionts in pyrrhocorid bugs as well as stink bugs, it has been shown that host individuals perform less well in the presence of heterospecific symbionts, suggesting an intimate association between the host and the bacterial symbionts (Hosokawa et al. 2007; Salem et al. 2012). Similarly, previous results demonstrated that beewolves share a close and long evolutionary history with their bacterial symbionts (Kaltenpoth et al, in revision), and a recent comparative analysis of the bacteria-produced antibiotics from various symbiotic *Streptomyces* strains throughout the world revealed that the antibiotic cocktail seems to be highly conserved even across distantly related beewolf species (Engl et al. unpubl data). Thus, over evolutionary time scales, the co-diversification of beewolf wasps with their bacterial symbionts may have lead to the evolution of an optimal composition of the *Streptomyces*-produced antibiotic cocktail which adapts to the extraordinary life style of beewolf digger wasps and, thus, ensures an efficient antimicrobial protection against a broad range of pathogenic microbes. (ii) The bacteria of the genus *Amycolatopsis* are not transmitted to the next generation, and the cocoons thus suffer from a lack of protective symbionts. As our results demonstrate, the secretion from the antennal gland reservoirs is lacking in brood cells from Amy re-infected females (Figures 7.5 and 7.6), although growth inside the reservoirs could be confirmed by qPCR and FISH (Figures 7.3 and 7.4). Mechanisms that may lead to a selective transmission of one bacterial strain over the other are not yet known in this system, but have been described in other symbioses, e.g. in squid and their bioluminescent *Vibrio* bacteria (Davidson et al. 2004; Nyholm and McFall-Ngai 2004). However, the amplification of Amy bacterial DNA from cocoons of females re-infected with the heterospecific symbionts, rather implies that the symbionts might either be transmitted from the female wasp, although less efficiently, or constitutes environmental contamination of beewolf cocoons with Amy bacteria, due to re-use of sand for the construction of the beewolf cages. Thus, sequencing of the samples that have been tested positive for Amy and screening for potential Amy-produced antibiotics may in future allow for a more detailed interpretation of this data set.

7.5.3 Maintenance of an association with a specific host

Despite the inconclusive results from our molecular analysis, the present study provides evidence that partner choice during symbiont transmission may indeed reinforce host-symbiont fidelity in the beewolf-*Streptomyces* symbiosis and thereby stabilizes the cooperative association in this system over long evolutionary timescales. However, the results can be expained by two independent hypotheses: (i) Heterospecific bacteria are not transmitted to the next generation of beewolf wasps, indicated by the lack of white substance in brood cells from females re-infected with Amy. Thus, partner choice would act during secretion of the symbionts from the antennal reservoirs, and positive qPCR results for cocoons from females re-infected with the heterospecific bacterial strain stem from environmental contamination. (ii) Amy is in fact secreted into the brood cell and transferred to the cocoon, but at lower efficiency than CaSP (Figure 7.7). Thus, partner choice would act during secretion of the white substance into the brood cell and/or during application of the bacteria to the cocoon. Also, if the heterospecific Amy is transmitted, it provides no or less efficient protection to the beewolf offspring (Figure 7.9), and thus, may experience lower transmission to the next generation, which would probably lead to a rapid loss of this new and inefficient symbiont strain over a few generations.

Physiological mechanisms that mediate partner choice in the beewolf-*Streptomyces* symbiosis still remain unclear, however, there are several hypotheses that might mechanistically explain a selective transmission of symbionts. First, the beewolves may provide selective conditions such as nutritional supply inside their antennal reservoirs, which favors the growth of their native symbiont, discriminating between the perfect match and ineffective bacterial strains. The strong growth of both tested bacterial strains inside the beewolf´s antennal reservoirs (Figures 7.3 and 7.4), however, suggests an alternative mechanism for selection than nutrient supplementation.

Second, the beewolves somehow regulate the process of symbiont transmission. In previous studies it could be demonstrated that the life time reproductive success (number of brood cells) significantly depends on the presence or absence of the *Streptomyces* symbionts. These surveys provided evidence that aposymbiotic females constructed significantly less brood cells during their whole life time as compared to symbiotic females (Koehler 2010). Based on this result, it has been hypothesized that the female beewolf senses the filling capacity of the reservoir which then triggers secretion of the white substance from the antenna into the brood cell (Koehler 2010). Interestingly, our current results show that females containing heterospecific Amy within the antennal gland reservoirs construct equal numbers of brood cells throughout their life as compared to females containing the native symbiont (Figure 7.8b). Thus, the signal triggering the brood cell construction behavior in beewolves appears to depend on the filling capacity of the reservoir rather than the identity of the contained symbionts. Despite the low survival probability for the progeny without symbionts (Kaltenpoth et al. 2005), one would expect that females anyway invest in the production of offspring, increasing the probability that at least some larvae survive to reach adulthood. By contrast, fitness would decrease to zero if the female does not construct brood cells at all. However, previous studies demonstrated that the growth rate of the *Streptomyces* symbionts of beewolves is low and it takes time for the bacterial population to fill the antennal reservoirs (Kaltenpoth et al. 2010). Thus, the inactivity of females with a low filling capacity of the reservoir might be adaptive for symbiont-containing individuals, as it would lead to the secretion of an optimal amount of symbionts into the brood cell to protect the offspring. In agreement with this hypothesis, earlier studies showed a positive correlation of bacterial cells with the amount of symbiont-mediated antibiotic substances (Koehler and Kaltenpoth 2013). However, for aposymbiotic females this adaptive regulation would be disadvantageous, reflecting in zero fitness. As such females are rare in the field (Kaltenpoth et al, in

revision), this maladaptive side effect of an otherwise adaptive behavior likely is not heavily selected against.

Although full antennal gland reservoirs appear to be necessary for eliciting the brood cell construction behavior in females, the filling status alone does not seem sufficient to trigger the secretion of the bacteria present in the antennal gland reservoirs, since we found white substance secretion in the brood chamber only for females re-infected with con- but not heterospecific symbionts. This suggests additional mechanisms discriminating between beneficial and ineffective bacterial strains. Recent transcriptome analyses of *Philanthus triangulum* antennae elucidated a gene coding for lysozyme, which we hypothesize may play a role in the secretion process of the bacteria from the antennal reservoirs (Kaltenpoth, Koehler et al. unpublished data). QPCR quantifications revealed that the gene expression level in the antennae tends to be higher in the presence of the *Streptomyces* symbionts as compared to aposymbiotic antennae (Kaltenpoth, Koehler et al. unpublished data). Additionally, culture-dependent experiments demonstrated that in the presence of lysozyme the *Streptomyces* grow in a loose, single cell form as compared to the dense mycelium they usually form (Nechitaylo et al. unpublished data). This change in bacterial colony growth may alleviate the excretion of the bacteria into the brood cell via the small reservoir opening. However, further experiments, including transcriptomic analyses of antennae from females that have been re-infected with con- vs. heterospecific symbionts, are necessary to disentangle these hypotheses and elucidate potential mechanisms that mediate a selective inhibition of ineffective symbionts.

Finally, beewolves may be able to specifically recognize their native symbiont and block the transmission of ineffective symbiotic strains. This recognition process might involve complex signaling between the two symbiosis partners as is already well known from plants (Pellmyr and Huth 1994; Denarie et al. 1996; Huth and Pellmyr 2000; Kiers et al. 2003; Gherbi et al. 2008; Kiers et al. 2011; Maillet et al. 2011) and bioluminescent *Vibrio* bacteria of squid (Nyholm and McFall-Ngai 2004; Nyholm and Graf 2012).
Transcriptome analyses of beewolf antennae recently elucidated a candidate gene coding for PGRP (peptidoglycan recognition protein) which may be responsible for the recognition of bacterial symbionts in the antennal reservoirs (Kaltenpoth, Koehler et al. unpublished data). PGRPs are known to recognize lipopolysaccharides of bacteria and fungi, initiating the insects immune system (Dziarski and Gupta 2006). In tsetse flies a specific PGRP has been proposed to scavange symbiotic peptidoglycan in juvenile individuals, thereby preventing the induction of the flies´ immune pathways (Wang and Aksoy 2012). Moreover, the experimental reduction of PGRP has been shown to diminish the female´s fecundity and damages the symbionts through induction of antimicrobial peptides (Wang and Aksoy 2012).
Also, the antibiotics which are produced in low amounts in the antennae of beewolf females (Engl et al. unpublished data) may play a role in the signaling process and mediate the communication between the wasp host and the symbiotic bacteria. Especially in low concentrations, antibiotics have been hypothesized to act as intra- and intercellular signaling molecules inducing changes in gene expression profiles (Goh et al. 2002; Linares et al. 2006; Yim et al. 2007). Although to our knowledge there is no evidence for antibiotics as signaling or communication molecules with macroorganisms, the produced substances may aid in detection and discrimination between effective and ineffective bacterial strains, facilitating the formation of beneficial associations.

In summary, the present study provides strong evidence for partner choice during symbiont transmission in the beewolf-*Streptomyces* symbiosis, which reinforces host-symbiont fidelity, and thereby stabilizes the cooperative association over long evolutionary timescales. However, the underlying mechanisms remain to be elucidated. One possible starting point may involve

transcriptome analyses of female beewolves re-infected with *Amycolatopsis* and/or other heterospecific bacterial strains. Additionally, further re-infection experiments, e.g. with lysozyme-resistant and sensitive strains, may provide new insights into the regulation and specificity of the symbiont secretion. Therewith, the beewolf-*Streptomyces* association provides an ideal model system to yield new insights on evolutionary as well as mechanistic aspects regarding the maintenance and specificity of insect-bacteria symbioses.

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CHAPTER 8

GENERAL DISCUSSION

8.1 ECOLOGY OF *STREPTOMYCES*-MEDIATED DEFENSE

8.1.1 Origin of the beewolf-*Streptomyces* symbiosis

As all ground nesting insects, the larvae of digger wasps generally have to cope with an increased risk of infection by pathogenic microorganisms from the surrounding soil during their development in subterranean brood cells (Janzen 1977; Strohm and Linsenmair 2001). Additionally, the storage of nutritional resources inside the nest increases the pathogenic threat to the developing offspring that feeds on these provisions. To protect the nourishment and, particularly, the offspring itself, beewolves evolved several mechanisms to reduce pathogen infestation of the provisioned honeybees (Rathmayer 1962; Strohm and Linsenmair 2001; Herzner and Strohm 2007) and the beewolf larva (Kaltenpoth et al. 2005; Kroiss et al. 2010; Engl 2011). The symbiosis with antibiotic producing bacteria of the genus *Streptomyces*, which provide protection for the beewolf progeny against pathogen infestation, thus, may represent a crucial evolutionary innovation for these ground-nesting and mass-provisioning insects.

The initial steps in the evolution of symbiotic interactions are often difficult to reconstruct, and the origin of mutualistic associations still remains a conundrum throughout the field of symbiosis research (Szathmary and Smith 1995). Traditionally, two hypotheses regarding the origin of mutualisms have been discussed (Ewald 1987). The first hypothesis assumes that microbial symbionts evolved from a parasitic ancestor, which requires both the symbiotic bacteria and the host to constrain adaptive interactions, such as bacterial virulence and a host immune system which tolerates co-operative bacteria, respectively. The second hypothesis implies that microbial mutualists evolved from environmental microorganisms, where a free-living ancestor possessed traits that could offer benefits to the host immediately upon contact. Because of their subterranean nesting sites, where the wasps continuously encounter soil dwelling bacteria, European beewolves may have been predisposed to evolve a symbiosis with *Streptomyces* bacteria. Additionally, the presence of glands in the beewolf antennae might have been a preadaptation favoring the formation of a bacterial symbiosis. The original function of these glands might have been to provide directional information to the newly emerged beewolf wasp to exit the brood cell and, subsequently, their mother´s nest (Strohm and Linsenmair 1995). However, it is not yet evident what came first, the glands or the bacteria. At an early stage of the association, different bacterial strains might have exclusively been colonizing the antennal surface as commensals, using nutrients provided by the wasp, similar to the symbiotic *Pseudonocardia* bacteria in attine ants (Currie et al. 2006). In a later step, the beewolf wasps may have gained advantages from this interaction such as the incorporation of the bacteria into their progeny´s cocoon for means of protection. Only thereafter, an invagination, such as a reservoir like structure, may have been formed. Later on, this reservoir might have been invaded by soil-dwelling bacteria being capable of utilizing a wide range of nutrient sources. In this scenario, the assimilation of bacteria from the genus *Streptomyces* seems plausible, since *Streptomyces* are abundant soil bacteria

(Kutzner 1981; Goodfellow and Williams 1983) and they can exploit an enormous variety of different carbon and nitrogen sources (Kutzner 1981; Goodfellow et al. 1983; Goodfellow and Williams 1983; Holt et al. 1994). Furthermore, several taxa produce endospores, which allow for persistence even under inhospitable conditions in the soil for very long periods of time (Holt et al. 1994).

Similarly, a recently described association of *Streptomyces* bacteria with mud dauber wasps from the family Sphecidae (Poulsen et al. 2011) might represent an early evolutionary stage of such a symbiosis. Comparable to the hypothesized early stage of the beewolf-*Streptomyces* symbiosis evolution, where different bacterial strains might have colonized the beewolf´s cuticle, including the antennae, a high diversity of different *Streptomyces* has been identified to be associated with mud dauber wasps (Poulsen et al. 2011). In the beewolf-*Streptomyces* symbiosis, the former loose association with soil bacteria might have developed into a highly specialized symbiosis via the colonization of cuticular cavities in the antennae, which then could have further evolved into a complex symbiont cultivation organ. Thus, in addition to the benefits for the host, the symbiotic bacteria also gained an advantage by obtaining a secure ecological niche for growth and at the same time a basic mechanism to enable a successful vertical transmission to the next generation of beewolf wasps.

8.1.2 Benefits for the beewolf host

Animals, particularly insects, have evolved a wide range of mechanisms to offset the detrimental effects of predators, parasites, and pathogen infections (Cane et al. 1983; Ortius-Lechner et al. 2000; Bot et al. 2002; Fernandez-Marin et al. 2006; Cremer et al. 2007; Fefferman et al. 2007; Burse et al. 2009; Evans and Spivak 2010; Tragust et al. 2013). Some of these strategies involve the utilization of the antimicrobial properties of symbiotic bacteria to protect the host and/or host resources from pathogens and parasites (Currie et al. 1999; Kellner 2002; Scott et al. 2008; Liu et al. 2009). The currently best studied example of defensive insect-bacteria symbioses is depicted by the tripartite mutualism between fungus-farming ants and their mutualistic bacteria of the genus *Pseudonocardia*, where the antibiotic-producing symbionts protect the host´s fungal cultivar against a specialized parasitic fungus (Currie et al. 1999; Currie 2001a; Currie 2001b; Currie et al. 2003; Cafaro and Currie 2005; Currie et al. 2006; Cafaro et al. 2011).

Similarly, European beewolves engage in a mutualism with bacteria of the genus *Streptomyces*. The bacterial symbionts provide protection to the wasp progeny against pathogen infestation (Kaltenpoth et al. 2005; Kaltenpoth et al. 2006). Spinning a cocoon, the *Streptomyces* cells are incorporated into the silken walls, where they have been demonstrated to produce a "cocktail" of at least nine different antibiotic substances, which efficiently ward off antagonistic microbes (Kroiss et al. 2010) and significantly enhance the progeny´s survival probability (Kaltenpoth et al. 2005). Although the protective effect of the symbiont-mediated antibiotics for the beewolf offspring has been demonstrated earlier (Kaltenpoth et al. 2005), information regarding the duration, stability and potential factors affecting the antimicrobial protection was so far lacking. This thesis provides evidence that the symbiont-mediated defense in beewolves, although established already during larval cocoon spinning, serves as a reliable long-term protection for the beewolf offspring, mediated by morphological differentiation of the symbiotic bacteria and, coincidently, the production of highly stable antibiotic compounds on the cocoon surface (Chapter 2). In contrast to *in vitro* studies which demonstrated that antibiotic substances can be affected by different abiotic and biotic factors (Higashide 1984; James et al. 1991; Barratt and Oliver 1994; Wiener 1996; Hassan et al. 2001; Slattery et al. 2001; Oskay 2011), the symbiont-produced compounds on beewolf cocoons appeared to be

independent of factors such as temperature, humidity and pathogen load (Chapter 3). Moreover, the detected long-term stability of these substances over several months of larval development provides an efficient perpetual antimicrobial prophylaxis for beewolf larvae against pathogen infestation (Chapter 2). This is particularly relevant with regard to the females' brood care strategy of mass provisioning, and the prolonged and vulnerable phase of hibernation of their progeny in humid, underground brood cells. During this time, the beewolf´s offspring as well as the remains of the prey items provide a rich nutrient source for antagonistic soil bacteria and fungi (e.g. Janzen 1977; Keller and Zimmermann 1989), and, unlike progressive provisioning insects, mass provisioning species, like the European beewolf, lack the possibility to continuously enter the nest and apply defensive chemicals to defend their progeny (Wilson 1971; Bohart and Menke 1976; Clutton-Brock 1991). Thus, the application of chemicals which provide a long-lasting and environmentally stable protection to the offspring during the sensitive period of larval development may depict an adaptive mechanism of parental investment (Chapter 2).

Despite the stability of the antibiotic substances across environmental conditions, we observed temporal changes in the composition of the symbiont-produced antibiotic "cocktail" (Chapter 2). This variation in the production time of individual substances and/or the production of compounds with different stabilities may represent a pre-adaptation either towards the prevention of resistant antagonistic strains (Mouton 1999), or an alternative strategy to ward off different soil fungi and bacteria (Challis and Hopwood 2003). The combination of several antibiotic substances has previously been shown to reduce the formation of resistant bacterial strains (Mouton 1999; Challis and Hopwood 2003) and in human medicine combination prophylaxis is commonly used to antagonize multiple pathogens (e.g. Mebis et al. 1998; Gionchetti et al. 1999; van Zanten et al. 2000; Ohkusa et al. 2005). Thus, the symbiont-mediated antibiotic production in the beewolf-*Streptomyces* mutualism seems to be finely tuned towards providing antimicrobial protection against a particularly broad spectrum of pathogenic microbes, according to the subterranean life style of these digger wasps.

As mentioned before, the production of antimicrobials coincides with morphological differentiation in the *Streptomyces* symbionts (Chapter 2), as has been demonstrated for various *Streptomyces* strains (Berdy 1984; Champness and Chater 1994; Hopwood 2007; Chater et al. 2010). Morphological differentiation into dormant spores usually enables bacteria to subsist in inhospitable conditions, e.g. limited nutrients, which may provide advantages over competitors during re-colonization of an ecological niche once the conditions improve again. Thus, the production of antibiotic compounds during spore formation may aid in the protection of the bacteria against competitors during the vulnerable phase of resource allocation into spores, and may provide them with superiority over future antagonists competing for the same niche (Wiener 1996; Slattery et al. 2001). In the beewolf-*Streptomyces* symbiosis, however, the production of spores not only initiates antibiotic production, which is essential for the protection of the wasp offspring, but allows for subsistence of the bacterial cells in the likely inhospitable conditions on the cocoon surface and, thus, facilitates the successful acquisition of symbionts by the next generation of beewolf wasps (Chapters 2+5).

In summary, the symbiont-mediated defense in the beewolf-*Streptomyces* symbiosis with multiple antibiotic substances appears to be optimized to provide an efficient long-term protection to the wasp´s progeny by antagonizing a broad range of pathogenic microorganisms, and to enhance the efficiency of vertical symbiont transmission to the next generation of beewolf wasps.

8.1.3 Costs for the beewolf host

Many organisms defend themselves against predators, parasites or pathogens by mounting an immune response, which, according to the life history theory, is supposedly energetically costly, and identifying the nature, magnitude and impact of these costs as well as the conflicts they may create, is essential for understanding the evolution of immune systems. In addition to the organism´s personal immune defenses, some protective mechanisms can benefit others than the challenged organism itself, e.g. its offspring. Also, the defensive agents are often utilized from bacterial symbionts, not only providing benefits to the host, but adding to the costs of defense via the maintenance of the symbiont culture.

Costly defensive strategies may manifest their expenses in different ways, however, the ultimate cost reflects in the reduction of the organism´s individual fitness. Though, defenses may abate the organism´s fitness to a certain degree, these mechanisms usually aim at ensuring survival and reproduction of the individual, and thus, increase fitness on average. Generally, mounting defensive mechanisms pays off if the overall gain in fitness mediated via defenses is higher than the overall costs (Maynard-Smith 1974), and, thus, selection will favor adaptations that increase the representation of a set of genes in the gene pool of the population over generations. To ensure a successful survival and future reproduction of their offspring, many species evolved specific behaviors, which are known as parental care (Clutton-Brock 1991), and although, parental care strategies are versatile, including offspring provision, nest construction etc., a key component of parental care involves the protection of the next generation against predators, parasitoids and pathogenic microorganisms (Cane et al. 1983; Marchini et al. 1997; Field and Brace 2004; Kaltenpoth et al. 2005; Cardoza et al. 2006; Herzner and Strohm 2007; Rozen et al. 2008; Cotter and Kilner 2010; Herzner et al. 2011). However, in most of the aforementioned model systems it is not clear whether the performed behaviors match the definition of parental investment (Trivers 1972), meaning that they cause a trade-off between current and future reproduction in their parents. If the fitness of the current progeny is increased but at the same time the parent´s investment in future offspring is reduced, this may constrain the allocation of resources in such defensive mechanisms.

The reproductive investment of the European beewolf is complex and multifaceted, and the nesting behavior comprises several components that qualify as parental investment to ensure a successful development of the progeny. Besides the establishment of a nest and the excavation of the brood chambers, prey hunting is an extremely costly behavior for the females (Strohm and Marliani 2002). Additionally, the subterranean nesting site of beewolf wasps demands the necessity of defensive strategies against potential soil microorganisms, especially fungi, which may peril the development of the beewolf larvae. As has been shown, many organisms evolved chemical defensive strategies, particularly aiming at the protection of their nutritional resources as well as their offspring. In leaf cutting ants, the metapleural gland secretion provides protection against pathogenic bacteria and fungi competing with their symbiotic fungus (Ortius-Lechner et al. 2000; Bot et al. 2002; Fernandez-Marin et al. 2006; Yek et al. 2012). Bark beetles and termites have been shown to coat their nesting chambers with antifungal oral secretions and faecal pellets, respectively (Cardoza et al. 2006; Fernandez-Marin et al. 2006), and similarly, stingles bees spread a sticky resin around the opening of their nest, thus preventing antagonists from settling near the nest´s entrance (Howard 1985; Patricio et al. 2002; Lehmberg et al. 2008). In European beewolves, parental care strategies include the embalming of the prey items with a secretion from the postpharyngeal gland that reduces fungal germination on the larval provisions (Herzner and Strohm 2007), and recently, a study by Herzner et

al. (2011) provided evidence that this embalming of the bees with the hydrophobic secretion entails costs to the female beewolf wasps (Herzner et al. 2011).
The cultivation of the symbiotic *Streptomyces* inside the antennal reservoirs of female beewolves may add to these costs. The female wasps likely supply the symbiotic bacteria with nutrients via the gland cells surrounding the reservoirs (Goettler et al. 2007) or via the hemolymph (Goettler et al. 2007; Kaltenpoth et al. 2009). The nutritional requirements and, as a result, the costs of the cultivation of the symbiotic *Streptomyces* inside the antennal gland reservoirs are not yet clear. However, bacterial growth minimally requires a carbon and nitrogen source. The hydrocarbons in the antennal gland secretions have been demonstrated to be positively correlated with the number of *Streptomyces* bacterial cells (Chapter 3), suggesting that they may provide a potential carbon source for the nourishment of the symbiotic bacteria (Kaltenpoth et al. 2009). As a product of the lipid metabolism, the hydrocarbons in the antennal gland secretion, thus, may compete for lipids involved in egg production or flight metabolism (Canavoso et al. 2001; Howard and Blomquist 2005; Arrese and Soulages 2010). However, the costs of allocating hydrocarbons for means of culturing the symbiotic bacteria might be comparatively low, since hydrocarbons are already produced by beewolves as part of their cuticle as well as for the PPG secretion (Strohm et al. 2008b). Also, the hydrocarbon amount found in the antennal gland secretion (Koehler and Kaltenpoth 2013) is marginal as compared to the amount of cuticular and PPG hydrocarbons (Strohm et al. 2008b; Herzner et al. 2011). In addition, nitrogen is generally assumed to be a limited resource for many organisms (Prosser and Douglas 1992; Hongoh and Ishikawa 1997; Wilkinson et al. 2007; Lopez-Sanchez et al. 2009; Fiore et al. 2010). Nitrogen containing compounds, e.g. amino acids, proteins etc., make up a major part of all organisms and are of primary importance in determining the structural organization, metabolic control, energy transfer and physiological capacities of every species. Thus, the symbiotic *Streptomyces* might compete for the same resources with different components of the beewolf´s metabolism, such as proteins or lipids, which are essential for egg production and maturation as well as the functionality of the wasps metabolism (Corrigan 1970; Canavoso et al. 2001; Howard and Blomquist 2005; Arrese and Soulages 2010).

Finally, it might be costly for the beewolf females to confine the bacteria inside the antennal gland reservoirs. Several pieces of evidence suggest that beewolves restrict their symbiotic *Streptomyces* to the antennal reservoirs. (i) The symbiotic bacteria have not been detected in other beewolf tissues than the antennae, and (ii) preliminary transcriptome analyses of symbiotic vs. aposymbiotic beewolf antennae revealed an up-regulation of several immune genes, such as the antimicrobial peptides hymenoptaecin and defensin as well as lysozyme, in the presence of symbionts (Kaltenpoth, Koehler et al, unpublished data). The costs of mounting an immune system has been shown previously (Ahmed et al. 2002; Bonneaud et al. 2003; Cotter et al. 2003; Freitak et al. 2003; Vainio et al. 2004; Rantala and Roff 2005; Schmid-Hempel 2005; Ardia et al. 2012), thus, it is likely that in the beewolf-*Streptomyces* symbiosis the maintenance and restriction of the bacterial symbionts to the antennal reservoirs, mediated via the up-regulation of immune functions, is costly for the beewolf host. Likewise, it has been demonstrated for weevils that co-evolution between insect hosts and their symbionts may have shaped special immune effector genes which control the growth of the symbionts, thereby preventing the invasion of different host tissues other than the bacteriomes (Login et al. 2011).

Hence, in addition to the aforementioned parental care strategies, the cultivation of the *Streptomyces* symbionts might add to the costs of parental investment (Strohm 1995; Herzner et al. 2011) and may reflect a female's quality to allocate resources between current and future offspring.

8.1.4 Benefits for the *Streptomyces* symbionts

In mutualistic interactions, by definition, both partners benefit from the association with the respective ally. For the microbial partner, there are generally three major benefits from engaging in mutualistic associations: (i) The eukaryotic host provides a novel ecological niche that can be colonized by the microbes, (ii) the host provides nutrition to the symbionts, and (iii) the host facilitates a successful transmission of the symbiotic microorganisms to the next generation.

In European beewolves, the symbiotic bacteria colonize specialized reservoirs in the antennae of female wasps (Kaltenpoth et al. 2006), providing an unusual but secure and, generally, competition-free location for the symbiotic microbes to grow in, and there are several morphological characteristics which suggest that the beewolf hosts supply nutrients to the symbiotic bacteria within these reservoirs. First, ultrastructural analyses of the antennal reservoirs revealed that they are surrounded by gland cells containing a high density of rough endoplasmatic reticulum (Goettler et al. 2007), which implies that they may produce nutritional components such as proteins that may be secreted into the reservoir lumen, serving as a nourishment for the symbiotic *Streptomyces*. Second, one part of the antennal gland reservoir consists of a reticulated, mesh-like wall with a thin epicuticle (Goettler et al. 2007), which may facilitate the nutrient transfer from the wasp´s hemolymph into the antennal gland reservoirs. This is further supported by chemical analyses of the beewolf´s antennal gland secretion and hemolymph, which show a high similarity in the hydrocarbon composition (Strohm et al. 2008b; Kaltenpoth et al. 2009). Finally, female beewolves usually construct more than one brood cell per day (Strohm and Linsenmair 1995), which they supply with large amounts of symbiotic bacteria (Kaltenpoth et al. 2005; Kaltenpoth et al. 2006). To replenish the bacterial population for further brood cells, the bacteria have to grow quickly within the reservoirs, wherefore they are in need of a sufficient nutrient supply from the beewolf host.

Conversely, the growth rate of the *Streptomyces* symbionts on the cocoon has been demonstrated to be very low (Chapter 2), which might be attributed to limitations for nutrients and space on the cocoon surface. Available resources might be restricted to proteins from the cocoon silk or remaining hydrocarbons or other nutrients contained in the antennal gland secretion, which allow for minimal growth within the first days on the cocoon (Chapter 2). Alternatively, metabolic byproducts from the larva´s gut excretions could be recycled and used as carbon and nitrogen sources by the bacteria. However, the cessation of symbiont growth on the cocoon surface even in the presence of the larva´s metabolic waste products refutes the hypothesis that nutrient supply promotes the bacteria´s growth via the recycling of larval excretory byproducts (Chapter 4).

Chemical analyses, however, revealed an increase in symbiont-mediated antibiotic substances on the cocoon surface in the presence of the larva´s gut excretions (Chapter 4). Thus, excretory byproducts might either be metabolized by the bacteria and directly channeled into the production of antimicrobial compounds or they could act as trigger as well as control agents for the antibiotic production in the *Streptomyces* symbionts (Mendez et al. 1985; Bibb 2005; Rigali et al. 2008; van Wezel and McDowall 2011). As has been demonstrated previously, antibiotics can provide an advantage to the producing organism in preventing the invasion of competing microorganisms (Wiener 1996; Slattery et al. 2001). Thus, the increase in antibiotic production not only benefits the host offspring against pathogens, but also provides an advantage to the symbiotic *Streptomyces* against competing microbes, facilitating their competitive superiority over present and future antagonists on the cocoon for a prolonged period of time, and subsequently, a successful transmission to the next beewolf generation.

8.2 EVOLUTIONARY HISTORY AND SPECIFICITY OF THE BEEWOLF-*STREPTOMYCES* SYMBIOSIS

8.2.1 Symbiont transmission

In insects, vertical transmission from mother to offspring appears to be the prevalent mode of symbiont transfer (Buchner 1965; Baumann and Moran 1997; Bourtzis and Miller 2003; Bright and Bulgheresi 2010), although examples of environmental symbiont acquisition have been reported recently for stinkbugs (Kikuchi et al. 2007) and whiteflies (Caspi-Fluger et al. 2011). Given their extraordinary life style inside the host´s cell, especially intracellular, primary symbionts have been intensively studied with regard to their transmission route. Lacking the ability to survive outside of the insect host, symbiont transmission in these cases appears to be maintained inside the mother´s body, e.g. via the ovaries during oogenesis (Buchner 1965; Schröder et al. 1996; Douglas 1998; Sauer et al. 2002; Nardon 2006). However, in many insect-bacteria associations the symbionts are extracellularly transmitted, often including an environmental persistence of the symbionts during part of the transmission cycle, such as egg-smearing (Buchner 1965; Prado et al. 2006; Kikuchi et al. 2009), coprophagy (Buchner 1965), or the deposition of symbiont-containing capsules (Fukatsu and Hosokawa 2002; Hosokawa et al. 2005).

In European beewolves, the symbionts have been shown to be vertically transmitted from the mother to their offspring via the cocoon surface (Kaltenpoth et al. 2005; Kaltenpoth et al. 2006; Kaltenpoth et al. 2010a). After secretion of the bacteria into the brood cell, the beewolf larva incorporates the bacteria into the cocoon walls, and during eclosion from the cocoon, female wasps subsequently assimilate a few bacterial cells into their specialized gland reservoirs (Chapter 5). This uptake of very low numbers of symbiont cells from the cocoon into the antennal reservoirs causes a severe bottleneck effect to the *Streptomyces* symbiont population during transmission (Chapter 5), which, for many obligate mutualists, has been shown to strongly shape the genome evolution of the bacterial symbionts (Baumann et al. 2002; Feldhaar and Gross 2009; Moran et al. 2009). Specifically, the symbiotic bacteria experience a significant genome reduction as well as shifts in base composition towards AT nucleotide enrichment (Moran 1996; Shigenobu et al. 2000; Akman et al. 2002; Gil et al. 2003; Tamas and Andersson 2003; van Ham et al. 2003; Moran and Plague 2004; Degnan et al. 2005; Wu et al. 2006; McCutcheon and Moran 2007). Given their external lifestyle outside the insect host for part of their life cycle, it seemed unlikely that extracellular symbionts experience similar genome erosion, and thus, many of the above mentioned specific genome adaptations have traditionally been assigned to an intracellular lifestyle. Surprisingly, however, genomic studies on γ-proteobacterial symbionts in stinkbugs recently provided evidence of genome erosion in extracellularly transmitted symbionts (Kikuchi et al. 2009), indicating that population bottlenecks during vertical transmission and the coincident increase in genetic drift are responsible for genome erosion, rather than the intracellular lifestyle *per se* (Salem et al, in prep).

Since genomic analyses of beewolf symbionts and a comparison to their free-living relatives are still lacking, we can only speculate on consequences of the symbiotic life style inside the beewolf´s antennal reservoirs for the evolution of the symbiont´s genome. The difficulty of cultivating the symbiotic bacteria *in vitro* (Kaltenpoth et al. 2006) suggests that they lost some biosynthetic capabilities, which is indicative for a certain degree of genome erosion. The extracellular life style, which requires ´*Candidatus* Streptomyces philanthi` to survive not only in the antennal gland reservoirs but on the beewolf cocoon, however, suggests that the symbiotic bacteria might not be able to spare as many genes as is the case for intracellular symbionts such as *Buchnera* and *Blochmannia*.

In summary, successful symbiont transmission from one host generation to the next is essential in most intimate symbiotic associations, and one can assume that selection favors adaptations towards the maintenance of beneficial associations in the host as well as in the bacterial symbionts. Particularly, symbionts with an obligate intracellular lifestyle are tightly integrated into the host's metabolism (e.g. McCutcheon and von Dohlen 2011) as well as development (Koga et al. 2012), and the mutual interdependence of both partners coincides with perfect vertical symbiont transmission. Over evolutionary timescales, the high integrity of symbiotic associations may then results in host symbiont co-cladogenesis and congruent branching patterns of the phylogenic trees for both host and symbiont (Moran et al. 1993; Bandi et al. 1995; Baumann and Baumann 2005; Moran et al. 2008), which, subsequently, can be assessed and visualized, yielding insights into the ecology and evolutionary history of mutualistic insect-bacteria associations.

8.2.2 Phylogeny of beewolf wasps and their symbiotic bacteria

Decades ago the phylogenetic position of beewolf wasps still was elusive and based on morphological data by Alexander (1992). However, recent studies investigating the phylogeny of these solitary digger wasps using molecular methods shed new light on the evolutionary history of Philanthini wasps and the origin of their unique symbiotic association with bacteria of the genus *Streptomyces* (Chapter 6).
An earlier phylogenetic tree of the subfamily Philanthinae based on morphological characters branches into four major tribes, the Philanthini, Cercerini, Pseudoscolini and Aphilanthopini (Figure 8.1), which again subdivide into different genera (Alexander 1992). The recent detection of the symbiosis with protective *Streptomyces* bacteria in one species of the genus *Philanthus* by Kaltenpoth et al. (2005), however, raised the question of the evolutionary origin of this extraordinary association with regard to the other Philanthinae tribes.

To answer this question, phylogenetic analyses have been performed for the Philanthini wasps (*Philanthus, Trachypus, Philanthinus)* as well as for several outgroup taxa. With regard to the morphology-based tree by Alexander (1992) the genera *Philanthus, Trachypus* and *Philanthinus* were expected to form a monophyletic group and the genus *Philanthinus* was proposed to be closely related to the *Philanthus* /*Trachypus* complex within the Philanthini (Figure 8.1). The comparative genetic analyses by Kaltenpoth and colleagues confirmed both hypotheses and, furthermore, verified the previously proposed paraphyly the genus *Philanthus* with respect to the genus *Trachypus* (Chapter 6, Alexander 1992).

In order to complement the current knowledge about the evolutionary history of beewolf wasps with their bacterial symbionts representatives of all genera in the subfamily Philanthinae (Alexander 1992) have also been analyzed for the presence of *Streptomyces* bacterial symbionts, with two exceptions: The genus *Eucerceris* as well as the very rare genus *Pseudoscolia* have not yet been analyzed with regard to the presence of bacterial symbionts. However, the phylogenetic analysis revealed that the symbiotic bacteria of the genus *Streptomyces* are present in the antennal gland reservoirs of all three genera of Philanthini wasps (Chapter 6, Kaltenpoth et al. 2006; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). By contrast, the genera *Cerceris, Clypaedon* and *Aphilanthops*, do not possess bacterial symbionts, suggesting that the symbiotic relationship with the bacteria of the genus *Streptomyces* evolved along the branch leading to the Philanthini (Chapter 6). Assuming the most parsimonious scenario that the emergence of the symbiosis was a unique event, the placement of *Philanthinus* as a sister group of *Philanthus* and *Trachypus* (Alexander 1992) is preferred over Bohart and Menke´s (1976) placement of

the genus within the tribe Aphilanthopini, which is also confirmed by the molecular phylogeny (Chapter 6).
The two remaining genera, *Eucerceris* and *Pseudoscolia*, are not expected to harbor *Streptomyces* symbionts due to the close relationship to the genus *Cerceris* which has already been demonstrated to lack symbiotic *Streptoymces* (Chapter 6).

Furthermore, novel data using molecular clock analyses allowed to date the origin of the beewolf-*Streptomyces* symbiosis to approximately 68 million years ago (Chapter 6). Comparably, the evolutionary age of the symbiosis between aphids and *Buchnera* bacteria has been calculated to date back 160-280 mya, and similarly, the symbiosis between cockroaches as well as termites and their bacteria of the genus *Blattabacterium* has been approximated to be over 140 mya old (for review see: Moran et al. 2008). Hence, in evolutionary timescales and compared to many intimate nutritional insect-bacterial symbioses, the association between beewolves and their *Streptomyces* bacteria appears to be relatively young. However, the beewolf-*Streptomyces* symbiosis seems to be more ancient than the defensive symbiosis between fungus-farming ants and their *Pseudonocardia* bacteria, given the evolution of fungi-culture in ants around 50 mya ago (Schultz and Brady 2008). Thus, the association between beewolf wasps and *Streptomyces* bacteria constitutes the oldest defensive symbiosis known at present.

In addition to the molecular data, there are morphological differences in the antennal reservoirs of different Philanthini wasps, which strongly support the basal position of the rare genus *Philanthinus* within the tribe Philanthini. The recent survey of *Philanthinus quattuordecimpunctatus* discovered an additional sixth antennal reservoir (Kaltenpoth et al. 2012) as compared to the normally five that can be found in *Philanthus* and *Trachypus* antennae (Goettler et al. 2007; Kaltenpoth et al. 2010b), which are also more complex in structure. Thus, during the evolution of this unique symbiosis, the complexity of the beewolf´s bacteria cultivation organ appears to have increased (Kaltenpoth et al. 2012). The complex structure of the antennal reservoirs from the genera *Philanthus* and *Trachypus* may increase the reservoir´s volume, providing more space for the symbionts to grow in, which, subsequently, would facilitate an efficient antimicrobial defense to the wasps progeny via an increase in symbiont numbers (Chapter 3, Kaltenpoth et al. 2012). Furthermore, the complex structure may allow for the retention of symbiont cells in the lateral lobe of the reservoir after secretion of the main proportion of the reservoir´s content into the brood cell prior to ovipositon. This increase in the residual population of symbionts might facilitate the growth and replenishment of the bacteria in the antennal reservoirs, and therefore, might minimize the time between current and future brood cells (Kaltenpoth et al. 2012). Conversely, the sixth antennal reservoir in *Philanthinus* spp. may compensate for the missing increase in volume and complexity as it has been demonstrated for the other two genera, and, thereby, in a different way ensure an efficient symbiont-mediated protection for the developing larvae. However, it remains unsolved whether the common ancestor of *Philanthus* and *Trachypus* has lost, or whether *Philanthinus* has gained a reservoir (Kaltenpoth et al. 2012).

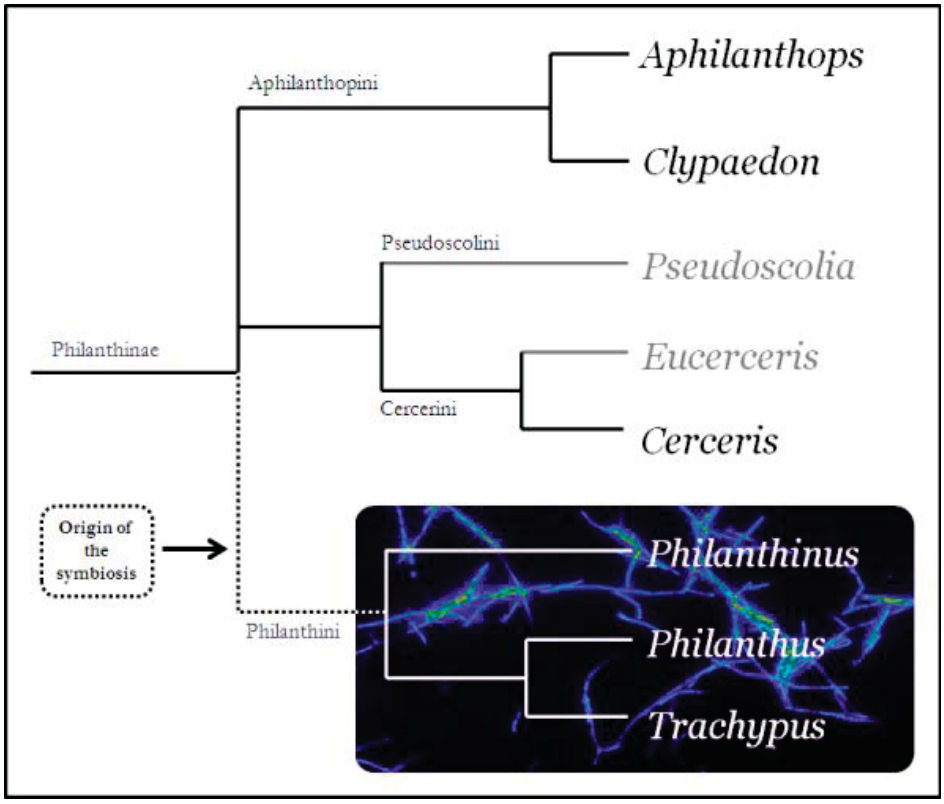

Figure 8.1 Distribution of symbiotic *Streptomyces* bacteria in wasps of the Crabronid subfamily Philanthinae. Phylogenetic tree modified from Alexander´s (1992) tree based on morphological data. Genera containing antennal gland reservoirs and symbiotic *Streptomyces* are underlayed with a FISH picture of the symbionts (in pseudo-colors), and genera that have not been analysed yet are depicted in grey. Genera highlighted in bold black have been analysed but do not possess bacterial symbionts.

8.2.3 Specificity of the beewolf-*Streptomyces* symbiosis

Although the reconstructed phylogenetic trees of beewolves and their symbiotic bacteria show some degree of co-cladogenesis, suggesting an ancient and co-evolved relationship (Chapter 6), the comparison of both phylogenies also revealed various discrepancies between the two trees, indicating horizontal transmission of symbionts among host species, which might be attributed to the unusual way of vertical symbiont transmission in beewolf wasps.
As described before, the beewolf larva incorporates the *Streptomyces* symbionts into the cocoon walls, where they remain for a prolonged period of time during beewolf hibernation. After successful development, eclosing adult females re-acquire the bacteria from the cocoon surface (Kaltenpoth et al. 2010a), depicting a vertical transmission mode from mother to offspring. Thus, the unique transmission route of the *Streptomyces* symbionts, includes a long period of persistence in the environment, which may facilitate horizontal transfer or *de novo* uptake of symbiotic bacteria from their environment. This is additionally supported by the observed pattern of diffuse co-cladogenesis of beewolves and their protective bacterial symbionts (Chapter 6).

Given the external mode of transmission via the cocoon horizontal symbiont replacement may occur either via (i) nest reuse which has been reported for some beewolves and could facilitate horizontal transfer via spores that subsist in beewolf habitats (Evans and O'Neill 1988; Koehler et al. 2013), (ii) interspecific predation among sympatrically occurring beewolf species (Evans and O'Neill 1988) and

subsequent uptake of symbiotic bacteria from the prey items in the nest or (iii) the transmission of symbiotic bacteria or bacterial spores via parasitoids visiting the nest (Kimsey and Bohart 1990; Strohm et al. 2008a).

However, despite these possible routes for horizontal symbiont transmission or *de novo* uptake of bacterial strains, and despite the rare examples of Actinobacteria other than the symbiotic *Streptomyces* in female beewolf antennae (Chapter 6), mostly closely related strains of ´*Candidatus* Streptomyces philanthi` have been detected in the beewolf genera *Philanthus*, *Trachypus* and *Philanthinus,* suggesting a not only ancient, but highly specific association between these two organisms (Kaltenpoth et al. 2006; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012).

Two major hypotheses have been proposed to explain the persistence and specificity of cooperative association: (i) partner fidelity, where a symbiont-mediated fitness benefit to the host feeds back as returned benefits to the symbionts, rewarding beneficial symbionts while harmful symbionts experience reduced fitness (Bull and Rice 1991; Simms and Taylor 2002; Sachs et al. 2004; Sachs et al. 2011a), or (ii) partner choice, where hosts preferentially reward beneficial symbionts and/or sanction cheaters, thereby selecting for beneficial associations (Bull and Rice 1991; Denison 2000; Sachs et al. 2004; Sachs et al. 2011a). In general, partner choice has been proposed to select for co-operative strains among environmentally acquired microbial symbionts, as is the case in the nitrogen-fixing rhizobia of legumes (Denison 2000; Kiers et al. 2003), mycorrhizal fungi of plants (Kiers et al. 2011; Maillet et al. 2011), the yucca moths associated with yucca trees (Pellmyr and Huth 1994; Huth and Pellmyr 2000), and the bioluminescent *Vibrio fischeri* bacteria of squids (Nyholm and McFall-Ngai 2004; Nyholm and Graf 2012). By contrast, partner fidelity is commonly assumed to facilitate the widespread and ecologically important vertically transmitted symbioses of insects (Sachs et al. 2011b).

However, in contrast to other insect symbioses which rely on partner choice rather than fidelity (Zhang et al. 2007; Cafaro et al. 2011; Kikuchi et al. 2011), it has been shown that only a distinct monophyletic clade of *Streptomyces* symbionts appears to be able to successfully establish a long-term association with the host. This is additionally supported by behavioral and molecular data, which provide evidence that the transmission of non-symbiotic bacteria to the next beewolf generation may be somehow selectively hindered and/or less efficient (Chapters 6+7). A selective blockade of heterospecific bacteria in the beewolf-*Streptomyces* symbiosis would suggest that partner choice might act inside the antennal gland reservoirs of female beewolves via growth-inhibition of non-symbiotic bacteria over symbiotic strains and/or during secretion of the white substance into the brood cell. Additionally, partner choice could act during application of the bacteria to the cocoon walls by the beewolf larva. Although, underlying molecular mechanisms of a selective symbiont transmission in the beewolf-*Streptomyces* symbiosis are unknown, the behavioral evidence suggests that control of transmission may facilitate partner choice and thereby contribute to the stability of the beewolf-*Streptomyces* mutualism over evolutionary timescales (Chapter 7). To efficiently discriminate the native symbionts versus ineffective bacterial strains, however, the hosts must either provide selective conditions which favor the growth or transmission of ´*Candidatus* Streptomyces philanthi` over other bacterial strains, or must be able to recognize their beneficial bacterial symbiont and sanction non-cooperating microbes.

The hypothesis of a selective host environment that favors establishment of certain antibiotic-producing symbionts has recently been proposed to shape the association between leaf-cutter ants and their actinobacterial community (Scheuring and Yu 2012). Although Actinobacteria other than the native ´*Candidatus* Streptomyces philanthi` have been shown to grow in beewolf antennae, it is possible that their long-term persistence and/or transmission into the offspring´s brood cell are prevented by

the selective conditions provided by the host (Chapter 7). Potentially, beewolf females may somehow physiologically regulate and control the process of symbiont transmission. As demonstrated before, life time reproductive success (number of brood cells) of beewolf females significantly depends on the presence or absence of the *Streptomyces* symbionts (Koehler 2010). Thus, female beewolves may sense the filling capacity of the reservoir, which then triggers secretion of the white substance from the antenna into the brood cell (Koehler 2010). However, in the presence of heterospecific symbionts the number of brood cells throughout their life was comparable to females containing the native ´*Ca.* Streptomyces philanthi` symbiont. Thus, the signal triggering the brood cell construction behavior in beewolves appears to depend on the filling capacity of the reservoir rather than the identity of the contained symbionts, and may depict an adaptive trait for symbiont-containing individuals, as it would lead to the secretion of an optimal amount of symbionts into the brood cell to protect the offspring. Concordantly, previous studies showed a positive correlation of bacterial cells with the amount of symbiont-mediated antibiotic substances (Chapter 3). Although full antennal gland reservoirs appear to be necessary for eliciting the brood cell construction behavior in females, however, the filling status alone does not seem sufficient to trigger the secretion of the bacteria present in the antennal gland reservoirs, suggesting additional mechanisms discriminating between beneficial and ineffective bacterial strains.

Specific partner-recognition is already known from plants (Denarie et al. 1996; Gherbi et al. 2008; Kiers et al. 2011; Maillet et al. 2011) as well as from marine symbioses such as the bioluminescent *Vibrio* bacteria in squids (Nyholm and McFall-Ngai 2004; Nyholm and Graf 2012) and dinoflagellates in jellyfish (Colley and Trench 1983; Fitt 1985). After the first recognition process, a subsequent activation of a specific host immune response resulting in the production of reactive oxygen species (Davidson et al. 2004) or cystein-rich peptides (van de Velde et al. 2010) may provide the host with the potential to select for symbiotic bacterial strains, while at the same time sanctioning ineffective microbes. However, both mechanisms of selection require the transport of host-specific factors into the antennal gland reservoirs, which likely occurs from the hemolymph (Kaltenpoth et al. 2009) or the surrounding gland cells (Goettler et al. 2007).

In summary, the observed pattern of co-evolution between beewolves and their defensive *Streptomyces* symbionts suggests that despite their localization in specialized antennal gland reservoirs, their extracellular lifestyle and their unique environmental transmission route allow for horizontal symbiont replacement. Thus, the beewolf-*Streptomyces* mutualism presents an interesting transitional case between vertically transmitted primary symbionts and more loosely associated secondary symbionts. Host control over symbiont identity, and domestication of an increasingly dependent symbiont, may be factors that promoted the long-term stability of an association with a specific clade of symbionts.

8.3 CONCLUDING REMARKS

Our results establish the beewolf-*Streptomyces* symbiosis as an ancient and co-evolved mutualism that has served to efficiently protect the wasp offspring against pathogenic microorganisms since the Cretaceous, constituting the oldest dated defensive symbiosis known at present.
Producing a cocktail of different antibiotic substances that are highly stable and independent of environmental changes, the symbiotic *Streptomyces* provide an efficient and reliable long-term antimicrobial protection to the beewolf wasp progeny during their long and vulnerable phase of hibernation. Moreover, host control over symbiont-mediated antibiotic production as well as symbiont transmission is important to ensure the perpetual antimicrobial defense and to stabilize the cooperative association between beewolves and streptomycetes, suggesting that partner choice may reinforce partner fidelity, thereby playing a pivotal role in the maintenance and persistence of the beewolf-*Streptomyces* symbiosis.

In summary, this thesis contributes to the current knowledge of the ecology and evolution of defensive insect-bacteria symbioses and their potential for the utilization of novel antimicrobial substances in biotechnological and medical applications, and thus, may provide human medicine with new strategies in the ongoing arms race with increasingly resistant pathogens.

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CHAPTER 9

SUMMARY

9.1 ANTIBIOTIC-PRODUCING SYMBIONTS IN BEEWOLF WASPS

It is common for animals, plants and fungi to participate in a wide range of associations with microorganisms, and symbioses have been demonstrated to play a crucial role in the evolution of complexity and adaptation for various organisms. Being the most diverse animal class on earth, particularly insects are associated with an extraordinary variety of symbiotic microorganisms. In many insects the symbionts patronize the host´s metabolic potential by producing essential dietary supplements, thereby promoting the survival of their host. However, recent studies yielded evidence that symbiotic microorganisms can play an essential role for the protection of the insect host, its nutritional resources and it´s offspring against pathogenic bacteria and fungi.

This thesis investigates the unique symbiotic association of beewolf wasps with antibiotic producing *Streptomyces* bacteria. Solitary digger wasps of the genera *Philanthus, Philanthinus* and *Trachypus,* engage in a highly specific symbiosis with bacteria of the genus *Streptomyces* that provide protection to the wasp´s progeny. Female wasps cultivate the symbionts in specialized antennal gland reservoirs and secrete them into the subterranean brood cells as a white substance prior to oviposition. Subsequently, they are taken up by the beewolf larva and are incorporated into the silken walls of the cocoon, where they provide an antimicrobial defense against pathogen infestation by producing a cocktail of different antibiotic substances.

The symbiont-produced antibiotic substances on the cocoon surface have been shown to efficiently ward of soil-dwelling fungi and bacteria, thereby significantly enhancing the survival probability of the beewolf´s progeny during their long and vulnerable phase of development, and GC-MS (Gas chromatography-mass spectrometry) analyses yielded new insights into the production dynamics and ecology of these symbiont-mediated compounds. The results revealed that the antibiotics are produced within the first two weeks after cocoon spinning. Establishing a high concentration of antibiotic substance on the cocoon surface, which remains stable over time regardless of changing environmental conditions, the *Streptomyces* symbionts provide an efficient and reliable long-term antimicrobial protection to the beewolf wasp´s progeny. Moreover, the chemical analysis revealed temporal differences in the composition of the antibiotic cocktail, implying that the antibiotic compounds are differentially produced and/or degraded over time, which might bestead the defense against various pathogens and prevent the evolution of resistant strains.
In addition, we could demonstrate that, in the larval stage, the beewolf host can directly influence the symbiont-mediated antibiotic production and thereby its own protection against pathogenic microorganisms during the long and unpredictable phase of development. The mechanisms leading to the enhancing effect on symbiont-mediated antibiotic production by the beewolf larva are not yet clear, however, several pieces of evidence suggest that a larval excretion, most likely from the larva´s gut, triggers the production of antibiotics in the bacterial symbionts: (i) Cocoons without larva have significantly less antibiotics on their surface, and (ii) this effect can be rescued by adding larval gut content to cocoons without larva. To our knowledge the beewolf-*Streptmyces* symbiosis constitutes the

first example of a defensive association where the host itself can influence the amount of antimicrobial substances produced. Thus, host control over antibiotic production may be essential to ensure an efficient antimicrobial protection for the host offspring against pathogenic microoranisms, and facilitates the successful transmission of the bacterial symbionts to the next generation of beewolf wasps.

9.2 EVOLUTIONARY HISTORY AND SPECIFICITY OF THE BEEWOLF-*STREPTOMYCES* SYMBIOSIS

In European beewolves, symbiotic *Streptomyces* bacteria are transmitted vertically from the mother to their offspring via the cocoon surface. After secretion of the *Streptomyces* symbionts into the brood cell, the beewolf larva incorporates the bacteria into the cocoon walls, and during eclosion of the adult beewolf the bacteria are assimilated from the cocoon into the antennal gland reservoirs of female wasps. After application of a high number of symbiont cells to the cocoon walls, the *Streptomyces* population size remains stable for the whole time of beewolf development. However, during eclosion only a few of the *Streptomyces* cells are taken up by the emerging female, leading to an extreme bottleneck, that may have significantly affected the evolution of this unique symbiotic association via increasing genetic drift, the accumulation of mildly deleterious mutations and genome erosion.

To investigate the evolutionary history of the symbiotic association between *Streptomyces* and Philanthini digger wasps, phylogenetic analyses of the bacterial symbionts as well as the host species have been conducted, and the analyses revealed that beewolves cultivate a distinct clade of *Streptomyces* in their antennal reservoirs for protection of their progeny against pathogenic microorganisms. Molecular clock analyses yielded evidence that the beewolf-*Streptomyces* association evolved from a soil-dwelling ancestor at least 68 million years ago, thus constituting the oldest dated defensive symbiosis known at present.
However, the observed pattern of diffuse co-evolution between beewolves and defensive *Streptomyces* symbionts indicates that, despite their localization in specialized antennal gland reservoirs, their extracellular lifestyle and external route of transmission allow for horizontal symbiont replacement and *de novo* uptake of non-symbiotic Actinobacteria from the environment. Indeed, our results show that strains of symbiotic bacteria were exchanged among host species, possibly through predation or nest reuse, and that other Actinobacteria are occasionally taken up by beewolves.

Concordantly, controlled re-infection experiments of aposymbiotic female beewolves with con- and heterospecific actionbacterial strains verified that different Actinobacteria can grow inside the beewolf´s specialized antennal gland reservoirs. However, behavioral observations as well as molecular and chemical analyses revealed that the transmission of heterospecific bacteria may be selectively hindered, resulting in a discontinuous vertical transmission of the bacteria to the next generation of beewolf wasps. Thus, partner choice during symbiont transmission reinforces host-symbiont fidelity in the beewolf-*Streptomyces* symbiosis, and thereby stabilizes the cooperative association over long evolutionary timescales.

CHAPTER 10

ZUSAMMENFASSUNG

10.1 ANTIBIOTIKA-PRODUZIERENDE SYMBIONTEN IN BIENENWÖLFEN

Tiere, Pflanzen und Pilze leben im Allgemeinen in sehr enger Beziehung mit einer großen Vielfalt an Mikroorganismen und Symbiosen spielen bewiesenermaßen eine entscheidende Rolle in der Evolution von Komplexität und Anpassung verschiedener Organismen. Als artenreichste Tier-Klasse der Erde, sind besonders Insekten mit einer außerordentlichen Vielfalt an symbiotischen Mikroorganismen assoziiert. In den meisten Insekten unterstützen die Symbionten den Metabolismus ihres Wirtes durch die Herstellung von essentiellen Nährstoffkomponenten und gewährleisten damit dessen Überleben. Neue Studien zu diesem Thema ergaben aber kürzlich, dass symbiotische Mikroorganismen ebenfalls eine wichtige Rolle für den Schutz des Wirtes, dessen Nahrungsressourcen sowie seinen Nachkommen gegen pathogene Bakterien und Pilze spielen können.

Die vorliegende Arbeit untersucht die einzigartige Assoziation von Bienenwolf-Wespen mit Antibiotika produzierenden Actinobakterien. Solitäre Grabwespen der Gattungen *Philanthus*, *Philanthinus* sowie *Trachypus* unterhalten eine hochspezifische Symbiose mit Bakterien der Gattung *Streptomyces*, welche Schutz für den Wespennachwuchs bieten. Das Wespen-Weibchen kultiviert die symbiotischen Bakterien in spezialisierten Antennendrüsenreservoiren und sezerniert sie vor der Eiablage in großen Mengen in die Brutkammer als eine Art „Weiße Substanz“. Später werden die Bakterien von der Larve aufgenommen und während des Spinnens ihres Kokons in dessen Wände eingesponnen, wo sie den Wespen-Nachwuchs durch die Produktion unterschiedlicher antibiotischer Substanzen vor Krankheitserregern schützen.

Die von den Symbionten produzierten antibiotischen Substanzen wehren nachweislich wirkungsvoll pathogene Bodenbakterien sowie im Boden lebende Pilze ab, wodurch sich die Überlebenswahrscheinlichkeit der Bienenwolf-Nachkommen in der sensiblen Phase der Überwinterung maßgeblich erhöht. GC-MS (Gas Chromatograph-Massenspektrometer) Analysen haben zusätzlich neue Einblicke in die Produktionsdynamik sowie die Ökologie dieser symbionten-produzierten Antibiotika ermöglicht. Die Ergebnisse zeigen, dass die Antibiotika besonders innerhalb der ersten zwei Wochen nach dem Spinnen des Kokons produziert werden. Mit der auf diese Weise erzeugten, hohen Konzentration der Antibiotika auf der Kokonoberfläche, welche unabhängig von sich ändernden Umweltbedingungen und über einen langen Zeitraum hinweg stabil bleibt, gewährleisten die Symbionten einen effizienten und zuverlässigen antimikrobiellen Langzeitschutz für die Bienenwolf-Nachkommen während deren Entwicklung und Überwinterung. Des Weiteren ergab die chemische Analyse temporäre Unterschiede in der Zusammensetzung des antibiotischen „Cocktails“, was darauf hinweist, dass verschiedene Antibiotika zu unterschiedlichen Zeitenpunkten produziert werden, oder im Laufe der Zeit differenziell abgebaut werden können. Eine alternierende Produktionsdynamik der verschiedenen antibiotischen Substanzen dient möglicherweise der Abwehr unterschiedlicher Pathogene oder stellt einen effizienten Mechanismus zur Vermeidung von resistenten Bakerienstämmen dar.

Zusätzlich kann die Bienenwolflarve im Kokon die Menge der von den Symbionten produzierten Antibiotika steigern, wodurch es dem sich entwickelnden Wirt möglich ist den antimikrobiellen Schutz aktiv zu beeinflussen. Wie die Larve die Antibiotikaproduktion der Symbionten beeinflusst, ist noch unklar. Jedoch gibt es Hinweise, dass die sich entwickelnde Bienenwolflarve durch Abgabe eines Sekrets, wahrscheinlich aus dem Larvendarm, die Produktion antibiotischer Substanzen in den bakteriellen Symbionten anregt: (i) Kokons ohne Larve haben nachweislich weniger Antibiotika auf ihrer Oberfläche, und (ii) Kokons ohne Larve haben höhere Antibiotikamengen, wenn das Darmsekret der Larve nachträglich auf die Oberfläche des Kokons gegeben wird. Damit ist die Assoziation zwischen Bienenwolf Wespen und *Streptomyces* Bakterien, nach derzeitigem Wissenstand, das erste Beispiel einer Verteidigungssymbiose, bei der es dem Wirt möglich ist die Menge der Bakterien-produzierten, schützenden Substanzen selbst zu beeinflussen.

10.2 EVOLUTIONSGESCHICHTE UND SPEZIFITÄT DER BIENENWOLF-STREPTOMYCETEN SYMBIOSE

Die bakteriellen Symbionten des Bienenwolfs werden vor allem vertikal von der Mutter zu den weiblichen Nachkommen weitergegeben. Dies geschieht beim Schlupf des adulten Weibchens, während dessen die Bakterien vom Kokon in die Antennendrüsenreservoire aufgenommen werden. Trotz, auch nach Überwinterung, unverändert hoher Bakterien-Zahl auf dem Kokon, werden nur wenige Streptomyceten-Zellen von den Weibchen assimiliert. Dies führt zu einer signifikanten Reduktion der Bakterien-Population, einem sog. „Bottleneck"-Effekt. Diese drastische Reduktion der Bakterienzahl mit jeder neuen Generation hat vermutlich im Folgenden die Evolution dieser einzigartigen Symbiose, durch genetische Drift, Akkumulation schädlicher Mutationen sowie durch fortschreitende Genomerosion, maßgebliche beeinflusst.

Genauere Untersuchungen der Evolutionsgeschichte der Symbiose zwischen Bienenwölfen und Bakterien der Gattung *Streptomyces* ergaben, dass die Symbionten aller analysierten Bienenwolf-Arten eine monophyletische Gruppe innerhalb der Gattung *Streptomyces* bilden, was darauf hindeutet, dass die Symbiose hoch spezifisch ist und wahrscheinlich das Ergebnis einer langen Co-Evolution und Co-Speziation darstellt. Eine ungefähre Berechnung des Ursprungs der Bienenwolf-Symbiose anhand von Sequenzunterschieden verschiedener Gene, lieferte Beweise dafür, dass die Assoziation zwischen Bienenwolf Wespen und *Streptomyces* Bakterien wahrscheinlich während der Abspaltung der Unterfamilie der Philanthinae entstand und mehr als 68 Mio. Jahre zurückreicht. Damit stellt die Symbiose zwischen Bienenwolf Wespen und ihren symbiotischen Bakterien der Gattung *Streptomyces* die älteste aller bisher datierten Verteidigungssymbiosen dar.

Des Weiteren liefern die phylogenetischen Analysen Hinweise darauf, dass trotz der strikt vertikalen Weitergabe der Symbionten, von der Mutter an die Nachkommen, die Möglichkeit existiert, dass symbiotische Bakterien horizontal zwischen verschiedenen Bienenwolfarten ausgetauscht bzw. aus der Umwelt aufgenommen werden können. Dies wird wahrscheinlich dadurch ermöglicht, dass die bakteriellen Symbionten, trotz ihrer ungewöhnlichen Lokalisation in den Antennendrüsenreservoiren der Bienenwolfweibchen, extrazellulär über die Kokonoberfläche des Nachwuchses weitergeben werden. Tatsächlich lässt sich anhand der phylogenetischen Untersuchung zeigen, dass symbiotische Streptomyceten zwischen verschiedenen Bienenwolfarten ausgetauscht werden, wahrscheinlich durch wiederholte Nutzung des gleichen Nestes von unterschiedlichen Individuen. Des Weiteren wurden

auch vereinzelt andere Bakterienstämme in den Antennen von Bienenwolfweibchen gefunden, was darauf hinweist, dass nicht-symbiotische Mikroorganismen aus der Umwelt aufgenommen und kultiviert werden können.

In einem kontrollierten Experiment wurden daraufhin symbionten-freie Bienenwolfweibchen mit ihrem nativen sowie einem nicht-symbiotischen Bakterienstamm reinfiziert. Die Ergebnisse liefern Beweise dafür, dass nicht nur die symbiotischen Streptomyceten, sondern auch artfremde Bakterienstämme in den Antennendrüsenreservoiren von Bienenwolfweibchen wachsen können. Zusätzlich haben die Analysen aber auch gezeigt, dass nicht-symbiotische Bakterien nicht, oder nur teilweise, an die Nachkommen weitergegeben werden. Daraus resultiert, dass die Wahl des Symbiose-Partners während der Weitergabe der Symbionten die „Partnertreue" in einer solchen Interaktion unterstützt und verstärkt, was über evolutionäre Zeiträume zu einer Stabilisierung der kooperativen Assoziation führt.

Mit diesen Ergebnissen bietet die molekulare Phylogenie der Philanthinae, nicht nur relevante Ergebnisse im Bereich der Systematik und Evolutionsbiologie, sondern erleichtert auch maßgeblich zukünftige Studien zur Co-Evolution der Bienenwolf-*Streptomyces*-Symbiose, wie zum Beispiel die vergleichende Analyse der von unterschiedlichen Symbionten-Stämmen produzierten Antibiotika.

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„Besser als tausend Tage fleißigen Lernens, ist ein Tag mit einem großartigen Lehrer."
- Japanisches Sprichwort –

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WEITERFÜHRENDE AUSBILDUNG

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WISSENSCHAFTLICHE PUBLIKATIONEN

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Koehler S (2011): "Dances with wolves - Production dynamics of symbiont-produced antibiotics on wasp cocoons". Qiagen Anwendertreffen, BioCentiv GmbH, Jena, Deutschland

Koehler S (2012): "The sleeping beauty - Long-term antimicrobial protection for hibernating beewolf larvae by *Streptomyces* symbionts". 11th IMPRS Symposium, Max-Planck-Institut für Chemische Ökologie, Dornburg, Deutschland

Koehler S (2012): "Joining forces in defense: Production dynamics of symbiont-mediated antibiotics reveal long-term antimicrobial protection for wasp offspring". 24th International Congress of Entomology (ICE), Daegu, Süd Korea

Koehler S (2013): „Brotherhood of the wolf - Production dynamics of symbiont-mediated antibiotics reveal long term antimicrobial protection for wasp offspring", Workshop "Innovative Morphologie und Phylogenie**,** Institut für Spezielle Zoologie und Evolutionsbiologie, Friedrich-Schiller-Universität Jena, Deutschland

Koehler S (2013): "Symbioses in insects", Johannes-Heidenhain-Gymnasium, Traunreut, Deutschland

Koehler S (2013): "Brotherhood of the wolf: Symbiotic *Streptomyces* provide antimicrobial combination prophylaxis to beewolf wasps". Lehrstuhlseminar, Institut für Zoologie, Universität Cambridge, England

POSTER PRESENTATIONEN

Kaltenpoth M, Goettler W, **Koehler S** and Strohm E (2009):
Transmission route and population dynamics of a defensive insect symbiont. Workshop Symbiotic Interactions, Helmholtz Zentrum München – Deutsches Forschungszentrum für Gesundheit und Umwelt, München, Deutschland

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Dynamics of antibiotic production in the defensive beewolf-*Streptomyces* symbiosis. SAB Meeting 2010, Max-Planck-Institut für Chemische Ökologie, Jena, Deutschland

Koehler S, Kroiß J, Hertweck C and Kaltenpoth M (2011):
Beewolves´ bodyguards: Production dynamics and activity of symbiont-produced antibiotics on wasp cocoons. 10th IMPRS Symposium, Max-Planck-Institut für Chemische Ökologie, Dornburg, Deutschland

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Der Pakt der Wölfe – Symbiotische Bakterien schützen Wespennachwuchs durch Produktion von Antibiotika. „Offener Landtag Thüringen", Erfurt, Deutschland

Koehler S, Kroiß J and Kaltenpoth M (2011):
Beewolves´ bodyguards: Antibiotic production dynamics in the defensive beewolf-*Streptomyces* symbiosis. 13th Congress of the European Society of Evolutionary Biology (ESEB), Tübingen, Deutschland

Koehler S (2011):
Symbiose – Eine Hand wäscht die andere. "Lange Nacht der Wissenschaften", Max-Planck-Institut für Chemische Ökologie, Jena, Deutschland

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From *in vitro* to *in silico* and back: genomic and physiological background of antibiotic production by symbiotic *´Candidatus* Streptomyces philanthi`. SAB Meeting 2012, Max-Planck-Institut für Chemische Ökologie, Jena, Deutschland

Koehler S and Kaltenpoth M (2013):
Nurture vs. nature: Maternal and environmental effects on symbiont-mediated antimicrobial defense in a solitary digger wasp. 12th IMPRS Symposium, Max-Planck-Institut für Chemische Ökologie, Jena, Deutschland

Koehler S and Kaltenpoth M (2014):
The brotherhood of the wolf: Symbiont-mediated antibiotics provide efficient long-term defense for wasp offspring in the ancient beewolf-*Streptomyces* symbiosis. Keystone Symposia on Molecular and Cellular Biology "Mechanisms and Consequences of Invertebrate-Microbe Interactions", Tahoe City, CA, USA

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Juli 2011	Einführungskurs „GC-MS Analyse“ für Schüler der 8. – 12. Klasse im Rahmen des Programms „Jun.iversity“, Faszination Begabung e.V.
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Betreuung von Praktikanten sowie einer Master-Arbeit zum Thema Insektensymbiosen / Evolutionsbiologie

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SS 2009	Übungen zur Systematik und Ökologie der Tiere für Lehramts- und Diplomstudenten
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WS 2009	Großpraktikum Zoologie, Anatomie und Cytologie der Tiere für Lehramts- und Diplomstudenten im Hauptstudium

WISSENSCHAFTLICHE AUSLANDSAUFENTHALTE

2008	Darwin, Australien Forschungspraktikum Zoologie zum Thema: “Development of the basal termite *Cryptotermes secundus*”
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EIGENSTÄNDIGKEITSERLÄRUNG

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Sabrina Koehler

"May your symbionts be with you!"

Angela Douglas – *The symbiotic Habit, 2009*