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Antimicrobial Defense Strategies in Two Solitary Wasp Species

Strategien antimikrobieller Verteidigung bei zwei solitären Wespen

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Summary: Microorganisms are ubiquitous and may act as pathogens as well as food competitors that continuously challenge the survival and well-being of all higher organisms. Owing to their various specific life-styles insects may be exposed to severe and diverse microbial hazards and have consequently evolved an impressive array of sophisticated antimicrobial defenses. In this paper some strategies of antimicrobial defense in two Hymenopteran model systems, the European beewolf *Philanthus triangulum* (Hymenoptera, Crabronidae) and the Emerald cockroach wasp *Ampulex compressa* (Hymenoptera, Ampulicidae) will be reviewed. Female *P. triangulum* provide their offspring with paralyzed honeybees (*Apis mellifera*) in subterranean nests, where the microclimatic conditions promote growth of mold fungi. To mitigate the risks of fungal infestations females embalm their prey bees with a secretion of unsaturated hydrocarbons that form an oily layer on the surface of the bees and reduce water condensation from the surrounding air. The induced alteration in microclimatic conditions significantly reduces growth of mold fungi on the provisioned bees and thus enhances survival of the wasp offspring. This benefit of prey embalming is not to be had for nothing, however, as it reduces the future reproductive ability of the beewolf mother. Larvae of *A. compressa* develop in cockroaches like the American cockroach *Periplaneta americana*. In order to ward off detrimental microbes that are naturally present on the cockroaches, *A. compressa* larvae sanitize their host cockroaches with large amounts of an antimicrobial secretion. This specific cocktail of substances has broad-spectrum activity against a wide range of microbes. Furthermore, the larvae implement the antimicrobials in a spatially and temporally coordinated manner in solution as well as in vapor form. This strategy provides them with a reliable long-term protection via three lines of defense against microbes invading from the nest environment. The examples given here clearly illustrate the ingenuity and diversity of nature's solutions to microbial hazards. While in beewolves the mothers make use of a physical effect to retard growth of mold fungi on larval provisions, in the emerald cockroach wasp the larvae themselves fight detrimental microbes by deploying a blend of antimicrobial chemicals. Further research on the as yet only scarcely investigated plethora of antimicrobial strategies of insects may inspire new approaches to microbial control in human food industry and medicine to counteract the proliferation of antimicrobial resistant microorganisms.

Keywords: Antimicrobial, antifungal, antibiotic, mold fungi, fumigation

Zusammenfassung: Mikroorganismen sind allgegenwärtig und können sowohl als Pathogene als auch als Nahrungskonkurrenten das Leben aller höheren Organismen gefährden. Aufgrund ihres großen Spektrums unterschiedlicher Lebensweisen können Insekten einer Vielzahl ernster Gefahren durch Mikroorganismen ausgesetzt sein. Folglich haben sie im Laufe der Evolution eine beeindruckende Vielfalt an ausgefeilten antimikrobiellen Verteidigungsstrategien entwickelt. In diesem Artikel werden am Beispiel zweier Modellorganismen, des Europäischen Bienenwolfs *Philanthus triangulum* (Hymenoptera, Crabronidae) und der Juwelwespe *Ampulex compressa* (Hymenoptera, Ampulicidae), einige antimikrobielle Abwehrstrategien beschrieben. Bienenwolf-Weibchen stellen ihren Nachkommen gelähmte Honigbienen (*Apis mellifera*) als Nahrung in unterirdischen Nestern zur Verfügung, deren mikroklimatische Bedingungen das Wachstum von Schimmelpilzen stark fördern. Um das Verschimmeln des Larvenfutters zu verzögern und zu vermindern, balsamieren die Weibchen ihre Beutebienen mit ungesättigten Kohlenwasserstoffen ein, die auf der Bienenoberfläche eine ölige Schicht bilden und so Wasserkondensation aus der Umgebungsluft verringern. Durch das auf diese Weise veränderte Mikroklima wird das Wachstum von Schimmelpilzen auf den Bienen signifikant reduziert und die Überlebenschancen der Bienenwolfnachkommen werden gesteigert. Diesen Nutzen der Beuteeinbalsamierung müssen sich die Weibchen allerdings mit einer verringerten zukünftigen Reproduktionsrate erkaufen. Larven von *A. compressa* entwickeln sich in Schaben wie der Amerikanischen Großschabe *Periplaneta americana*. Um sich gegen die zahlreichen schädlichen Keime auf den Schaben zu wehren, hygienisieren *A. compressa*-Larven ihre Wirte mit großen Mengen eines antimikrobiell wirksamen Sekrets. Diese spezielle Mischung von Substanzen besitzt Breitbandwirkung gegen die verschiedensten Mikroorganismen. Darüber hinaus setzen die Larven die Substanzen in einer räumlich wie zeitlich koordinierten Art und Weise, sowohl in Lösung als auch gasförmig, ein. Diese Strategie gewährleistet einen zuverlässigen Langzeitschutz durch drei Verteidigungslinien auch gegen Mikroben, die aus der Nestumgebung angreifen. Die hier gezeigten Beispiele verdeutlichen den Einfallsreichtum der Natur und die Mannigfaltigkeit möglicher Strategien im Kampf gegen Mikroben. Während sich bei Bienenwölfen die Mütter einen physikalischen Effekt zunutze machen, um Schimmelbildung auf der Larvalnahrung zu verhindern, sind es bei der Juwelwespe die Larven selbst, die mithilfe einer Mischung antimikrobieller Substanzen schädliche Mikroben auf ihren Wirten und in den Nestern bekämpfen. Zukünftige Untersuchungen der bisher kaum erforschten Fülle antimikrobieller Strategien von Insekten könnten neue Ansätze für den Kampf gegen Mikroorganismen in der Lebensmittelindustrie oder Humanmedizin liefern, um der Ausbreitung resistenter Mikroorganismen entgegenzuwirken.

Schlüsselwörter: Antimikrobielle Substanzen, antimykotische Substanzen, Antibiotika, Schimmelpilze, Desinfektion

1. Introduction

It is becoming increasingly evident that we live in a microbial world (McFall-Ngai et al. 2013). While microorganisms play essential roles in sustaining and promoting life, they also act as pathogens or food competitors and continuously challenge our survival and well-being (Janzen 1977; European Food Safety Authority 2011; World Health Organization 2014). Countering the problems caused by detrimental microbes has become more and more difficult, owing to the emergence and rapid spread of resistance to the commonly used antimicrobials (World Health Organization 2014). These difficulties have stimulated the search for and study of natural antimicrobials and antimicrobial strategies deployed in the natural environment (Haine et al. 2008; Rozen et al. 2008; Dossey 2010; Poulsen et al. 2011; Vilcinskas 2011; Röhrich et al. 2012). The results obtained from these studies may aid in exploring novel remedial measures and hygiene strategies with applications in human food industry or medicine.

Despite obvious differences between humans and insects, insects are equally defied by ubiquitous antagonistic microorganisms (e.g. Trienens et al. 2010; McLean et al. 2014). Since insects are the most successful group of eucaryotic organisms on earth in terms of species numbers and diversity as well as life history variety, they provide nearly infinite opportunities for the study of antimicrobial chemical compounds and strategies to ward off antagonistic microbes. Some insect groups can be considered especially prone to microbial attacks due to their life-style or choice of habitat and food resource. These insect species have evolved elaborate and effective defense mechanisms to protect themselves, their offspring and their nutritional resources from microbial pathogens and decomposers. They are thus especially promising study objects for the discovery and exploration of antimicrobial strategies deployed in natural systems.

Social insects like ants, termites, bees, and wasps for example often live in highly dense colonies, thus facing an increased risk of pathogen transmission within the group. Disease resistance mechanisms in social insects have been shown to include individual and social immunity (Cremer et al. 2007; Cremer & Sixt 2009; Wilson-Rich et al. 2009; Hamilton & Bulmer 2012; Rosengaus et al. 2013), as well as a so-called extended disease resistance (exosymbiosis with Actinobacteria; Chouvenc et al. 2013).

Furthermore, it has been proposed that the success of some invasive insect species can be at least partly attributed to their ability to mount strong anti-pathogen defenses. The invasive Asian ladybird or harlequin ladybird beetle *Harmonia axyridis*, for example, possesses a strong constitutive antimicrobial defense mechanism (the chemical compound Harmonine in the hemolymph; Gross et al. 2010; Röhrich et al. 2012) as well as an extended repertoire of inducible antimicrobial peptides (Schmidtberg et al. 2013; Vilcinskas et al. 2013).

Insects living or nesting in soil, an environment tremendously rich in entomopathogenic and opportunistic microbes, are under constant assault from diverse bacteria and fungi. Many of these soil-nesting insects provision their progeny with highly nutritious food resources like pollen, arthropod prey or small vertebrate carcasses that constitute attractive resources for competing, putrefactive microbes. Solitary bees from different genera line their subterranean brood cells with hydrophobic substances from their Dufour's glands to defend offspring and provisions from fungi and bacteria (Michener 1964; Cane 1981 and references therein; Hefetz et al. 1982).

Finally, insects that feed on microbe-laden food resources are at risk for acquiring infections from their contaminated food. Burying beetles of the genus *Nicrophorus* produce and emit oral and anal exudates to preserve the carcasses of small vertebrates that are buried in soil and used as larval food supplies (Rozen et al. 2008; Jacques et al. 2009; Cotter et al. 2010; Degenkolb et al. 2011).

In this paper various aspects of antimicrobial defense in two Hymenopteran model systems, the European beewolf *Philanthus triangulum* (Hymenoptera: Crabronidae), a ground-nesting digger wasp that has to cope with micro-climatic conditions favorable for the rapid growth of mold fungi, and *Ampulex compressa* (Hymenoptera: Ampulicidae), a cockroach hunting wasp that provides its offspring with high-risk food, will be reviewed. Since both species experience strong selective pressures by detrimental microbes they have evolved sophisticated prophylactic mechanisms.

2. Prey embalming in the European beewolf *Philanthus triangulum*

2.1. Life history of *Philanthus triangulum*

The European beewolf *Philanthus triangulum* provisions its offspring in underground nests with paralyzed Western honey bee workers (*Apis mellifera* L., Hymenoptera, Apidae) upon which their larvae feed (e.g. Strohm 1995; Strohm & Linsenmair 1997). Female *P. triangulum* hunt and paralyze honeybees usually on flowers, bring them to a nest in flight (Fig. 1), and deposit one to six prey bees in each brood cell of their nest as food for one larva. The larva hatches two to three days after oviposition and feeds on the bees for another five to eight days before it spins a cocoon inside the brood cell in which it usually overwinters. The size of the emerging adult beewolf depends on the amount of food, i.e. the number of bees, provided by the mother (Strohm 2000).

2.2. Benefits of prey embalming

In a *Philanthus triangulum* nest, which may consist of up to 34 brood cells that are subsequently provisioned via one main burrow (STROHM 1995), the majority of brood cells usually experience humid and warm conditions and the nutrient-rich larval provisions are consequently highly susceptible to spoilage due to microbial degradation, mostly by mold fungi (Fig. 2A). Fungal infestation of larval provisions compromises larval survival (HERZNER et al. 2011) and reduces the body size of the emerging adult beewolf (STROHM 2000). Beewolves should therefore have evolved defense mechanisms against fungal infestations of the provisions and their brood. In fact, only about 5% of the brood cells of the European beewolf are infested by molds under natural conditions (STROHM & LINSENMAIR 2001), suggesting that beewolves employ effective counteractive measures in their fight against fungi. As *P. triangulum* is a mass-provisioning species and provides no active post-oviposition care to its progeny, any measure of prey preservation employed by the mother has to be taken prior to brood cell closure.

The use of observation cages that allow the surveillance of beewolves inside their nests (STROHM & LINSENMAIR 1994/1995) revealed that female *P. triangulum* display a conspicuous behavior: During excavating a brood cell and before ovipositing on one of the bees in the brood cell, they repeatedly and extensively lick the surface of all bees (STROHM & LINSENMAIR 2001). As this “grooming” behavior delays fungus growth on provisioned bees as compared to control bees (Fig. 2B), STROHM & LINSENMAIR (2001) proposed that female *P. triangulum* apply an antifungal secretion to the bees that retards mold growth.

Following this hypothesis, surface extracts obtained from provisioned bees were tested for an antifungal activity by using inhibition zone assays (HERZNER et al. 2007a). Surprisingly, even very high concentrations of the extracts failed to impede the growth of mold fungi, namely an *Aspergillus* strain obtained from a beewolf brood cell, *Aspergillus fumigatus*, and *Penicillium roquefortii*, in vitro, precluding a direct chemical effect of the surface extracts against mold fungi (HERZNER et al. 2007a).

But do female *P. triangulum* actually apply a secretion to their paralyzed prey bees during “grooming”? A comparison of the chemical profiles on the cuticles of paralyzed, provisioned honeybees from beewolf brood cells and paralyzed, but not provisioned control bees by gas chromatography/mass spectrometry revealed that provisioned bees carry on average significantly higher amounts of hydrocarbons than control bees (HERZNER et al. 2007a; HERZNER & STROHM 2007, 2008). Furthermore, while the chemical profiles of control bees are dominated by saturated hydrocarbons, the major compound on provisioned bees is the unsaturated hydrocarbon (*Z*)-(9)-pentacosene. As this compound is also the major constituent of a large head gland of *P. triangulum* females, the postpharyngeal gland (STROHM et al. 2007, 2008, 2010), it can be reasoned that females apply the secretion of this gland to their honey bee prey during the “grooming” behavior.

The postpharyngeal gland has long been thought to be restricted to ants, where it serves several “social functions” such as nestmate recognition (SOROKER et al. 1995; LUCAS et al. 2004; EELEN et al. 2006). *P. triangulum* is the first solitary hymenopteran species for which a PPG has been described (HERZNER et al. 2007b; STROHM et al. 2007). The morphology and ultrastructure of the beewolf postpharyngeal gland shows close resemblance to the postpharyngeal gland of many ant species (STROHM et al. 2007, 2010). In *P. triangulum* females the postpharyngeal gland contains mainly hydrocarbons, with (*Z*)-9-pentacosene [or (*Z*)-9-heptacosene]

Fig. 1: A female European beewolf, *Philanthus triangulum*, carrying its honeybee prey (*Apis mellifera* worker) in flight to its nest.
Abb. 1: Ein Weibchen des Europäischen Bienenwolfs, *Philanthus triangulum*, bringt eine erbeutete Honigbiene (*Apis mellifera*-Arbeiterin) im Flug zum Nest.

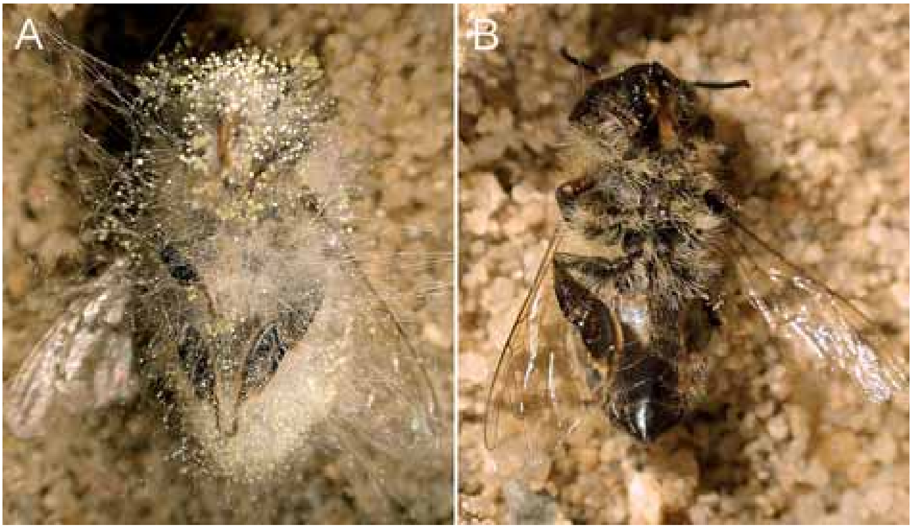

Fig. 2: Honeybees after three days in artificial brood cells under conditions mirroring those in *Philanthus triangulum* nests in the field. **A** Control honeybee (paralyzed but not provisioned by *P. triangulum*) completely overgrown by mold fungi. **B** Paralyzed and provisioned bee almost devoid of mold fungi.
Abb. 2: Honigbienen nach drei Tagen in künstlichen Brutzellen unter Bedingungen, wie sie in *Philanthus triangulum*-Nestern im Freiland herrschen. **A** Kontrollbiene (von *P. triangulum* gelähmt, aber nicht verproviantiert), die völlig von Schimmelpilzen überwachsen ist. **B** Gelähmte und verproviantierte Biene, fast völlig frei von Schimmel.

being the major compound (STROHM et al. 2008). In addition to the hydrocarbons, the gland contains some unusual long-chain saturated and unsaturated ketones. The fact that these ketones can also be found on provisioned bees (but not on conspecific control bees; HERZNER et al. 2007a) is in line with the hypothesis that females apply their postpharyngeal gland secretion to the surface of their prey.

In view of the above described findings that (1) fungus germination as well as conidia formation is significantly retarded on provisioned bees carrying the postpharyngeal gland secretion as compared to controls carrying simply their original epicuticular hydrocarbons (STROHM & LINSENMAIR 2001; HERZNER & STROHM 2007; HERZNER et al. 2011), but that (2) extracts of provisioned bees have no direct chemical effects on fungi (HERZNER et al. 2007a), the question of an alternative explanation for the antifungal effect of prey "grooming" arises.

A close examination of provisioned and not provisioned control bees revealed a conspicuous difference: While rather large water droplets were visible on control bees (Fig. 3A), provisioned bees appeared mostly dry (Fig. 3B). Quantitative analyses confirmed a significantly reduced water accumulation on provisioned bees (HERZNER & STROHM 2007). Further investigations of provisioned bees' and control bees' surfaces by scanning electron microscopy showed that female *P. triangulum* virtually embalm their prey with hydrocarbons that form a thick and continuous coating on the bee surface (Fig. 4) (HERZNER & STROHM 2007). This "food wrapping" covers microstructures such as the sockets of sensilla or tiny contaminants on the bee surface that could otherwise function

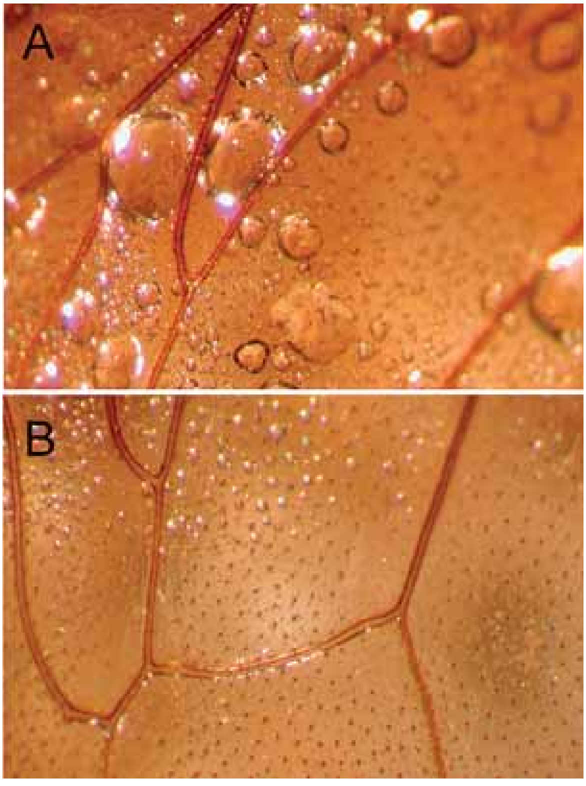

Fig. 3: Stereomicroscopic image of forewing of **A** a control honeybee (paralyzed but not provisioned by *Philanthus triangulum*), and **B** a paralyzed and provisioned honeybee, after three days in an artificial brood cell under conditions mirroring those in beewolf brood cells in the field. Note the rather large water droplets on the wing of the control bee that have formed due to condensation. By contrast, the surface of the provisioned bee appears mostly dry.

Abb. 3: Stereomikroskopische Aufnahme des Vorderflügels **A** einer Kontroll-Honigbiene (gelähmt, aber nicht verproviantiert von *Philanthus triangulum*), und **B** einer gelähmten und verproviantierten Honigbiene, nach drei Tagen in künstlichen Brutzellen unter Bedingungen, wie sie in Bienenwolfnestern im Freiland herrschen. Die großen Wassertropfen auf der Kontrollbiene haben sich durch Kondensation geformt. Die Oberfläche der verproviantierten Biene ist im Gegensatz dazu weitgehend trocken.

as nucleation sites for water condensation or could promote infectious processes by fungi (Boucias & Pendland 1991; Beysens 1995; Sosa-Gomez et al. 1997). Moreover, the embalming increases the proportion of unsaturated hydrocarbons on the bee surface about threefold so that the cuticular coating forms an oily, hydrophobic layer (Herzner & Strohm 2007). The resulting topographic and physico-chemical changes of the bee surface heavily reduce water condensation on the bees. The antifungal effect of prey embalming is thus, surprisingly, mediated by a physical effect: The reduced availability of water renders microclimatic conditions on embalmed bees unsuitable for fungal germination and growth. Other insects, like e.g. burying beetles of the genus *Nicrophorus*, are also known to preserve larval provisions (Rozen et al. 2008; Jacques et al. 2009; Cotter et al. 2010; Degenkolb et al. 2011). However, the indirect, physically mediated, mechanism of prey preservation by *P. triangulum* represents a unique adaptation of higher animals in their combat with fungi.

2.3. Costs of prey embalming

Female *Philanthus triangulum* allocate a substantial share of their resources to hydrocarbon production for prey embalming (Herzner & Strohm 2008; Herzner et al. 2011). It is unequivocal that the parental care behavior of prey embalming is beneficial in that it reduces fungal infestations of the larval provisions and enhances offspring survival (Strohm & Linsenmair 2001; Herzner & Strohm 2007; Herzner et al. 2011). However, when studying parental care behaviors, both benefits as well as costs to the

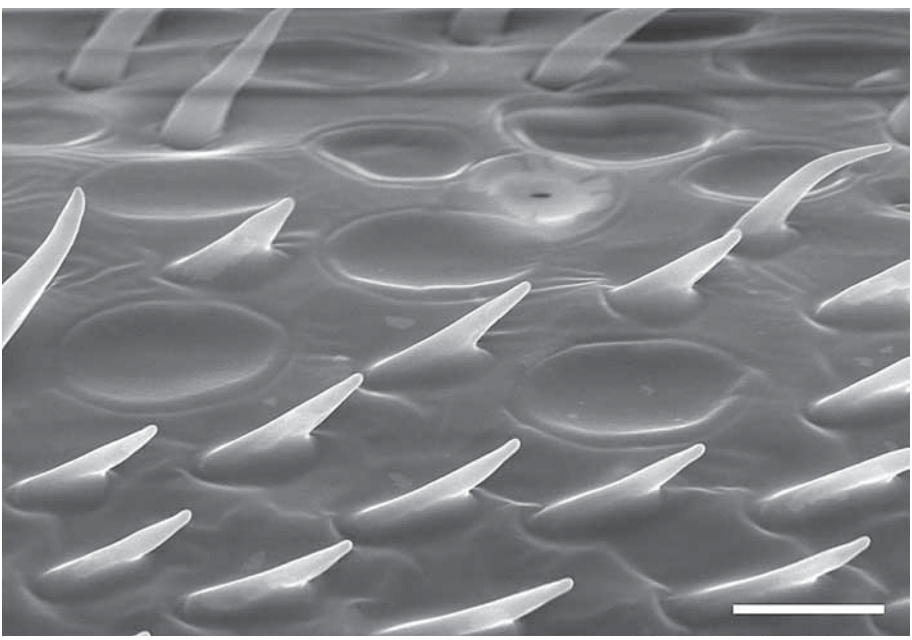

Fig 4: Scanning electron micrograph of a honeybee antenna embalmed by a *Philanthus triangulum* female (scale bar = 10 µm). The surface is coated with a continuous, oily layer of unsaturated hydrocarbons.
Abb. 4: Rasterelektronenmikroskopische Aufnahme einer Honigbienenantenne, die von einem *Philanthus triangulum* Weibchen einbalsamiert wurde (Maßstabsleiste = 10 µm). Die Oberfläche ist durchgehend mit einer öligen Schicht aus ungesättigten Kohlenwasserstoffen überzogen.

providing individual have to be considered. A quantification of hydrocarbons on freshly provisioned prey bees obtained from observation cages revealed that the mean amount of postpharyngeal gland secretion a female allocates to a given brood cell increases with the number of bees in the brood cell (Herzner et al. 2011). Brood cells containing many bees are thus costlier to produce in terms of prey embalming than brood cells containing fewer bees. Interestingly, the amount of secretion per single bee decreases significantly with an increasing number of bees in the brood cell, suggesting that the actual availability of the postpharyngeal gland secretion is limited (Herzner et al. 2011).

When females are experimentally forced to upregulate their embalming effort for one brood cell, the amount of postpharyngeal gland secretion applied to the subsequent brood cell is reduced (Herzner et al. 2011). This reduction in the amount of postpharyngeal gland secretion applied to the bees results in an elevated risk of fungal infestations and, thus, in higher larval mortality and reduced reproductive output of the beewolf mother (Herzner et al. 2011).

P. triangulum females that allocate large amounts of secretion for one brood cell have two options: They can postpone the next brood cell until the hydrocarbon stores in the postpharyngeal gland are replenished and incur a reduced rate of reproduction. Or they can allocate smaller amounts of hydrocarbons to the subsequent brood cell with the consequence that these bees will have a higher risk of becoming moldy. In each case, the embalming of the bees for the current offspring reduces the future reproductive success. Prey embalming in *P. triangulum* hence qualifies as parental investment in the sense of Trivers (1972) in that it provides benefits for current offspring but incurs costs in terms of a decrease in the parent's future reproductive ability (Herzner et al. 2011). To maximize their lifetime reproductive success female beewolves have to balance their investment of hydrocarbons in current and future offspring.

2.4. Summary and Outlook: Prey embalming in *Philanthus triangulum*

The above described example shows that mold fungi may be warded off by other means than antifungals that chemically interfere with their germination and growth. By embalming their prey bees with large amounts of unsaturated hydrocarbons, female *Philanthus triangulum* alter the microclimatic conditions on the bee surface and by doing so deprive fungi of the water they need to thrive. The ultimate evolutionary and ecological significance of the parental care behavior of prey embalming with the postpharyngeal gland secretion is beyond doubt. If beewolf females allocate their resources accordingly, they can increase the survival or fitness of their progeny and, thus, their own reproductive output under conditions that could otherwise cause high mortality or reduced progeny size (Strohm 2000; Herzner et al. 2011). Recently, it has been shown that females of the neotropical beewolf genus *Trachypus* also possess postpharyngeal glands and embalm their prey bees (Herzner et al. 2013a).

Remarkably, the European beewolf *P. triangulum* has evolved one other intriguing and effective antimicrobial defense mechanism: Its cocoons are protected by a cocktail of antibiotics produced by symbiotic *Streptomyces* bacteria (Kaltenpoth et al. 2005, 2014; Kroiss et al. 2010; Koehler et al. 2013). This protection is highly beneficial in terms of microbe suppression within the brood cells (Kaltenpoth et al. 2005; Kroiss et al. 2010). Since the bacterial symbionts are maintained in specialized antennal glands of females (Goettler et al. 2007), this antimicrobial defense mechanism might as well be costly. It will be interesting to see whether these two mechanisms interact in their action against microbes and whether the expenditures for the two components of parental care are somehow correlated. In any case, owing to the two antimicrobial defense mechanisms, beewolf offspring are protected from the egg stage on until emergence of the adult wasp (Strohm & Linsenmair 2001; Herzner & Strohm 2007; Koehler et al. 2013).

3. Antimicrobial defense in larvae of the Emerald cockroach wasp *Ampulex compressa*

3.1. Life history of *Ampulex compressa*

The emerald cockroach wasp *Ampulex compressa* is, like *Philanthus triangulum*, a parasitoid wasp sensu lato that stores insect prey for future consumption by its larvae (Williams 1942; Heitmans 1990; Libersat 2003; Keasar et al. 2006; Libersat & Gal 2014; Weiss et al. 2014). It depends on cockroaches like the peridomestic American cockroach *Periplaneta americana* (Blattaria, Blattidae) as larval food. Female *A. compressa* catch cockroaches (Fig. 5) and put them into a lethargic state by injecting their venom into the supraesopharyngeal ganglion ("brain") of the cockroach (Libersat & Gal 2014). The docile cockroach is then guided to a nest, where the female attaches one single egg to the coxa of one of the mesothoracic legs. The female subsequently closes the nest with twigs, leaves, stones etc. and thus virtually immures the cockroach inside the nest. When the egg hatches, the larva remains at the oviposition site on the outer cockroach cuticle for six to seven days and drinks hemolymph of the still living host through a hole in the cuticle. Subsequently, the larva migrates inside the cockroach to feed on the inner tissues, causing the death of the host. After eroding the cockroach completely the larva spins its cocoon inside the then empty cockroach carcass. Until its emergence as adult wasp about six weeks after oviposition (at 27 °C) the developing individual remains surrounded by the cocoon and the cockroach cuticle (Weiss et al. 2014).

3.2. Host sanitation by *Ampulex compressa* larvae

Cockroaches like *Periplaneta americana* are known to pick-up, carry and transfer microorganisms from their environment (Figure 6). Microorganisms isolated from cockroaches include various entomo- und human-pathogenic strains like e.g. *Serratia marcescens*, *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus flavus*, *Aspergillus nomius*, and *Candida* sp. (Chaichanawongsaroj et al. 2004; Pai et al. 2004, 2005; Lemos et al. 2006; Fakoorziba et al. 2010; Herzner et al. 2013b; Weiss et al. 2014). Since *Ampulex compressa* offspring spent the whole developmental phase on their cockroach hosts, which concomitantly are their sole food source, these wasps have to deploy effective hygienic measures against microbes.
As there were no indications of any prey preservation behavior by *A. compressa* mothers, it seemed reasonable to assume that the larvae themselves exert some kind of antimicrobial defense. By carefully cutting holes in the abdomens of parasitized cockroaches and covering these holes with glass cover slips *A. compressa* larvae can be monitored through a "window" inside their cockroach hosts (Herzner et al. 2013b). Such observations disclosed that *A. compressa* larvae secrete a clear liquid with which they impregnate their cockroach hosts (Fig. 7A; Herzner et al. 2013b). Chemical analyses revealed that this secretion contains nine relatively polar compounds, mainly (***R***)-(-)-mellein [(***R***)-(-)-3,4-dihydro-8-hydroxy-3-methylisocoumarin] and micromolide [(4***R***,9***Z***)-octadec-9-en-4-olide] (Fig. 7B), and some hydrocarbons (Herzner et al. 2013b). The amounts of antimicrobials applied by a single larva to its cockroach host are astonishingly large (about 5 mg; Herzner et al. 2013b; Weiss et al. 2014).

Fig. 5: A female emerald cockroach wasp, *Ampulex compressa*, with its cockroach prey, *Periplaneta americana*.
Abb. 5: Ein Weibchen der Juwelwespe *Ampulex compressa* mit ihrer Schabenbeute, einer *Periplaneta americana*.

Fig. 6: A *Periplaneta americana* on nutrient agar in a Petri dish. Note the red bacterial colonies (*Serratia marcescens*) and the mold fungi (*Aspergillus* sp.) emerging from the cockroach and growing on the agar.
Abb. 6: Eine *Periplaneta americana* auf Nähragar in einer Petrischale. Rote Bakterienkolonien (*Serratia marcescens*) und Schimmelpilze (*Aspergilllus* sp.) wachsen von der Schabe ausgehend auf dem Agar.

Antimicrobial assays revealed that the polar fraction of the secretion is active against the Gram-negative, entomopathogenic bacterium *S. marcescens*, which had previously been isolated from *P. americana* cockroaches, as well as against the Gram-positive *Staphylococcus hyicus* (Herzner et al. 2013b). Similar assays with synthetic (*R*)-(-)-mellein and micromolide showed that the activity of the larval secretion against *S. marcescens* can be attributed mainly to the activity of (*R*)-(-)-mellein. The effect against *S. hyicus* could only be explained by the combined activity of (*R*)-(-)-mellein and micromolide.

In addition to the two major compounds (*R*)-(-)-mellein and micromolide the secretion contains smaller amounts of the mellein derivatives 7-hydroxymellein, 4-hydroxymellein, and 5-hydroxymellein, the γ-lactones (*R*)-hexadecan-4-olide, heptadecan-4-olide,

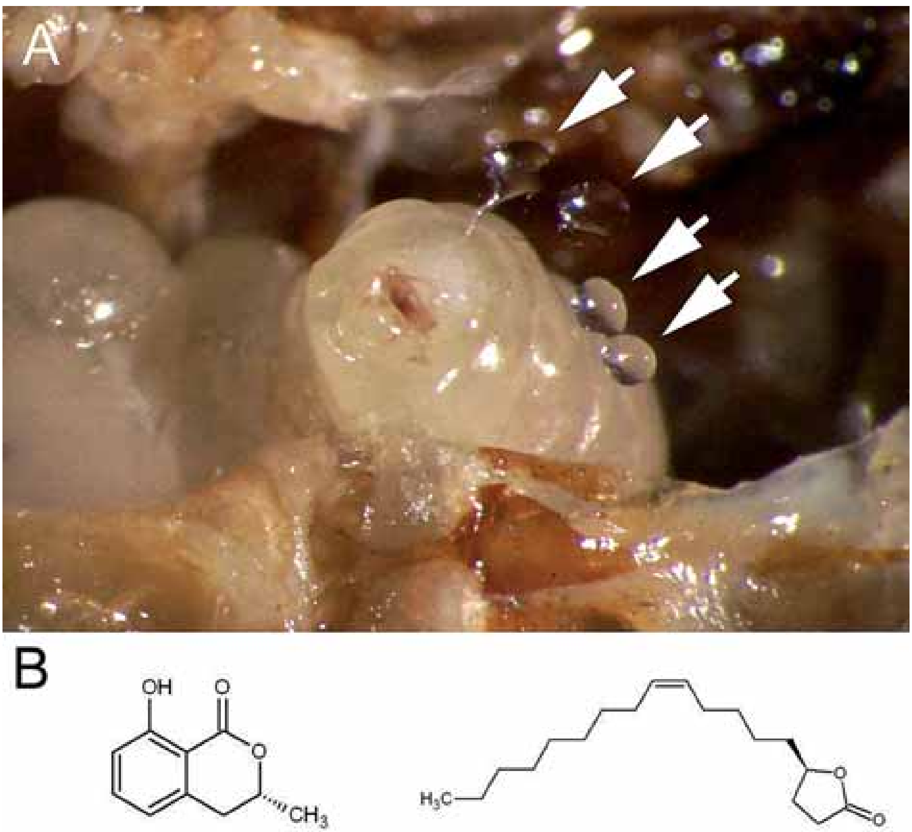

Fig. 7: **A** A larva of *Ampulex compressa* inside its *Periplaneta americana* host applying droplets of antimicrobial secretion (arrows) to a cover slip that covers an artificial opening in the abdominal cuticle of the cockroach. **B** Chemical structures of the two major constituents of the antimicrobial larval secretion, the methylisocoumarin (*R*)-(-)-mellein and γ-lactone micromolide.

Abb. 7: A *Ampulex compressa*-Larve im Inneren ihrer Wirtsschabe *Periplaneta americana* bei der Abgabe des antimikrobiellen Sekrets (Pfeile) auf ein Deckgläschen, das ein künstliches Loch im Abdomen der Wirtsschabe abdeckt. **B** Strukturformeln der beiden Hauptbestandteile des antimikrobiellen Larvalsekrets, des Methylisocumarins (*R*)-(-)-Mellein und des γ-Laktons Micromolid.

octadeca-9,12-dien-4-olide, and (***R***)-octadecan-4-olide (Herzner et al. 2013b; Weiss et al. 2014). As (***R***)-(-)-mellein, micromolide and the other constituents of the secretion of *A. compressa* larvae seem to have antimicrobial activity against a wide range of different microbes like Gram-negative and Gram-positive bacteria, mycobacteria, fungi, and a virus (Schulz et al. 1995; Krohn et al. 1997; Höller et al. 1999; Dai et al. 2001; El-Mehalawy et al. 2005; Ma et al. 2005; Yuan et al. 2008; Rukachaisirikul et al. 2009; Feng et al. 2010; Oliveira et al. 2011; Zhao et al. 2012; Herzner et al. 2013b; Weiss et al. 2014), it can be reasoned that *A. compressa* larvae sanitize their cockroach hosts with a blend of antimicrobials that provide reliable broad-spectrum antimicrobial activity.

The microbial hazards developing *A. compressa* immatures may encounter are manifold. Investigating the antimicrobial strategy of the wasp larvae in more detail revealed that they have evolved a sophisticated and intriguing strategy to face these diverse threats (Herzner et al. 2013b; Weiss et al. 2014). The first danger certainly emerges from the cockroach host itself, as cockroaches represent a high risk food naturally contaminated with pathogenic and putrefactive microbes (Chaichanawongsaroj et al. 2004; Pai, et al. 2004, 2005; Lemos et al. 2006; Fakoorziba et al. 2010; Herzner et al. 2013b; Weiss et al. 2014). Chemical analyses of different developmental stages of *A. compressa* and their host cockroaches showed that larvae start sanitizing their food with their antimicrobial secretion as soon as they have entered their host to feed on its inner tissues (Herzner et al. 2013b; Weiss et al. 2014). In this way the larvae prevent decomposition of their sole food source as well as infection by food borne microbes. Subsequently, the larvae have to take care that no detrimental microbes end up inside the cocoon, where they could harm or kill them. The larvae therefore thoroughly disinfect the inside of the cockroach by virtually soaking it with their antimicrobial secretion to create a germfree environment for cocoon spinning (Weiss et al. 2014). After successful cocoon formation *A. compressa* progeny must protect themselves against opportunistic microbes that may invade from the nest surroundings during the pupal phase and metamorphosis. The antimicrobials are therefore also incorporated into the cocoon walls to establish a second defensive barrier against microbes in addition to the cockroach shell (Weiss et al. 2014). Interestingly, the antimicrobial profiles of the two protective layers surrounding the wasp offspring, the cockroach and the cocoon, differ markedly with cockroaches bearing higher proportions of (***R***)-(-)-mellein and cocoons bearing higher proportions of micromolide.

Studies on the persistence of the antimicrobials on the parasitized cockroaches and cocoons revealed that the amount of the non-volatile micromolide remains rather constant after the cocoon has been completed, whereas about 80% of the volatile (***R***)-(-)-mellein evaporate from the cockroach surface and accumulate in the nest space (Weiss et al. 2014). Microbial challenge assays revealed that the gaseous (***R***)-(-)-mellein surrounding the parasitized cockroach hampers the growth of entomopathogenic and opportunistic bacteria (*S. marcescens*) and fungi (*Aspergillus sydowii*, *Metarhizium brunneum*) in *A. compressa* nests (Weiss et al. 2014). Owing to its volatility (***R***)-(-)-mellein can permeate the whole nest space and affect microbes at some distance from the cockroach and wasp offspring as well as those hidden in otherwise inaccessible places. Sanitizing the nest space with vaporous (***R***)-(-)-mellein thus represents a kind of front line defense. (***R***)-(-)-mellein seems to have a triple function as food preservative during feeding, protective antimicrobial cover on cockroach shell and cocoon, as well as fumigant of the nest space.

3.3 Summary and Outlook: Host sanitation by *Ampulex compressa* larvae

The synthesis and secretion of antimicrobials is without doubt crucial to the survival and well-being of many insect species. The specific combination of substances in an antimicrobial blend and the way in which they are deployed are likely to be critical to the success of an antimicrobial strategy.

In the emerald cockroach wasp *Ampulex compressa*, the larvae apply a cocktail of nine different substances to their hosts and cocoons, most of which have proven antimicrobial activity (Schulz et al. 1995; Krohn et al. 1997; Höller et al. 1999; Dai et al. 2001; El-Mehalawy et al. 2005; Ma et al. 2005; Yuan et al. 2008; Rukachaisirikul et al. 2009; Feng et al. 2010; Oliveira et al. 2011; Zhao et al. 2012; Herzner et al. 2013b; Weiss et al. 2014). As a result of the spatially and temporally coordinated deployment of several antimicrobials with combined antimicrobial activity and different physico-chemical properties, developing *A. compressa* are protected by three lines of defense until adult emergence: the cocoon and cockroach cuticle impregnated with antimicrobials and the vaporous (*R*)-(-)-mellein in the surrounding nest space. This multifaceted defense strategy has apparently evolved to match the need for a dependable and enduring protection against an unpredictable spectrum of detrimental microbes. The excretion and usage of antimicrobials outside their bodies is without much doubt only one facet of the immune defense strategy of *A. compressa* larvae. Future studies will most likely reveal other components of immune defense in these wasps.

4. Overall Conclusion

Like humans, insects face strong selective pressures to cope with microbial pathogens and food competitors, because these microbes compromise their own and their offspring's survival and well-being. As an adaptive response to these pressures insects have evolved potent antimicrobial measures. Since the hazards that the diverse insect species experience during their ontogenies are manifold, so are the remedies that have evolved to rise up to these challenges.

In both beewolves and cockroach wasps, the specific life-style of the larvae entails certain risks in terms of adverse effects by microbes. Beewolves are ground-nesting species that store Hymenopteran prey under microclimatic conditions that favor fungal growth. To overcome these microbial threats the beewolf mothers preserve the larval food by applying copious amounts of oily lipids. They aim at the Achilles heel of most fungi and make use of the physical effect of reduced water condensation to retard fungal growth.

The cockroach wasp choses food resources for its offspring which have a high probability of being contaminated with detrimental microbes. The larvae themselves practice food hygiene in that they impregnate their hosts with large amounts of a blend of several antimicrobial compounds that directly interfere with bacterial and fungal growth. Moreover, the larvae implement the different antimicrobials in a spatially and temporally coordinated manner and mount three lines of defense to secure their survival during the especially vulnerable stages of their development.

The examples described here clearly demonstrate the sophistication and ingenuity of nature's solutions to microbial hazards. Considering that *Philanthus triangulum* and *Ampulex compressa* are only two out of a myriad of species of insects on earth, most of which have not been investigated as yet, it becomes clear what a cornucopia of undiscovered antimicrobial strategies the insect world still is. And even though research on these topics is fascinating and worthwhile by itself, it might well be that the antimicrobial defenses already disclosed and those still to be discovered in nature, continuously reassessed and refined by evolutionary processes as they are, may inspire new approaches to microbial control in human food industry and medicine to counteract the proliferation of antimicrobial resistant microorganisms.

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