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Postprint version\*

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- Published in:** **Functional Ecology**
- Reference:** **Kaltenpoth, M., Engl, T. (in press). Defensive microbial symbionts in Hymenoptera. Functional Ecology**
- Web link:** <http://onlinelibrary.wiley.com/journal/10.1111/%28ISSN%291365-2435>
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\*The manuscript edited after peer review (see: <http://oa.mpg.de/lang/en-uk/glossar/>)



# Defensive microbial symbionts in Hymenoptera

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## Summary

1. In all stages of their life cycle, insects are threatened by a multitude of predators, parasites, parasitoids, and pathogens. The lifestyles and feeding ecologies of some hymenopteran taxa render them especially vulnerable to pathogen infestation. Specifically, development in subterranean brood cells, mass provisioning of resources for the offspring, and the life of social insects in large communities can enhance the risk of pathogen infestation and/or the spread of disease among conspecifics.
2. To counteract these threats, insects have evolved mechanical, chemical and behavioral defenses as well as a complex immune system. In addition to the host's own defenses, some Hymenoptera are associated with protective symbionts. Leaf-cutting ants, bees and bumblebees, and solitary digger wasps engage in symbiotic interactions with bacteria that protect the adult host, the developing offspring, or the food resources against microbial infections. In the well-studied cases of ants and wasps, the protective activity is mediated by the production of antimicrobial secondary metabolites. In other symbiotic interactions, however, competitive exclusion and immune priming may also play an important role in enhancing protection. Phylogenetic studies indicate that the defensive associations in Hymenoptera are generally more dynamic than the intimate nutritional mutualisms, with horizontal transfer or *de novo* uptake of the symbionts from the environment occurring frequently.
3. Mutualistic microorganisms can also significantly influence the outcome of host-parasitoid interactions. Some insects are protected by symbiont-produced toxins against parasitic wasps. Ichneumonid and braconid parasitoids, on the other hand, are associated with symbiotic viruses that are injected into the caterpillar host during oviposition and suppress its immune system to the advantage of the parasitoid.
4. The increasing affordability of next-generation sequencing technologies will greatly facilitate the analysis of insect-associated microbial communities and undoubtedly uncover a plethora of as yet unknown protective symbioses. However, a detailed understanding of the host's natural history is indispensable for elucidating the fitness benefits of the symbionts and the molecular basis of symbiont-conferred protection.

## Key-words

Actinobacteria, beewolf, defensive mutualism, honeybee, immune system, leaf-cutting ants, parasitoid, pathogen defense, polydnavirus, protective symbiosis.

## Introduction

The Hymenoptera represent one of the four megadiverse holometabolous insect orders, with more than 110,000 described species to date, and an estimated total number of up to 2.5 million species worldwide (Grissell 1999; Whitfield 2003; Heraty et al. 2011). The evolutionary origin of the Hymenoptera dates back to the Triassic, and they have since undergone one of the most successful adaptive radiations among arthropods (Wiegmann et al. 2009). The order is generally divided into the paraphyletic “Symphyta” (sawflies) and the monophyletic Apocrita (Peters et al. 2011). The latter comprises the ecologically as well as economically important groups of bees, wasps, and ants, which play key roles in terrestrial ecosystem functioning, e.g. as plant pollinators, predators, scavengers and parasitoids (Wilson 1971).

A plethora of different lifestyles and feeding ecologies occur within the Hymenoptera. Herbivory as the presumed ancestral condition is still found in the larvae of some lineages of sawflies that feed on leaves, shoots, or pollen. Other symphytan groups are wood-boring, which probably represents the lifestyle from which ectoparasitism has evolved in the ancestor of all extant Orussoidea and Apocrita (Dowton & Austin 2001; Whitfield 2003). While the Orussoidea maintained the ectoparasitic lifestyle and remained relatively species-poor, the Apocrita have experienced an immense radiation with multiple transitions from ecto- to endoparasitism, predation, omnivory, mycophagy, or secondary reversals to herbivory (Whitfield 2003). The evolution of nest-building and provisioning behavior in some groups has probably laid the foundation for the subsequent evolution of differential degrees of social behavior, ultimately culminating in the complex societies of eusocial bees, wasps, and ants (Andersson 1984). The nature of the provisions in social insects ranges from dead arthropods (in many wasps and ants) to pollen and nectar (in most bees) and to actively tended fungal crops that are cultivated by leaf-cutting ants in specialized gardens equivalent to human agriculture (Mueller et al. 2005). However, although the social species have been studied most intensively, it has to be noted that the vast majority (around 75%) of extant hymenopteran species are parasitoids (Whitfield 2003).

The diversity in lifestyles not only requires physiological adaptations towards the effective utilization of different food sources, but also the evolution of defense mechanisms against other organisms. While pathogens, predators and parasitoids pose a universal threat that insects in general have to cope with, the ecological characteristics of some Hymenoptera likely increased the selective pressures on evolving particular defense mechanisms. Specifically, parasites and parasitoids have to continuously protect

themselves against the immune system of the host or evade detection in the first place (Strand & Pech 1995). In many non-parasitic taxa, on the other hand, the completion of larval development in underground nests, especially in combination with the provisioning and storage of nutrient-rich food resources, entails a significant risk of pathogen infestation from the surrounding soil (Janzen 1977; Jurkevitch 2011). This problem is likely exacerbated in social species, due to the storage of particularly large amounts of resources and the facilitation of within-colony transfer of detrimental microbes by contact of nestmates (Currie, Mueller & Malloch 1999a; Cremer, Armitage & Schmid-Hempel 2007). While behavioral or chemical defenses in insects have traditionally received considerable attention, we are only beginning to appreciate the significant and diverse roles that symbiotic microorganisms can play in protecting the host against detrimental organisms (e.g. Brownlie & Johnson 2009; Kaltenpoth 2009).

Here we review the defensive symbiotic alliances with bacteria and viruses that are currently known in Hymenoptera, and we discuss these alliances in the light of the hosts' ecology to identify ecological characteristics that may predispose certain taxa towards engaging in defensive symbioses. After a brief introduction into alternative defense strategies, we will focus on symbiont-mediated protection of the adult insect or the developing offspring against pathogens, parasites, parasitoids and predators, consider mutualistic bacteria that protect the host's nutritional resources against detrimental fungi, and discuss the complex roles of symbiotic bacteria and viruses in host-parasitoid interactions (Fig. 1). Our aim is not only to provide an overview of this emerging field, but also to suggest novel directions for future research in order to gain a better understanding of the importance and diversity of defensive mutualisms in Hymenoptera.

### **Defense strategies in insects**

To protect themselves against antagonists, insects evolved a range of defensive mechanisms. A simple solution to the problem is to evade or avoid the antagonist temporally or spatially by behavioral adaptations (Strohm, Laurien-Kehnen & Bordon 2001). If this is not possible, mechanical protection, physical contest with a predator or parasitoid, or active removal of pathogens or parasites can constitute efficient defense strategies. Noxious chemicals constitute another common means for protection, and they can act in a variety of ways. Defensive compounds can serve as repellents that deter enemies but have no actual harmful effect (Evans & Schmidt 1990). Alternatively, they can distract

attackers, by creating a sensory overload or by physically inhibiting mouthparts, legs or wings (Gross 1993). Finally, toxic substances directly interfere with the enemy's metabolism and have reversible or irreversible ill effects on its physiology (e.g. Bot et al. 2002). Generally, defensive substances can be produced *de novo* or sequestered from the environment (Cane, Gerdin & Wife 1983).

Once in contact, the next line of defense is the insect's immune system. As several excellent recent reviews are available on this topic (e.g. Schmid-Hempel 2005; Siva-Jothy, Moret & Rolff 2005), we will only provide a brief summary here to enable an understanding of the contributions of symbiosis to insect immunity. The insect immune system consists of several mechanisms that are complementary or act in concert to provide protection against pathogens and parasites (Schmid-Hempel 2005). The first layer of defense is the cuticle, a tough, flexible and waterproof barrier. The outer layer of the cuticle, the epicuticle, primarily consists of lipids and hydrocarbons, which reduce desiccation but provide no real protection against pathogens. The inner (endo-)cuticle is composed of chitin and proteins that gain their rigidity by cross-linking, melanization and sclerotization. Thus, the endocuticle provides mechanical protection against pathogens, and only a few specialized entomopathogenic fungi evolved the ability to actively penetrate this layer. However, the cuticle is not only a passive barrier, it also possesses active immune components. Antimicrobial peptides have been found in both the epicuticle and the endocuticle, and the latter additionally contains phenoloxidase, an enzyme involved in the sclerotization process but also in the defense against invading pathogens (Siva-Jothy et al. 2005).

Once the cuticle is breached, the insect's immune system must recognize the invading pathogen. Pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides, peptidoglycan, beta-1,3-glucans and mannans are recognized by a set of receptor proteins (Schmid-Hempel 2005; Siva-Jothy et al. 2005). These activate the humoral and cellular immune responses which include opsonization, phagocytosis, melanization, encapsulation, coagulation, the production of reactive oxygen and nitrogen species, antimicrobial peptides and proteins with lytic activities (Schmid-Hempel 2005). These processes attack or isolate and – if successful – ultimately kill the invading pathogen or parasite.

In addition to the cuticle, there are three main entry routes for pathogens and parasites into the insect's body (Siva-Jothy et al. 2005): the digestive system, the reproductive tract, and the tracheae. In all three organs, the cuticle is partially very thin or completely absent, because there is a trade-off between protective function and permeability for nutrients and gases. Although the gut is still protected by a thin cuticular layer, which can also be sloughed to remove attached pathogens, it is especially prone to microbial infestation as it constantly comes into contact with microbes that are ingested with the food.

Consequently, the gut epithelium is immunologically very active. Several defensive antimicrobial peptides (AMPs) like defensins, Gram-negative binding proteins, chitinase-like proteins, serine proteases and lectin-like proteins as well as phenoloxidase and small cytotoxic molecules like nitric oxide, radical hydroxide and peroxide are produced for defense against pathogens and parasites (Siva-Jothy et al. 2005). The epithelium of the tracheae seems to be similarly active, and hemocytes are intimately associated with these tissues (Siva-Jothy et al. 2005). Likewise, different mechanisms have been described that protect the genitalia from infections, e.g. antimicrobial peptides in the seminal fluid of fruit flies (*Drosophila melanogaster*; Lung, Kuo & Wolfner 2001) and bed bugs (*Cimex lectularius*; Otti et al. 2009).

As social insects live in large colonies that facilitate the transmission of pathogens and thereby increase susceptibility to infections, many social taxa have evolved specialized mechanisms to prevent pathogens from spreading within their colonies, which are collectively referred to as “social immunity”. Hygienic behaviors, like allogrooming, control of individuals entering the nest, separating groups with different tasks and infection risks, and waste control reduce the risk of pathogen infestation in the colony (Cremer & Sixt 2009). Furthermore, ants, termites, bees and wasps apply antimicrobial substances to colony members (e.g. Baracchi, Francese & Turillazzi 2011) or incorporate materials that contain such compounds into their nests (e.g. Batra 1980; Rosengaus, Guldin & Traniello 1998; Chapuisat et al. 2007).

### **Protection of the adult insect against pathogens and parasites**

In addition to the host’s own defenses, several insect taxa are known to team up with symbiotic microorganisms for protection. There are three main mechanisms by which symbionts can provide protection: (1) Symbiotic microorganisms can produce chemical compounds that have direct harmful effects on antagonists, (2) they can colonize vulnerable niches in or on the host and competitively exclude pathogens from successfully establishing an infection, or (3) they can interact with the host immune system and thereby enhance resistance to pathogens or parasites.

The production of antimicrobial compounds is the most common way by which mutualistic microbes participate in the protection of adult insects. As preventive measures, those antimicrobial compounds can act even before the pathogens come in contact with or enter the insect body. Attine ants use not only the secretion from their metapleural glands (Ortius-Lechner et al. 2000; Bot et al. 2002) but also antibiotics produced by symbiotic Actinobacteria growing on their cuticle for pathogen defense (Oh et

al. 2009a; Mattoso, Moreira & Samuels 2011). Both the gland secretions and the symbiont-produced antimicrobial compounds protect their fungal gardens from parasitic fungi (see below), but also provide protection for the adult ants themselves against the entomopathogenic fungus *Metarhizium anisopliae* (Mattoso et al. 2011).

Alternatively, symbionts can modulate the host's immune system in a way that enhances the efficiency of protection against pathogens. The immune system is generally stimulated by contact with low levels of pathogenic bacteria (Evans & Lopez 2004) or fungi (Konrad et al. 2012). Previously pathogen-exposed individuals show an activated immune system upon second exposure, which leads to a more efficient defense against the pathogen (Konrad et al. 2012). Interestingly, the immune system cannot only be primed by pathogens, but also by the presence of symbionts: The encapsulation response of *Camponotus fellah* ants increases significantly with a higher number of intracellular *Blochmannia* bacteria as compared to ants which have been treated with antibiotics to eliminate their symbionts (de Souza et al. 2009). Likewise, probiotic *Lactobacillus* bacteria induce the expression of the antibacterial peptide abaecin in honeybee (*Apis mellifera*) larvae (Evans & Lopez 2004). Thus, symbiotic bacteria can activate the immune system of the insect host and thereby increase the efficiency of pathogen defense.

It has long been hypothesized that an important function of the native gut microbiota of insects is to competitively inhibit pathogens from colonizing the gut (Berg 1996; Dillon & Dillon 2004), especially as the gut represents one of the most sensitive entry routes for pathogens (Siva-Jothy et al. 2005). The first empirical support for this hypothesis came from studies on the locust *Schistocerca gregaria* (Dillon & Charnley 1995). Locust feces contain at least three different quinines, which significantly inhibit the germination of *M. anisopliae* conidia (Dillon & Charnley 1995). These quinines are absent from germ-free locusts, but present in individuals that are infected exclusively with the dominant gut microbe *Pantoea agglomerans* (Dillon & Charnley 1995), indicating that the protective quinines are produced by this bacterial symbiont.

In Hymenoptera, we are only beginning to understand the composition of microbial gut communities and their possible importance for the host. Due to the recent concerns about the decline of honeybee populations and the concomitant decrease in pollinating services, the microbiota of social bees and its significance for colony health has been subject to especially intense research efforts. Compared to other Hymenoptera and Diptera, honeybees possess a strongly reduced set of immune-related genes (Evans et al. 2006), which may be compensated by the presence of protective microbial symbionts, a strategy that has also been suggested for the pea aphid (Moran et al. 2005; Gerardo et al. 2010). Additionally, the low



number of genes encoding lysozyme (Kunieda et al. 2006) and the low expression of lysozyme in the gut epithelium (Anderson et al. 2011) may support the establishment of a distinct gut microbiota that takes part in immune defense. In both honeybees and bumblebees, a consistent gut microbiota comprising about nine different phylotypes has been found across geographical regions (Gilliam 1997; Cox-Foster et al. 2007; Martinson et al. 2011).

In bumblebees (*Bombus terrestris*), a betaproteobacterial symbiont in the gut has been implicated in the resistance against the trypanosomatid intestinal parasite *Crithidia bombi*. The bacteria are transferred between nestmates via the feces, but the mechanisms underlying the protective activity against *C. bombi* are not known yet (Koch & Schmid-Hempel 2011). In honeybees, some evidence for protective activities of the gut microbiota was provided by a series of *in vitro* and *in vivo* studies. Specifically, several lactic acid bacteria (Forsgren et al. 2010) as well as *Escherichia coli*, *Providencia* and *Sphingomonas* strains isolated from the honeybee gut (Yoshiyama & Kimura 2009) were found to have inhibitory effects on the brood pathogen *Paenibacillus larvae* and to thereby significantly enhance the survival probability of larvae in infected colonies (Forsgren et al. 2010). Other microbial symbionts in honeybee hives, notably strains of the genus *Bacillus*, have been implicated in the defense against fungal pathogens, especially *Ascosphaera apis*, the causative agent of chalkbrood. As *Bacillus* strains have been repeatedly isolated from various bee species including 25-40 million year-old amber-preserved specimens (Cano et al. 1994; Cano & Borucki 1995), an intimate association between bees and protective *Bacillus* symbionts has been hypothesized (Gilliam 1997). It has to be noted, however, that the mechanistic basis of the protective activity conferred by both the lactic acid bacteria and the *Bacillus* strains remains largely unknown, although the involvement of organic acids as well as antimicrobial peptides and fatty acids has been hypothesized (Gilliam 1997; Vasquez et al. 2012).

In addition to the gut of a single bee, the entire hive can be a source of symbionts providing resistance or defense (Cremer & Sixt 2009). Concordantly, bacteria with the ability to inhibit bee pathogens have been isolated throughout the bee hive (Gilliam et al. 1988; Anderson et al. 2011), and Anderson et al. (2011) even hypothesized that there might be a sub-caste of bees that is best-suited to nurture the symbiotic bacteria for the aid in food preservation and protection from disease. The bacterial community in bee hives is continuously exchanged between the adult individuals, stored food and larvae, and it is subjected to an incoming flow of bacteria from the environment. In addition, not only bacterial but also fungal symbionts with bioactive potential have been reported: Mucorales, but also

Aspergilli and Penicilli, inhibit the highly pathogenic fungus *Nosema apis* (Gilliam et al. 1988). Thus, the social bees represent a complex symbiotic system whose individual components are only beginning to be explored. Similar mechanisms of pathogen defense by a beneficial gut microbiota can be expected to occur in the other social hymenopteran communities of wasps and ants, where first studies already provided evidence for stable gut communities associated with certain taxa (in ants: Anderson et al. 2012).

### **Protection of the developing offspring against pathogens**

Juvenile stages of insects are often more susceptible to pathogen infection than adults, because some developmental stages are immobile or not able to protect themselves due to the lack of physical defenses, an incompletely developed immune system, or limited resources that need to be apportioned to both growth and immunity. In addition to defensive chemicals provided by parents, nestmates, or the developing insect itself, the offspring of social insects is usually tended by a special subcaste of workers that is separated from individuals which have more contact to pathogens and therefore pose a higher infection risk (Cremer et al. 2007). Furthermore, the microbial community associated with social insects and their nests can contribute significantly to pathogen defense. As indicated above, the microbiota of bees has been implicated in the protection of honeybee larvae against several specialized bee pathogens (Forsgren et al. 2010; Vasquez et al. 2012).

However, to our knowledge the only specific symbiotic protection of hymenopteran offspring has been described for solitary beewolves of the genera *Philanthus*, *Trachypus*, and *Philanthinus* (Crabronidae) (Kaltenpoth et al. 2005; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). Adult females cultivate the Actinobacterium '*Candidatus Streptomyces philanthi*' in specialized antennal gland reservoirs (Goettler et al. 2007). In their underground nests, they secrete the bacteria into the larval brood cells (Strohm & Linsenmair 1995; Kaltenpoth et al. 2010a). Around a week later, when the larva has fed on the stored provisions and starts to spin its cocoon, it takes the bacteria up from the brood cell and incorporates them into the cocoon silk (Kaltenpoth et al. 2010a). Within the first two weeks after cocoon-spinning, the *Streptomyces* bacteria produce a cocktail of at least nine different antibiotic substances on the cocoon surface (Kroiss et al. 2010; Koehler, Doubský & Kaltenpoth 2013). These compounds provide an efficient protection to the beewolf larva against microbial infestation during the long and vulnerable phase of hibernation (Kaltenpoth et al. 2005; Kroiss et al. 2010). The symbionts of various beewolf

species form a monophyletic group within the genus *Streptomyces*, suggesting an intimate and highly specific symbiotic association (Kaltenpoth et al. 2006; Kaltenpoth et al. 2010b; Kaltenpoth et al. 2012). However, recent studies also provide evidence for horizontal transmission of symbionts among host species, as phylogenetic analyses of streptomycetes among Philanthini neither showed the expected placement of *Philanthinus* symbionts as a sister group to the symbionts of *Philanthus* and *Trachypus* (Kaltenpoth et al. 2012), nor a monophyletic clade of *Trachypus* bacteria (Kaltenpoth et al. 2010b).

Many other social and solitary Hymenoptera (as well as a multitude of other arthropods) develop within the soil, but for most taxa it is currently unknown how the developing offspring is protected against pathogenic microorganisms. Since eggs, larvae, and pupae generally provide a rich source of nutrients for competing and pathogenic bacteria and fungi that are ubiquitous in the soil (Janzen 1977; Keller & Zimmermann 1989), it seems likely that protective alliances with antibiotic-producing microorganisms are much more common than is currently recognized.

### **Brood care and the protection of nutritional resources**

Many female hymenopterans provide brood care to their offspring. This is not only true for social bees (Apidae, Halictidae), wasps (Vespidae), and ants (Formicidae), but also for many solitary species within the Apoidea, Vespidae, Eumenidae, Masaridae, Sphecidae, Ampulicidae, Crabronidae, and Pompilidae (Gauld & Bolton 1988). Provisioning behaviors vary in their complexity across Hymenoptera, with mass provisioning being the most common type among nonsocial species as well as in some eusocial bees (Halictidae, Xylocopinae, and Meliponinae), in which a brood cell is supplied with enough resources for the complete larval development and closed after oviposition. Progressive provisioning involves the mother accessing the brood cell and supplying resources to the larva during development, which occurs in a small proportion of several solitary wasp families and in most eusocial taxa (Field 2005). The most complex forms of provisioning can be found in social Hymenoptera, most of which collect the food resources for their offspring and store them in sophisticated nests (Wilson 1971). Notably, leaf-cutting ants are among the few animals that evolved an active form of agriculture, growing fungal cultivars on leaf material and using them as nourishment for the developing offspring as well as for the adult ants (Hölldobler & Wilson 1990).

All insects that store nutritional resources for their offspring have to cope with competing microorganisms that could not only devour the provisions but also infest the developing offspring. Most

social species actively tend the provisions and remove microbial contamination by biting off fungal hyphae and removing contaminated materials (e.g. Currie & Stuart 2001). Additionally, many solitary as well as social Hymenoptera have evolved chemical defenses to reduce the risk of microbial infestation. For example, colletid and halictid bees apply a dense lining to their nest, which may inhibit the invasion of detrimental microorganisms (Batra 1968; Batra 1980), and metapleural gland secretions of leaf-cutting ant workers have antimicrobial activity and thereby probably contribute to the protection of the fungus garden from pathogenic and competing microbes (Ortius-Lechner et al. 2000; Bot et al. 2002). Similarly, the jewel wasp *Ampulex compressa* sanitizes its cockroach prey with a mixture of antimicrobial compounds (mellein and micromolide) to prevent pathogen infestation (Herzner et al. 2013). The solitary European beewolf and related digger wasps apply a secretion mainly consisting of long-chain unsaturated hydrocarbons from a specialized head gland directly to their paralyzed prey (Herzner et al. 2007; Strohm et al. 2008), which prevents water condensation and thereby delays the onset of fungal germination (Strohm & Linsenmair 2001; Herzner & Strohm 2007). However, instead of – or in addition to – producing such protective chemical cocktails themselves, some insects engage in symbioses with microorganisms that serve to ward off detrimental microbes (Kaltenpoth 2009).

In honeybees, a healthy microbial community is essential for the well-being of a colony and its defense against pathogens. While we already discussed the role of microbial symbionts for the protection of the adult and larval honeybees themselves, mutualistic bacteria are also important for the protection of the bees' food resources from spoilage. It is well established that propolis and honey of honeybees (Simone, Evans & Spivak 2009; Simone-Finstrom & Spivak 2010; Kwakman & Zaat 2012) as well as stingless bees (Temaru et al. 2007; Umthong, Puthong & Chanchao 2009) have antimicrobial properties, and some bees line the nest walls with propolis, possibly to reduce microbial infections (Anderson et al. 2011). Interestingly, a community of lactic acid bacteria (*Lactobacillus* and *Bifidobacterium*) has been repeatedly isolated from the honey crop as well as the propolis of different honeybee species (Vasquez et al. 2012). These bacteria as well as other microbial partners appear to be involved in the fermentation of the bee bread, which plays an important role for the preservation of the food resources (Gilliam 1997; Vasquez & Olofsson 2009). Apart from honeybees, this lactic acid bacterial community is also present in stingless bees (Meliponini) (Vasquez et al. 2012) and in lower abundance also in bumblebees (Olofsson & Vasquez 2009).

Arguably the best-studied symbiosis for the defense of nutritional resources in Hymenoptera is the association between leaf-cutting ants and Actinobacteria. The ants' monocultural fungus gardens are prone to infection by a specialized parasitic fungus of the genus *Escovopsis* (Currie et al. 1999a; Currie 2001). Infestation by this parasite can be detrimental for whole colonies, so there is a high selective pressure on ants to evolve effective defenses (Currie et al. 1999a). In addition to active removal of pathogens (Currie & Stuart 2001) and the use of metapleural gland secretions for antimicrobial defense (Bot et al. 2002), the ants are associated with protective Actinobacteria (Currie et al. 1999b). In most attine ant genera, bacteria of the genus *Pseudonocardia* grow on species-specific regions of the cuticle that are probably supplied with nutrients from underlying exocrine glands (Currie et al. 2006). *In vitro*, the *Pseudonocardia* symbionts have been demonstrated to produce antimicrobial compounds (Oh et al. 2009a; Barke et al. 2010; Carr et al. 2012) that inhibit the growth of *Escovopsis* (Currie et al. 1999b; Poulsen et al. 2010; Cafaro et al. 2011). Although the *Pseudonocardia*-produced antibiotics have not yet been detected *in vivo*, bioassays provided evidence that the bacteria enhance the fitness of ant colonies by suppressing the growth of the parasitic fungus (Currie, Bot & Boomsma 2003). Interestingly, the symbiont-produced bioactive compounds can also impair the growth of the ants' cultivar fungus *in vitro*, but this does not appear to have any negative effects on fungus garden biomass *in vivo* (Poulsen & Currie 2010).

Despite intensive research on the multipartite leaf-cutting ant symbiosis, the specificity and evolutionary history of the ant-*Pseudonocardia* association remains controversial (Mueller 2012). While Poulsen et al. (2005) found little or no genetic variation among *Pseudonocardia* isolates within colonies of *Acromyrmex octospinosus* and *A. echinaior*, another study reported multiple *Pseudonocardia* strains in individual colonies of *Trachymyrmex septentrionalis* (Ishak et al. 2011). Broader phylogenetic studies across leaf-cutting ants and their *Pseudonocardia* symbionts revealed some degree of specificity, but also frequent horizontal transmission and *de novo* uptake of symbionts from the environment (Cafaro & Currie 2005; Mueller et al. 2008; Mueller et al. 2010; Cafaro et al. 2011), suggesting that partner choice plays an important role in maintaining the association over evolutionary timescales. Concordantly, bioassays with four *Acromyrmex* species indicated that the ants can differentiate their native microbial symbiont from other *Pseudonocardia* strains (Zhang, Poulsen & Currie 2007). Additionally, there is some evidence that the ants prefer closely related over phylogenetically more distant *Pseudonocardia* strains, regardless of whether the bacteria were previously ant-associated or free-living (Poulsen et al. 2011a). In an attempt to reconcile the contrasting concepts of partner choice and partner fidelity in the ant-

*Pseudonocardia* system, Scheuring and Yu (2012) developed a mathematical model demonstrating that nutrient-rich provisioning by the host can selectively favor antibiotic-producing bacterial symbionts by stimulating competition among a community of acquired microbes. Although this model still needs to be tested empirically, it might provide an elegant explanation for the prevalence of both vertical transmission and horizontal exchange of symbionts in the leaf-cutting ant-*Pseudonocardia* association as well as in the beewolf-*Streptomyces* symbiosis.

In addition to *Pseudonocardia*, several other actinobacterial taxa, including members of the genus *Streptomyces* (Kost et al. 2007; Haeder et al. 2009; Barke et al. 2010; Schoenian et al. 2011; Zucchi, Guidolin & Consoli 2011) and *Amycolatopsis* (Sen et al. 2009), as well as a beta-proteobacterial *Burkholderia* sp. (Santos et al. 2004) have also been discovered in attine ant nests. Although the importance of this diverse microbial community for ant fitness is not yet clear, the presence of *Streptomyces*-produced antibiotics *in situ* on the cuticle of ant workers suggests that they may also contribute to nest hygiene (Schoenian et al. 2011). Interestingly, as in the beewolf-*Streptomyces* symbiosis (Kroiss et al. 2010), ant-associated bacteria appear to produce multiple antimicrobial compounds (Barke et al. 2010; Schoenian et al. 2011; Seipke et al. 2011). From *Pseudonocardia* isolates of different ant species, dentigerumycin (Oh et al. 2009), five angucyclines (Carr et al. 2012), and a nystatin-like compound (Barke et al. 2010) have been isolated, and ant-associated *Streptomyces* species have been found to produce candicidin (Haeder et al. 2009; Barke et al. 2010) as well as valinomycin, antimycins, and actinomycins (Schoenian et al. 2011). Thus, both beewolves and leaf-cutting ants may employ a strategy that is comparable to the combination prophylaxis used in human medicine to ward off detrimental microorganisms (Kroiss et al. 2010).

Similar defensive symbioses as in the attine ants have been suggested for two other fungus-growing insect taxa: A recent study isolated antibiotic-producing *Streptomyces* and *Amycolatopsis* species from a non-attine ant genus (*Allomerus*) (Seipke et al. 2012a). These ants are associated with a fungal cultivar that is a major component of gallery-like structures that are used to trap prey (Dejean et al. 2005; Ruiz-Gonzalez et al. 2011). Although specificity, prevalence, and function of the association between *Allomerus* ants and Actinobacteria is not known yet, the bacterial symbionts may be involved in the protection of the fungal galleries against pathogenic fungi. Outside of the order Hymenoptera, fungus-growing pine beetles (*Dendroctonus frontalis*) are associated with diverse *Streptomyces* bacteria (Scott et al. 2008; Hulcr et al. 2011). One isolate was found to produce a compound termed mycangimycin,

which inhibits the growth of fungal competitors of the beetles' cultivar (Oh et al. 2009b). In termites, the third large group of fungus-growing insects, defensive symbionts have not been discovered yet, despite targeted efforts directed at detecting specific actinobacterial mutualists with protective activities (Visser et al. 2011). Likewise, it is currently unknown whether microorganisms play a role in other wood-dwelling insects that live in close association with nutritional fungi, e.g. wood wasps of the genus *Sirex*.

As many Hymenoptera provide stored provisions for their offspring over more or less extensive time periods, it is tempting to speculate that microbes present a widespread solution to the risk of fungal infestation of mass provisions, especially in social insects that live in big colonies and store large amounts of food. However, attine ants currently constitute the only well-documented case of a symbiosis for the protection of the nutritional resources, with the *Allomerus* ants representing a possible second defensive symbiotic system (Seipke et al. 2012a). As similar symbionts need not necessarily be associated with the insect itself and could instead be present only in the nest material, their discovery is challenging and requires large-scale metagenomic or culture-based analyses of insects and their nests. A recent study by Poulsen et al. (2011b) reported on the isolation of a large number of *Streptomyces* strains (Actinobacteria) from two solitary wasp species (*Sceliphron caementarium* and *Chalybion californicum*, Hymenoptera, Sphecidae). The isolated Actinobacteria produced a range of bioactive compounds, but their functional role *in vivo* remained enigmatic (Oh et al. 2011; Poulsen et al. 2011b). Other previous efforts to discover defensive symbionts in insects have also focused on Actinobacteria (e.g. Visser et al. 2011), as their abundance in the soil, their metabolic versatility, and their ability to produce a wide range of antimicrobial secondary metabolites probably predispose them towards engaging into protective symbiotic interactions with soil-living insects (Kaltenpoth 2009; Seipke, Kaltenpoth & Hutchings 2012b). However, after identifying Actinobacteria in nest material or associated with an insect and demonstrating their antimicrobial activity, efforts need to be directed at elucidating their function *in vivo* and the specificity of the association with the host.

### **Defensive symbiosis in host-parasitoid interactions**

Symbiotic bacteria cannot only provide protection against pathogenic microorganisms, but also against eukaryotic parasitoids (Oliver et al. 2003). An interesting example is *Hamiltonella defensa* in pea aphids (*Acyrtosiphon pisum*) and black bean aphids (*Aphis fabae*). This gamma-proteobacterial secondary symbiont confers protection against parasitic wasps (Oliver et al. 2003), probably by producing a toxin that targets and kills the developing wasp larva in the aphid (Moran et al. 2005; Degnan et al. 2009).

Interestingly, the toxin genes are located on a bacteriophage rather than in the *Hamiltonella* genome itself, and phage-free *Hamiltonella* clones fail to provide protection (Moran et al. 2005; Degnan & Moran 2008b; Oliver et al. 2009; Oliver et al. 2010). Thus, the three-partite association between aphid, bacterium, and phage is necessary for successful defense against a parasitoid wasp, and the efficiency of the protection depends on a complex interaction of host, symbiont, phage, and parasitoid genome (Degnan & Moran 2008a; Oliver et al. 2009; Sandrock, Gouskov & Vorburger 2010; Schmid et al. 2012). Recently, several other secondary symbionts of insects have also been implicated in the protection of the host against parasitoid wasps, i.e. *Regiella insecticola* in aphids (Vorburger, Gehrler & Rodriguez 2010; Hansen, Vorburger & Moran 2012), *Spiroplasma* in *Drosophila hydei* (Xie, Vilchez & Mateos 2010; Xie et al. 2011), and, based on correlational evidence, *Arsenophonus* in a psyllid (Hansen et al. 2007).

To our knowledge, microbial anti-parasitoid defense has not yet been described in Hymenoptera, although many of the solitary, social, and even of the parasitic taxa are threatened by dipteran or hymenopteran parasitoids. However, an intriguing example of a defensive symbiosis between the potter wasp *Allodynerus delphinalis* and the mite *Ensliniella parasitica* has been reported recently (Okabe & Makino 2008). The wasps house and transport mites in specialized structures called acarinarium (Makino & Okabe 2003). The symbiotic mites attack parasitoids that try to oviposit into the pupal or prepupal potter wasps. By biting and clinging to the parasitoid, they significantly reduce parasitization success and thereby enhance their hosts' – and thus also their own – survival probability (Okabe & Makino 2008). Since acarinarium are present in several hymenopteran taxa (i.e. in Eumeninae and Xylocopinae) (Makino & Okabe 2003; Klimov, Vinson & Oconnor 2007), it is possible that symbiotic mites constitute a more widespread defense against parasitoids in solitary Hymenoptera.

Interestingly, in host-parasitoid interactions not only the host, but also the parasitoid can team up with protective symbionts. Developing endoparasitoids generally have to survive in an extremely hostile environment, as the cellular immune system of an insect host has evolved to recognize, encapsulate and ultimately kill eukaryotic intruders (Strand & Pech 1995). Thus, in order to survive, the parasitoid needs to evade or suppress the host's immune response. Certain groups of ichneumonid and braconid wasps have independently evolved an especially intriguing mechanism to solve this problem: they are associated with symbiotic polydnviruses and inject them into the host during oviposition (Edson et al. 1981; Fleming 1992; Strand & Pech 1995; Strand 2010; Beckage & Drezen 2012; Strand & Burke 2012). While related non-symbiotic viruses replicate within the host tissue and cause pathological effects, the



mutualistic viruses of Braconidae (bracoviruses) only produce progeny virions in specialized calyx cells in the wasp ovaries (Strand 2010; Strand & Burke 2012). Upon oviposition of the parasitoid egg into the lepidopteran host, the virions deliver genes with immunosuppressive function that enhance parasitoid survival (Edson et al. 1981; Fleming 1992; Thoetkiattikul, Beck & Strand 2005). Expression of virus-transferred genes can induce apoptosis or clumping of host hemocytes or inhibit phenoloxidase activity and thereby protect the developing parasitoid against the host's immune system (Beckage 1998; 2012). Additionally, the virus can affect metabolic and developmental processes to the advantage of the parasitoid (Fleming 1992). The association between Braconidae and bracoviruses evolved around 100 million years ago from a nudiviral ancestor (Whitfield 2002; Murphy et al. 2008; Bezier et al. 2009) and subsequently experienced codiversification of hosts and symbionts (Whitfield & Asgari 2003). The long coevolutionary history has led to a high degree of integration, and the symbiotic viruses are more reminiscent of cell organelles like mitochondria and chloroplasts rather than representing independent entities (Whitfield & Asgari 2003). In addition to the polydnviruses of Braconidae and Ichneumonidae, entomopoxviruses, ascoviruses, cypoviruses, as well as the unclassified *Leptopilina boulardi* filamentous virus have been found to enhance the survival of certain parasitoid wasps by suppressing the host immune system (Bigot et al. 1997; Lawrence 2005; Renault et al. 2005; Martinez et al. 2012), indicating that protective symbiotic associations with viruses may be a widespread and common phenomenon in parasitoid wasps.

The examples of aphids and Braconidae/Ichneumonidae demonstrate that symbiotic microorganisms can affect host-parasitoid interactions in intricate ways, either by defending the host against parasitoid attack or by protecting the parasitoid against the host's immune system. As a large number of hymenopteran taxa are parasitic, defensive symbioses with bacteria, fungi or viruses are likely to play an important ecological and evolutionary role in this insect order by shaping the outcome of the ongoing arms race between hosts and parasitoids.

### **Protection against predators**

To our knowledge, only a single case of bacteria-provided anti-predator defense has thus far been reported in insects. Staphylinid beetles of the genus *Paederus* harbor *Pseudomonas* symbionts and transfer them vertically to their offspring via the egg shell (Kellner 2003). The symbiont genome encodes a mixed polyketide synthase/non-ribosomal peptide synthetase gene cluster (Piel 2002) that is responsible for the production of the toxin pederin (Kellner 2001; 2002), which deters wolf spiders as

potential predators of the beetle larvae (Kellner & Dettner 1996). Incidentally, the toxin also causes severe cutaneous problems for humans that happen to come into contact with the beetles. As the molecular pathways for the synthesis of noxious chemicals used to deter predators and their eukaryotic origin are unknown in many cases, it is conceivable that other insects including Hymenoptera employ bacterial symbionts for their own protection.

### **Conclusions and future perspectives: Where and how to look for novel defensive symbioses in Hymenoptera?**

Symbiotic microorganisms can provide protection to insects or their nutritional resources against pathogens, parasites, parasitoids, or predators. The Hymenoptera are an especially interesting order in which to investigate such relationships, due to the large diversity of different lifestyles and the ecological and economical relevance of many taxa, specifically the social ants, bees, and wasps. To date, only a limited number of defensive alliances involving Hymenoptera have been described, but it seems likely that many associations have so far been overlooked. The leaf-cutting ants and their exosymbiotic antibiotic-producing *Pseudonocardia* bacteria provide a prominent example for a symbiosis that has long awaited discovery, despite the facts that these ants have been studied intensively for decades and the symbionts of several species are easily visible with the unaided eye. The conspicuous white coating on species-specific regions of the cuticle was assumed to be a waxy layer until Currie and colleagues investigated it in detail by scanning electron microscopy and identified it as a dense cover of actinobacterial symbionts (Currie et al. 1999b). Similarly, the protective symbionts of bees were discovered only recently (Kaltenpoth et al. 2005), although the symbiont-containing antennal gland secretion has been described in the 1990s (Strohm & Linsenmair 1995), and the gland reservoirs themselves had been discovered even earlier. Likewise, virtually all other defensive symbionts in insects have been described only during the last one or two decades.

Why are defensive symbioses so much more elusive than nutritional ones, for which numerous examples across most major insect orders have already been known since the seminal work of Paul Buchner (1965)? In our view, the reasons for this are at least threefold. First and foremost, defensive symbionts can be localized in unexpected places within or on the host's body (e.g. Currie et al. 2006; Goettler et al. 2007). While nutritional symbionts are usually located in close association of the digestive tract or in specialized abdominal bacteriomes, defensive symbionts have been found as endosymbionts in diverse tissues, e.g. in the hemolymph (Oliver et al. 2003), in the gut (Koch & Schmid-Hempel 2011), in

the antennae (Kaltenpoth et al. 2005), or as exosymbionts on the cuticle of insects (Currie et al. 1999b), which makes them harder to detect by conventional screening efforts. Second, protective symbionts are often facultative, so their infection rate in any given species can vary (e.g. Oliver et al. 2008), which complicates their discovery. And third, the fitness benefits conferred by defensive symbionts may be context-dependent. Thus, elimination of a defensive symbiont by antibiotic treatment will not necessarily reveal its functional role and the fitness benefits it confers to the host, if the target antagonist is absent, or if the wrong life stage of the host is investigated. The lack of antagonists may be particularly problematic for the detection of defensive symbioses when studying insect populations in the laboratory. Hence, detailed field studies on the natural history of the organism of interest are necessary to identify antagonistic taxa that may be targets of defensive symbionts and, thus, should be investigated specifically under controlled laboratory conditions.

The first two issues can in part be overcome by the increasing feasibility and affordability of high-throughput next-generation sequencing technologies. Massively parallel amplicon sequencing of microbial 16S rRNA genes by 454 or Illumina technology can be used to comprehensively characterize microbial communities associated with insects, even if some of the microorganisms are rare or inconsistently present (e.g. Sudakaran et al. in press). Additionally, transcriptome analyses of non-model organisms by RNAseq can yield important insights into the molecular interactions of hosts, symbionts, and their antagonists. Screening such high-throughput sequencing datasets may reveal the occurrence of putative defensive symbionts (i.e. taxa from bacterial groups that are known as potent producers of antibiotic compounds, e.g. Actinobacteria, Bacilli, Burkholderiales) or the expression of candidate toxin or antibiotic genes (e.g. polyketide synthase or non-ribosomal peptide synthase genes) with possible defensive functions. But although high-throughput sequencing and DNA barcoding provide powerful tools that can also contribute significantly towards understanding animal ecology (e.g. Clare et al. 2009), none of these techniques can act as a substitute for detailed observations on the natural history of the study organisms. In order to find novel defensive symbioses, knowledge on the behavior and ecology of insects is necessary to identify the most vulnerable life stages that need to be protected, as well as potential enemies against which the host needs to be defended. This information will allow designing bioassays to elucidate the function of putative defensive symbionts, which can be complemented by deep sequencing and chemical analyses of symbiont-produced bioactive compounds. Unfortunately, in most defensive symbioses the identification of such compounds has so far been restricted to *in vitro* analyses or *in silico* predictions. Efforts should be directed towards identifying the presence and activity

of candidate compounds *in situ* or even *in vivo* (Kroiss et al. 2010; Schoenian et al. 2011), and the development of high-resolution mass-spectrometric techniques (e.g. MALDI-imaging, nanoSIMS, DESI-imaging) provides the powerful tools that are necessary to localize target substances directly within the host tissue.

### **Acknowledgements**

We would like to thank Erhard Strohm and Michael Poulsen for critical comments on the manuscript, and Michael Strand and Michael Poulsen for providing the pictures of *Microplitis demolitor* and *Acromyrmex octospinosus*, respectively. We gratefully acknowledge financial support from the Max Planck Society and the German Science Foundation (MK, DFG KA2846/2-1).

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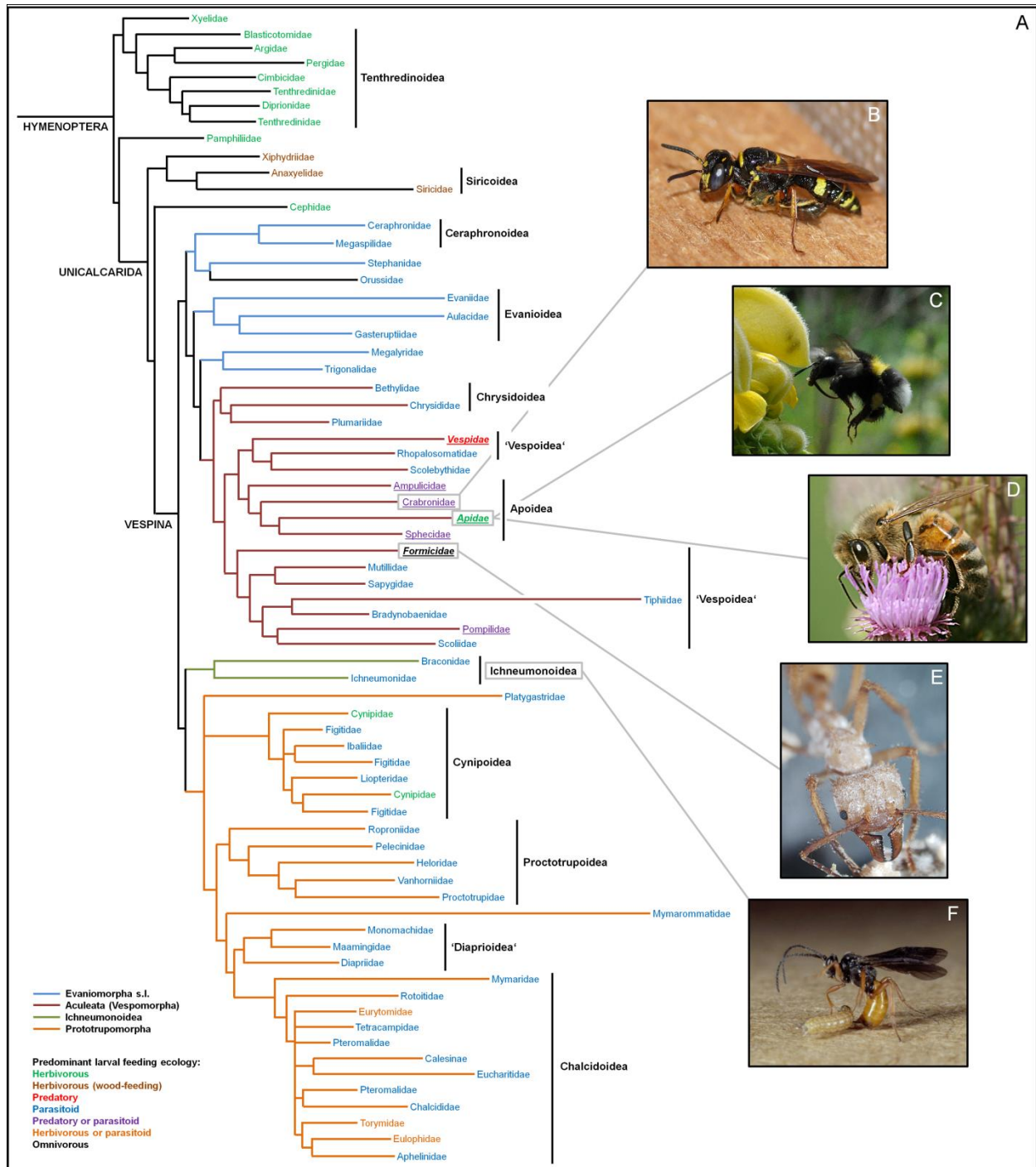


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# Figures



**Figure 1:** Defensive microbial symbioses in Hymenoptera. (A) Phylogenetic tree of major hymenopteran families (modified from Heraty et al. 2011, with permission). Branches are color-coded according to supergroups. Coloring of family names indicates the predominant feeding ecology of the larvae (green=herbivorous, brown=wood-feeding, red=predatory, blue=parasitoid, purple=predatory or

parasitoid, orange=herbivorous or parasitoid, black=omnivorous). Names of families containing social species are highlighted in bold italics, those containing mass-provisioning species are underlined. (B-F) Hymenopteran families or superfamilies which are known to contain taxa with defensive microbial symbionts. (B) Beewolves (here: *Philanthus gibbosus* with halictid bee as prey) cultivate '*Candidatus Streptomyces philanthi*' in antennal gland reservoirs and on the larval cocoon, where the symbionts produce antibiotics and thereby provide protection for the larva against pathogenic fungi. (C) A betaproteobacterial symbiont in bumblebees (*Bombus terrestris*) has been implicated in the defense against the gut parasite *Crithidia bombi*. (D) The microbial community associated with honeybees (*Apis mellifera*) plays an important role for the protection against fungal and bacterial pathogens (picture from Kaltenpoth 2011). (E) On specific regions of their cuticle, *Acromyrmex octospinosus* and other leaf-cutting ants grow *Pseudonocardia* bacteria for the defense of their fungus gardens (picture kindly provided by Michael Poulsen). (F) *Microplitis demolitor* (Braconidae) and other parasitoid wasps within the families Braconidae and Ichneumonidae are associated with symbiotic viruses that are injected into the host along with the parasitoid egg and protect the developing parasitoid from the host's immune system (picture kindly provided by Michael Strand).