BOOK: Evolution of Venomous Animals and Their Toxins

CHAPTER: Venom as a component of external immune defense in Hymenoptera

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ABSTRACT

An intriguing feature of most hymenopteran venoms is that they display broad antimicrobial activity. In particular, the venoms of social Hymenoptera (ants, wasps and bees) represent a most conspicuous source of antimicrobial secretions. In solitary and parasitic hymenopteran species, venom is used to immobilize or kill prey and to preserve them as stored food for their immature brood. In social hymenopteran species, venom is frequently also externalized both onto the cuticle and the nest surface. This indicates that venom use in Hymenoptera is not just restricted to hunting activities or to deter predators, but is also actively used as an externalized defensive agent, providing a first chemical barrier against microorganisms present in the environment. This chapter will discuss the importance and biological significance of venom as part of an external immune defense in Hymenoptera with special emphasis on social species. In addition ecological and environmental factors constraining the use of venom as external immune defense will be highlighted.

1 Introduction

A variety of venom systems have evolved across the animal kingdom. This taxonomic diversity highlights the importance of venom as an evolutionary innovation (Casewell et al. 2013). Unsurprisingly, many studies have been conducted to understand the evolutionary processes that drove the generation of these venomous systems and of venom complexity. From this wealth of data the insight emerged that the complex composition and targeting of venom reflects the multiple functions and biological roles venom has in different animals. From an evolutionary perspective, venoms are commonly regarded as either foraging adaptations to subdue prey or as defensive adaptations against predators (Casewell et al. 2013). Venoms found in the insect order Hymenoptera are certainly not an exception from this point of view (Piek 1986). As in other venomous animals, the composition and function of venom in Hymenoptera is well adapted to immobilize or kill prey, and in many other cases, it serves as a defensive adaptation against enemies such as invertebrate and vertebrate predators. Defense is often also a common secondary function of venom in many species in which foraging is its primary purpose. This conception has led to neglect the fundamental role that venoms play in the interactions with pathogenic, parasitic, commensal or mutualistic microorganisms. Yet, these microorganisms certainly also represent a strong selective pressure for the maintenance of venom for defensive purposes (Moreau 2013). Indeed, a characteristic of venomous secretions in Hymenoptera is the strong antimicrobial activity that they exert (Kuhn-Nentwig 2003; Moreau 2013). Although this characteristic of venom is broadly distributed among distant hymenopteran species, it has so far been considered to be only of secondary importance. Only recently it became clear that many hymenopteran species, whatever their life styles, have evolved venom features that actively participate in the regulation of microbial infections. This view has come from the recognition that many insects deploy antimicrobials to their immediate environment in order to manipulate the composition of the microbial community surrounding them. These antimicrobials often originate from exocrine glands, especially from venom glands (Otti et al. 2014).

In this chapter the importance and biological significance of venom as part of an external immune defense in Hymenoptera will be highlighted with special emphasis on those species characterized by social habits. Venom of vertebrates and invertebrates is thought to be metabolically costly and the energetic cost of venom might constrain both its synthesis and use (Casewell et al. 2013; Nisani et al. 2012; but see Smith et al. 2014). Despite that, most social hymenopterans use considerable quantities of venom to sanitize themselves, related group members and the nest surface, implying that the advantages overcame the metabolic cost.

2 Immune defenses in solitary and social hymenoptera

Like all animals, Hymenoptera enlist a variety of immune defenses against disease agents (Schmid-Hempel 2011). From a molecular perspective the insect immune system involves three core signal transduction pathways, two of which are regulated by pattern recognition receptors (Toll and Imd) and the third one by stress signals from tissues (JAK/STAT). These pathways orchestrate a huge number of molecular effectors, including antimicrobial peptides, reactive oxygen species and lectins. The system, however, also involves physical barriers to infection such as the integument and the gut. Furthermore coordinated responses of several subpopulations of haemocytes are activated in the hemolymph when these barriers are breached by a putative pathogen.

Apart from these internally expressed immune defenses, there are several other defense mechanisms existing outside of what is traditionally considered to be part of the immune system. Those mechanisms involve for example changes in life-history traits (Michalakis 2009) or behavioral avoidance and self-medication (de Roode and Lefèvre 2012; Moore 2002) and clearly contribute to an organism’s defense against parasites and pathogens. Social insects also benefit from the fact that they show cooperative defenses that complement the defense of the individual. Thus insects living in a society can rely on both individual and collective defenses with selection for immunity acting simultaneously on both these levels, which encompass complex interactions and different selective constrains. One of the most illustrative examples of cooperative defense is the social fever exhibited by honeybees, where an increase of comb temperature is induced by adults in response to infestation by the fungal pathogen *Ascosphaera apis*, preventing disease development (Starks et al. 2000). Other defenses in insect societies include organizational properties of the colony that are critical in shielding infectious diseases (Schmid-Hempel 1998; Stroeymeyt et al. 2014). For example, in the colonies of ants and bees, the inner region of the nest containing immature brood, young workers and the queen are spatially and behaviorally segregated from older workers, which are mainly active outside the nest foraging or in the nest periphery disposing of dead bodies and garbage (Baracchi and Cini 2014; Mersch et al. 2013). The spatial segregation emerging from division of labor and preferential age and task based interaction leads to a form of organizational immunity protecting the more important and delicate region of the nest from possible infections. Besides indirect effects of behaviors through organizational immunity, behaviors can have a more direct effect on immune defense. Behaviors targeted at decreasing disease transmission and increasing resistance to parasites and pathogens within a social insect colony have been referred to as antiseptic behaviors (Wilson-Rich et al. 2009). Antiseptic behaviors include a large repertoire ranging from the hygienic removal and undertaking of diseased brood and young adults in ants and bees (Baracchi et al. 2012a; Sun and Zhou 2013; Tragust et al. 2013a; Tragust et al. 2013b) to mutual grooming behavior (Evans and Spivak 2010; Tragust et al. 2013a).

The use of antimicrobials against parasites and diseases in insect societies is intimately linked to behavioral adaptations as they are required to apply and distribute antimicrobial compounds as a first line of defense. Antimicrobials acting in the environment of a social insect colony might be environment-derived, derived from symbiotic relations or self-produced.

Ants and bees often disinfect their nest material with resins, i.e. complex plant secretions with diverse antimicrobial properties, derived from the environment. In the wood ant *Formica paralugubris* resins have been shown to inhibit the growth of microbes and nests rich in resins have fewer bacteria and fungi than ant nests containing only very little resin (Christe et al. 2003). Even if resin collection might be costly in term of time and effort there are indications that wood ants benefit directly from the antimicrobial property of resin as they survive longer if infected by bacteria or fungi (Chapuisat et al.2007). Similar behaviors are also common in the honeybee *Apis mellifera* and other honeybee species where resins are actively included into the wax of the nest to form what has been called propolis. This behavior is clearly an adaption to fight pathogens, as colonies of *Apis mellifera* increase resin foraging rate after a challenge with the fungal pathogen *Ascophaera apis*. Additionally, colonies experimentally enriched with resin had decreased infection intensities of this fungal pathogen (reviewed in Simone et al. 2009).

In addition to antimicrobial active plant resins, the antimicrobial immune defense of social insects also relies on antimicrobials gained through symbiotic relationships. It has recently been shown that members of all nine recognized honeybee species, plus stingless bee species, harbor diverse symbiotic lactic acid bacteria that are involved in food preservation. In addition those symbiotic bacteria likely also contribute to host defense against pathogens and parasites intercepted during foraging (Vásquez et al. 2012).

Besides antimicrobial compounds derived from the environment and from symbionts, social insects produce a variety of antimicrobial secretions in their exocrine glands, especially ants, and use them to sanitize their own body and their nest. Until recently, the role of venom as a major source of self-produced antimicrobial compounds has often been neglected, despite the fact that most venoms show a strong antimicrobial activity (Kuhn-Nentwig 2003).

Altogether, organizational, behavioral and physiological adaptations of social insects to prevent the establishment and spread of parasites and pathogens have been referred to as social immunity (Cremer et al. 2007). The key idea is that by acting collectively, individuals are better able to mount a defense than is possible acting independently. The idea of a social immune system has been later expanded to include immune services targeting one or more recipients not only in social insects but also in other animal family structures, in social microbes or in the context of herd immunity, i.e. the reduction of the risk of infection among susceptible individuals by the presence and proximity of immune individuals (Cotter and Kilner 2010). With the focus on immune defense of organisms in general, it was recently proposed to view any heritable trait acting outside an organism and improving the protection from pathogens, or manipulating the composition of the microbial community in favor of an organism, as external immune defense (Otti et al. 2014). This broad definition of immune defense integrates ideas on social immunity and proposes that the expression of internal or external immune defenses will depend on the ecological niche or life history of an organism. Furthermore it provides a framework in which costs and benefits of immune defense traits can be evaluated from an evolutionary and ecological perspective. In particular the framework proposes that variation in the level of microbe pressure present in a given environment and the temporal or spatial variation of the environment itself represent the two most important factors in the evolution of external immune defense and its effectiveness (Otti et al. 2014), (Figure 1). Focusing on antimicrobial active venoms, the following sections of this chapter will explore whether the evolution of external immune defense has indeed been favored due to life history traits found in solitary and social Hymenoptera, i.e. the storage of food, the use of a stable and confined nest and group living. However, first, the antimicrobial active venom of Hymenoptera and its biological role and function as external immune defense will be described.

3 Hymenoptera venoms: a complex multifunctional secretion

The majority of Hymenoptera have a venom gland associated with the ovipositor or the sting (Piek 1986), (Figure 2). Details on the function and composition of the secretions of these glands are known for only a part of the over 150.000 hymenopteran species, and for the sawflies (Symphyta) such knowledge is almost completely lacking. Hymenoptera venom glands produce extremely complex cocktails of diverse bioactive compounds. It is possible to distinguish at least three different groups of chemical substances according to their molecular weight (Kuhn-Nentwig 2003; Piek 1986). The first group of heavy compounds (higher than 10 kDa) consists of proteins, including several enzymes such as phospholipases (responsible for cleaving the membrane phospholipids), hyaluronidases (which degrade the matrix component hyaluronic acid), acid phosphatases (acting on organic phosphates) and sphingomyelinases (involved in sphingolipid metabolism reactions). The second group of intermediate molecular weight (around and lower than 10 kDa) is represented by a peptide fraction, including several cytolytic and neurotoxic compounds. A third group is composed of low molecular-mass substances such as ions, free amino acids, biogenic amines (commonly histamine, serotonin, dopamine and noradrenaline), neurotransmitters, polyamines, heterocyclic compounds and alkaloids. Understanding why venoms are such complex mixtures of compounds requires a clear understanding of what is the evolutionary history of venom and what functions it holds in living species.

- *The evolutionary history of venom in Hymenoptera*

Traditionally, the order of Hymenoptera has been taxonomically partitioned into two major groups: the Symphyta or sawflies, most of which are phytophagous, and the Apocrita, most of which are entomophagous. The Apocrita can be further divided into the Terebrantia and Aculeata that share common parasitic ancestral origins. Terebrantia have an ancestral ovipositor (terebra or drill) that is also used as venom duct, while Aculeata have an ovipositor (aculeus or sting) that is fully modified for injecting venom into a host and has lost its association with the reproductive system. Terebrantia use their stinging organ to transiently or permanently immobilize prey for their developing offspring and to deposit their eggs inside (endoparasitoids) or outside (ectoparasitoids) the prey’s body. In many solitary aculeate species, venom compounds retained their non-lethal paralytic function for the storage and capture of prey while acquiring a new one for use in self-defense (Hermann and Blum 1981). In the social Hymenoptera Aculeata, the venom, originally used as a tool for capturing and storing prey in solitary species, essentially became a weapon for defending the colony from predators and competitors. In addition to serve as injectable or topically applied defensive agent, ant venoms are used also as trail, alarm, sex, queen-recognition, aggregation, attractant-recruitment, and recognition pheromones, as repellents, and even as toxic agents for prey capture (Piek 1986).

- *Venom use in solitary and parasitic Hymenoptera*

Besides the well-studied venomous functions of prey capture and defense, the antimicrobial properties of hymenopteran venoms have often been considered of secondary importance although they constitute a function broadly distributed among distant hymenopteran species (Moreau 2013). A hypothesis that could explain the antimicrobial activity in hymenopteran venom is that it serves to prevent the contamination of the venom apparatus by opportunistic pathogens, contracted at the occasion of stinging events. Data in support of this hypothesis are however completely lacking except for a recent survey of bacteria, fungi and viruses associated with the venom apparatus of Hymenoptera. This survey revealed that the venom apparatus of Hymenoptera is a suitable organ for the development of viruses only and not for other microbes (Moreau 2013). An alternative hypothesis to explain the adaptive significance of antimicrobial venom in solitary and parasitic Hymenoptera is its use to control infection by opportunistic pathogens in stung prey. This makes intuitive sense, especially for parasitoid and solitary species, which need to keep the paralyzed prey alive or from decomposing during the development of their offspring. Furthermore, protection of stored food has been outlined as a likely selective pressure for the evolution of external immune defense traits such as antimicrobial active venom (Otti et al. 2014). Indeed, evidence points to the fact that Hymenoptera, especially parasitoids, appear to have evolved venom-based strategies that limit the opportunity for microorganisms to establish a secondary infection in their host (reviewed in Asgari and Rivers 2011; Moreau 2013). These include the injection of venom antimicrobial proteins and peptides, but also the selective manipulation of the host’s immune reactions to the benefit of the parasitoid’s offspring. For example, the venom components of the endoparasitic hymenopteran *Leptopilina boulardi* specifically target their dipteran host’s encapsulation and melanization responses but parasitized hosts keep their ability to produce antibacterial and antifungal peptides (Moreau 2013). Another example is the venom of the Jewel Wasp *Ampulex compressa*, which induces excessive grooming behavior in the stung prey (Libersat and Gal 2014). Both venom-based strategies presumably function to counteract the increased risk of infection, resulting from a complete suppression of the host's immune responses in the case of *Leptopilina boulardi* or from pathogens on the host's cuticle in the case of *Ampulex compressa*, which may be harmful for the wasp's egg or developing larva. Similar to parasitic Hymenoptera, several antimicrobial peptides in the venoms of solitary predatory Hymenoptera are known (Moreau 2013). Although the potential to regulate infections in animals they sting can be envisaged, the exact biological roles are still unclear.

Taken together, the venom in many solitary and parasitoid hymenopteran species holds functions as external immune defense in addition to that of paralyzing hosts. The following sections will show that the antimicrobial activity of venom has been retained in social Hymenoptera and that venom has a biological function as external immune defense also in social species.

- ***Rise of sociality and the threat of predators and pathogens***

In the escalation of parental care, we pass from species in which the females of parasitoid Hymenoptera lay their eggs on paralyzed prey, to species in which a solitary female builds a shelter before capturing a prey on which to lay an egg, and then to species in which the growing larvae are kept in a nest and progressively furnished with prey in social Hymenoptera. The nest provides social insects with an element of control over the environment, improving colony capacities for rearing the immature brood through storage of food reserves. Apart from cooperative brood care, living in a society has many other benefits. The fitness of each individual in a group is thought to increase by decreasing the costs associated with important life-history activities such as foraging efficiency, colonizing and competitive abilities, and the ability to adaptively modify the environment. In turn, the social life style requires highly developed defense abilities. The amount of resources offered by insect colonies is likely not only to attract a wide array of potential predators, notably mammals, birds and various other arthropods but also several microorganisms to take advantage of it. The high number of, often closely related, individuals living in high densities with frequent physical contact and the shared use of space is predicted to significantly increase the vulnerability of societies to the establishment and spread of infectious diseases. This hypothesis is generally supported by the observation across many different species that the prevalence of pathogens and parasites increases with the size of host social groups (Côté and Poulin 1995; Rifkin et al. 2012) and that numerous parasites and pathogens exist in social insect societies (Schmid-Hempel 1998).

4 Venom as externalized immune defense in social Hymenoptera

Several antimicrobial compounds acting against a wide range of bacteria and fungi have been described in the venom of eusocial bees, bumblebees, social wasps, hornets and ants. The presence of a range of antimicrobial peptides which are used also for internal immune defense is notable. For example, the venom of the honeybee *Apis mellifera* contains melittin, a basic 26-amino acid peptide that accounts for 45–50% of the venom dry weight and exhibits strong antimicrobial activity. Similarly, several antimicrobial peptides named mastoparans have been described in social wasp genera such as *Agelaia*, *Vespula*, *Protonectarina*, *Protopolybia*, *Parapolybia*, *Polybia* and *Polistes* Kuhn-Nentwig 2003; Moreau 2013). In ants the metapleural glands have long been considered to be one of the most important sources of antimicrobial compounds active against a wide range of bacteria and fungi (Yek et al. 2013). Nonetheless, several antimicrobial peptides have been described also in the venoms of ants; for example, in the Australian jumper ant *Myrmecia pilosula* and in the ponerine ant *Pachycondyla goeldii*. Furthermore, other venom compounds with strong antimicrobial activity (for example alkaloids or formic acid (Morgan 2008)) are known from ants such as the fire ant *Solenopsis invicta* (Storey et al. 1991) or species belonging to the ant subfamily Formicinae (Tragust et al. 2013a).

- ***Venom on the cuticle***

Interestingly, venom components can be found on the cuticle of social bees, wasps and ants. The primary function of the epicuticle, the most external layer of the insect cuticle, and the complex mixtures of lipids on it, is thought to protect against dehydration and to provide a mechanical barrier against invasion of foreign matter. The presence of venom compounds with strong antimicrobial activity on insect surfaces suggests that the venom acts also as a chemical barrier providing a first line of protection against microorganisms. Besides *Polistes* paper wasps (Turillazzi 2006; Turillazzi et al. 2006) the presence of venom components with strong antimicrobial activity on the epicuticle has been recently documented in Stenogastrinae wasps (Baracchi et al. 2010; Baracchi et al. 2012b). Stenogastrinae wasps are a sub-family of tropical facultative eusocial wasps, closely related to Polistinae and Vespinae, forming simple societies that are very small in size. The medium molecular weight polar substances found on the wasp epicuticle (roughly from 900 to 4000 Da) were identical to those found in the venom of all the ten studied species from four different genera, suggesting the venom reservoir as the primary source of cuticular polar substances. Support for the idea that the venom reservoir is the source of antimicrobial compounds on the cuticle comes also from the study of different social bees of the genus *Apis* (Baracchi et al. 2011; Baracchi and Turillazzi 2010). While venom peptides are present on the cuticle of females, irrespective of their colony duties, they can be found only in traces on the cuticle of drones, which lack the sting apparatus (Figure 3). The fact that newly emerged bees lack venom antimicrobial peptides both in the venom reservoir and on the cuticle further confirms this hypothesis. The presence of antimicrobial venom components on the cuticle of ants is known only for the fire ant *Solenopsis invicta*. In this ant species, small quantities of venom are dispensed on the brood surface during a behavior called “gaster flagging” (Obin and Vander Meer 1985), (Figure 4) and venom components are also deposited on eggs by queens during the egg-laying process (Vander Meer and Morel 1995), (Figure 5).

The behavioral mechanisms responsible for the presence of venom compounds on the cuticle of bees and wasps are still not completely clear. The most likely explanation is the use of cleaning movements during grooming to smear venom on the body. Self-grooming observations in Stenogastrinae wasps suggest the possibility that little drops of venom released from the sting can be collected with the legs by the wasps and applied all over the body surface (Baracchi et al. 2012b). The importance of grooming for the spread of antimicrobial active substances derived from the venom gland has recently also been shown in the ant *Lasius neglectus* (Tragust et al. 2013a). In this species, adults continuously apply antimicrobial venom onto their pupae. While direct spraying of their venom onto the pupae can be occasionally observed, the predominant mode of application is indirect. Venom is first taken up orally during a behavior called “acidopore grooming” and subsequently applied to pupae during grooming.

Although it is likely that antimicrobial venom components on the cuticle of adults and brood of social bees, wasps, and ants serve as a protection against microorganisms, direct evidence for this hypothesis exists only for ants. Blockage of the venom gland opening in the weaver ants *Polyrhachis dives*, in the fungus growing ant *Acromyrmex echinatior* and in the garden ant *Lasius neglectus* all resulted in a reduced survival of adults and of pupae cared by them when challenged with the entomopathogen *Metarhizium anisopliae* (Graystock and Hughes 2011; Tragust et al. 2013a; Tranter et al. 2014), (Figure 6).

In the ant *Lasius neglectus*, the authors could show that formic acid from the venom gland is the active agent inhibiting fungal growth and that venom depleted ants had a significantly reduced ability to do so (Figure 7). These authors could also show that application of venom on pupae is amplified under pathogen pressure indicating that it is an adaptive behavior.

Although, so far, brood care in the ant *Lasius neglectus* is the only example of therapeutic use of the venom in response to pathogens reported in all Hymenoptera, it is likely that future work will reveal that other species of social insects are also capable to therapeutically defend themselves and related group members from a wide array of pathogens using their antimicrobial secretions.

- *Venom on the nest surface*

Venom components are found not only on the cuticle of social bees, wasps, and ants, but also on the nest surface, likely also serving as a first line chemical barrier against microorganisms there. For example, the antimicrobial peptide melittin has been described from the nest surface of several species of the genus *Apis* (Baracchi et al. 2011; Baracchi and Turillazzi 2010) and the antimicrobial mastoparan peptides Dominulin A and Dominulin B have been described from the nest surface of the social paper wasp *Polistes dominula* (Turillazzi et al. 2006). In ants, there is only indirect evidence that antimicrobial active venom compounds are found on the nest surface, for example, greater fungal abundance but lower fungal species richness and diversity were detected in mounds of the fire ant *Solenopsis invicta* and in *Aphaenogaster texana* nests (Zettler et al. 2002). An involvement of venom compounds in the sanitation of nests is likely for the weaver ant *Polyrhachis dives*. In this species, the blockage of the venom gland opening resulted in an increased hazard for the nest material to be overgrown by fungi, compared with nest material that was tended by workers with a functional gland (Tranter et al. 2014), (Figure 8).

- *Venom on the cuticle and the nest surface as externalized immune defense*

Recently, venom components on the nest surface and on the cuticle of several species belonging to the genus *Apis* (*A. mellifera*, *A. dorsata*, *A. cerana* and *A. andreniformis*) have been investigated with respect to their nesting ecology and environmental constraints (Baracchi et al. 2011). According to their nesting habits, the species can be divided into two groups: cavity dwelling species (*Apis cerana* and *Apis mellifera*) and open nesting species (dwarf honey bees *Apis andreniformis* and giant honey bees *Apis dorsata*). Using an analytical survey of medium weight polar venom compounds it was found that the major difference between these *Apis* species corresponds to nesting habit, i.e. between the cavity dwelling and the open nesting species. While the former have venom compounds on the cuticle, venom peptides are almost absent on those of *A. dorsata* and *A. andreniformis*. Similarly, the antimicrobial venom compound melittin is present on the nest surface of both the cavity dwelling species but not evident on the nest surface of the open nesting giant honeybee and dwarf honeybee. This result is exactly what would be expected for the conditions favoring the evolution of external immune defense such as the use of externalized antimicrobial active venom suggested by Otti et al. (2014): i.e., a highly stable and confined environment with constant or high microbe pressure. In this context, it is interesting to note that extracts from the cuticle of social wasp species with paper nests, show a higher antimicrobial activity than those of solitary species which excavate burrows, while extracts of solitary mud nesting species show no antimicrobial activity at all (Hoggard et al. 2011) (Table 1). It might be argued that the environmental conditions found in excavated burrows and mud are much more variable than the conditions found in paper nests, thus not favoring the evolution of external immune defense. On the other hand, factors such as the relative contribution of social lifestyle and of phylogenetic relationships to the evolution of external immune defense clearly need to be considered and disentangled. For example, the primitive social hover wasps Stenogastrinae lack venom compounds on the nest surface, despite the fact that not a single species excavates burrows (Baracchi et al. 2012b). The following section of this chapter will explore whether life history traits of social insects, namely the high number of often closely related individuals living in high densities with frequent physical contacts, have indeed favored the use of antimicrobial active venom as external immune defense.

- *Social lifestyle and the evolution of venom as external immune defense*

Since the discovery of antimicrobial properties of hymenopteran venoms, it has been argued that the adaptive significance of this trait relies on protection from commensal pathogen infections during stinging events. However, experimental data supporting this hypothesis are lacking to date (Moreau

2013). Instead, researchers have started to shed light on the evolutionary significance of antiseptic venoms in social insects. Stow and co-workers (Stow et al. 2007) explored whether the evolution of sociality required the synchronous evolution of increased chemical defenses against pathogens in social bees. They found that the strength of antimicrobial compounds on the cuticle of bees was positively correlated to group size and genetic relatedness along a gradient of sociality ranging from solitary (*Amegilla bombiformi* and *Amegilla asserta*) and semi-social (*Exoneura robusta* and *Exoneura nigrescens*) to eusocial (*Exoneurella tridentate* and *Trigona carbonaria*). This indicates that the evolution of sociality was accompanied by the evolution of stronger antimicrobial compounds. The link between the levels of antimicrobial compounds on the cuticle and the levels of social complexity was also revealed by Hoggard and co-workers (Hoggard et al. 2011) in wasps. Besides trends of increasing antimicrobial activity along social complexity, within a single species, correlations between antimicrobial activity on the cuticle and both colony size and the level of within-colony genetic variation were also found (Hoggard et al. 2013). More precisely, in the paper wasp *Polistes humilis*, the effectiveness of antimicrobial activity on the cuticle increases with genetic diversity and decreases with colony size (i.e. the number of wasps forming the colony). It is most likely the venom that is responsible for the antimicrobial activity found on the cuticle, as venom components of bees and wasps are commonly found on the cuticle (see previous sections). Since the increase in antimicrobial strength on the cuticle found in the study of Stow and co-workers (Stow et al. 2007) was not linear, with the greatest increment being between smaller group sizes, it was suggested that selection pressure from microbial pathogens is so intense that even minimal sociality requires substantially stronger antimicrobials. Support for this hypothesis comes from the fact that even minimal societies such as those of the hover wasps *Metischnogaster drewseni*, whose colonies count a maximum of 2-3 females, have strong antimicrobial venoms (Baracchi et al. 2012b).

We have seen that the same link between the strength of antimicrobial compounds and level of sociality has been established in both wasps (Hoggard et al. 2011) and bees (Stow et al. 2007). The same information is lacking for ants. However it is known that in fungus-growing ants there is a positive correlation between the size of metapleural gland reservoirs, an important source of antimicrobial compounds on the cuticle of ants (Yek et al. 2013), and social complexity. The relationship between antimicrobials compounds and the level of sociality might thus hold throughout the social Hymenoptera.

5 Conclusion and future directions

This chapter has summarized the evidence that predatory and social lifestyles found in Hymenoptera have resulted in the increased use of venoms for defensive and offensive purposes. Intriguingly, a background antimicrobial function has been conserved or recruited in these venoms, indicating that microbial pressures have been important in shaping the evolution of the composition and the use of hymenopteran venoms. However, until recently this has almost never been taken into consideration. Recent research has proposed that any heritable trait acting outside an organism and improving protection from pathogens or manipulating the composition of the external microbial community should be viewed as external immune defense (Otti et al. 2014). As outlined in this chapter, antimicrobial venom of Hymenoptera is frequently externalized for the purpose of self-sanitation, sanitation of related group members and the nest, and for the preservation of stored food. Thus, there is no doubt that antimicrobial venoms represent an important component of external immunity in Hymenoptera.

Yet, many facets of the ecological immunology of the venom remain insufficiently understood. External immune defenses come at a cost and are often tightly linked to the physiology of an organism and its internal immune system. Elucidating the costs related to the use of venom as external immune defense is thus required to clarify potential trade-offs in a more precise way. For example, it is known that the use of environment derived antimicrobials as external immune defense in ants and bees reduces the expression of the internal immune response (Castella et al. 2008; Simone et al. 2009). Pros and cons of relying more on external rather than internal immunity clearly depend on different ecological and environmental factors, but this needs to be evaluated in more detail. Potential trade-offs between different external immune defense traits will also have to be taken into consideration, while recent advances in many technologies and analytical techniques will undoubtedly help researchers in this endeavor. However, insights from the fields of ecological immunology, chemical ecology, biochemistry and molecular biology clearly need to be combined in order to complete our understanding of hymenopteran venom compounds and functions.

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Figure and table legends

Figure 1 (Line 156): Selection for external immune defense. Three gradients of important ecological factors, in combination with microbe pressure and spatial or temporal variation in the environment, favor the evolution of external immune defenses. Selection pressure will increase: (i) from small to large group size; (ii) from temporary/open to permanent/confined nests; and (iii) from no food storage/slow decay to permanent food storage/fast decay. Reprinted from Otti et al. (2014) with permission of Cell Press.

Figure 2 (Line 165): A selection of types of glandular venom apparatus in Hymenoptera. All representatives show a venom gland, mostly paired and highly branched, and a venom reservoir. The venom reservoir is part of the ductus venatus, except in Braconidae (3). Nearly all show a second gland, the Dufour's gland, which is smaller, unpaired and not branched, except in some Apoidae (15, 16). In the Sphecoidea, a third gland is frequently present (7-10). In some groups the venom bladder is muscular 2, 3, 4, 12, 13, 14. Reprinted from Piek (1986) with permission of Academic Press.

Figure 3 (Line 294): Average mass spectrometry spectra of 950-4000 Da fraction of cuticular methanol extracts of individuals belonging to different sexes and castes of honeybee (*Apis mellifera*). The highest peaks at ~2000 Da (apamin) and ~ 2850 Da (melittin) of each spectrum accounts for ~ 45-50% and ~2 % of the venom dry weight respectively, but only melittin has proven antimicrobial activity (Baracchi et al. 2013). Reprinted from Baracchi and Turillazzi (2010) with permission of Elsevier.

Figure 4 (Line 301): Gas chromatogram demonstrating the presence of worker-derived venom alkaloids on the surface of *S. invicta* brood. (A) *S. invicta* venom alkaloids from dissected worker poison sac (B) *S. invicta* brood rise. Std = internal standard, un. pk. = unidentified peak. Reprinted from Vander Meer and Morel (1995) with permission of Springer.

Figure 5 (Line 302): Comparison of venom alkaloid gas chromatogram profiles: a) worker, b) queen, c) hexane rinse of eggs. QA= queen-specific piperidine alkaloid; WA = worker-specific alkaloids. Chromatograms (a) and (b) are from worker and queen venom sac extracts, respectively, and are very concentrated compared to chromatogram (c). Reprinted from Vander Meer and Morel (1995) with permission of Springer.

Figure 6 (Line 320): Survival of *Acromyrm exechinatior* leaf-cutting ants (A) and *Polyrhachis dives* weaver ants (B) that had either their venom gland (squares) or metapleural gland (triangles; *A. echinatior* only as *P. dives* lacks a metapleural gland) blocked with nail varnish, or had nail varnish applied to the pronotum as a control (circles), and which were then treated with either the *Metarhizium anisopliae* fungal parasite (solid lines, filled symbols) or with 0.05% Triton-X control solution (dashed lines, open symbols). Reprinted from Graystock and Hughes (2011) with permission of Springer.

Figure 7 (Line 323): (A) Workers of *Lasius neglectus* inhibited germination of conidio-spores on the surface of pupae, as revealed by germination checks of conidio-spores washed off after 24 hr of tending and subsequently plated on agar. MPG-blocked workers inhibited fungal growth to the same extent as control workers. In contrast, blockage of the acidopore and the mouth prevented this antifungal effect. (B) Venom-depleted ants also had a significantly reduced ability to inhibit fungal growth in comparison to control workers, but they still showed some antifungal effect compared to the worker-absence control. Bars in panels (A) – (C) show means + SEM. Different letters indicate statistically significant differences at $\alpha = 0.05$. Reprinted from Tragust et al. (2013) with permission of Cell Press.

Figure 8 (Line 343): Proportion of trials where foreign fungus overgrew leaf-cutting ant nest material, grouped by treatment. Foreign fungal species were *Aspergillus fumigatus* (white), *A. tamarii* (light gray), *A. nomius* (dark gray), *A. sclerotiorum* (black), *Fusarium sp.* (left ward diagonals), *Trichoderma sp.* (cross-hatched), and *Escovopsis sp.* (right ward diagonals). Reprinted from Tranter et al. (2014) with permission of Springer.

Table 1 (Line 363): Antimicrobial activity of cuticular extracts from several solitary, communal and social wasp species. n: number of individuals (number of colonies for social species); Sociality: social (Soc.), communal aggregator (Com.), solitary (Sol.); IC50: mean equivalent surface area (mm^2) of wasp cuticle required to kill or inhibit 50% of *S. aureus* growth; nr: number of replicates per species. Reprinted from Hoggard et al. (2011) with permission of Plos Library of Science.