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### **Venom as a Component of External Immune Defense in Hymenoptera**

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1 BOOK: Evolution of Venomous Animals and Their Toxins

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

3

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13

14 ABSTRACT

15 An intriguing feature of most hymenopteran venoms is that they display broad antimicrobial

16 activity. In particular, the venoms of social Hymenoptera (ants, wasps and bees) represent a most

17 conspicuous source of antimicrobial secretions. In solitary and parasitic hymenopteran species,

18 venom is used to immobilize or kill prey and to preserve them as stored food for their immature

19 brood. In social hymenopteran species, venom is frequently also externalized both onto the cuticle

20 and the nest surface. This indicates that venom use in Hymenoptera is not just restricted to hunting

21 activities or to deter predators, but is also actively used as an externalized defensive agent,

22 providing a first chemical barrier against microorganisms present in the environment. This chapter

23 will discuss the importance and biological significance of venom as part of an external immune

24 defense in Hymenoptera with special emphasis on social species. In addition ecological and

25 environmental factors constraining the use of venom as external immune defense will be

26 highlighted.

27

28 **1 Introduction**

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## 41 **1 Introduction**

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

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

74

## 75 **2 Immune defenses in solitary and social hymenoptera**

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

84 Apart from these internally expressed immune defenses, there are several other defense mechanisms  
85 existing outside of what is traditionally considered to be part of the immune system. Those  
86 mechanisms involve for example changes in life-history traits ([Michalakis 2009](#)) or behavioral  
87 avoidance and self-medication ([de Roode and Lefèvre 2012](#); [Moore 2002](#)) and clearly contribute to  
88 an organism's defense against parasites and pathogens. Social insects also benefit from the fact that  
89 they show cooperative defenses that complement the defense of the individual. Thus insects living  
90 in a society can rely on both individual and **collective defenses** with selection for immunity acting  
91 simultaneously on both these levels, which encompass complex interactions and different selective  
92 constraints. One of the most illustrative examples of cooperative defense is the social fever exhibited

93 by honeybees, where an increase of comb temperature is induced by adults in response to  
94 infestation by the fungal pathogen *Ascosphaera apis*, preventing disease development ([Starks et al.](#)  
95 [2000](#)). Other defenses in insect societies include organizational properties of the colony that are  
96 critical in shielding infectious diseases ([Schmid-Hempel 1998](#); [Stroeymeyt et al. 2014](#)). For  
97 example, in the colonies of ants and bees, the inner region of the nest containing immature brood,  
98 young workers and the queen are spatially and behaviorally segregated from older workers, which  
99 are mainly active outside the nest foraging or in the nest periphery disposing of dead bodies and  
100 garbage ([Baracchi and Cini 2014](#); [Mersch et al. 2013](#)). The spatial segregation emerging from  
101 division of labor and preferential age and task based interaction leads to a form of organizational  
102 immunity protecting the more important and delicate region of the nest from possible infections.  
103 Besides indirect effects of behaviors through organizational immunity, behaviors can have a more  
104 direct effect on immune defense. Behaviors targeted at decreasing disease transmission and  
105 increasing resistance to parasites and pathogens within a social insect colony have been referred to  
106 as antiseptic behaviors ([Wilson-Rich et al. 2009](#)). Antiseptic behaviors include a large repertoire  
107 ranging from the hygienic removal and undertaking of diseased brood and young adults in ants and  
108 bees ([Baracchi et al. 2012a](#); [Sun and Zhou 2013](#); [Tragust et al. 2013a](#); [Tragust et al. 2013b](#)) to  
109 mutual grooming behavior ([Evans and Spivak 2010](#); [Tragust et al. 2013a](#)).

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

114 Ants and bees often disinfect their nest material with resins, i.e. complex plant secretions with  
115 diverse antimicrobial properties, derived from the environment. In the wood ant *Formica*  
116 *paralugubris* resins have been shown to inhibit the growth of microbes and nests rich in resins have  
117 fewer bacteria and fungi than ant nests containing only very little resin ([Christe et al. 2003](#)). Even if  
118 resin collection might be costly in term of time and effort there are indications that wood ants

119 benefit directly from the antimicrobial property of resin as they survive longer if infected by  
120 bacteria or fungi ([Chapuisat et al.2007](#)). Similar behaviors are also common in the honeybee *Apis*  
121 *mellifera* and other **honeybee** species where resins are actively included into the **wax** of the nest to  
122 form what has been called propolis. This behavior is clearly an adaption to fight pathogens, as  
123 colonies of *Apis mellifera* increase resin foraging rate after a challenge with the fungal pathogen  
124 *Ascophaera apis*. Additionally, colonies experimentally enriched with resin had decreased infection  
125 intensities of this fungal pathogen (reviewed in [Simone et al. 2009](#)).

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

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

137

138 Altogether, organizational, behavioral and physiological adaptations of social insects to prevent the  
139 establishment and spread of parasites and pathogens have been referred to as **social immunity**  
140 ([Cremer et al. 2007](#)). The key idea is that by acting collectively, individuals are better able to mount  
141 a defense than is possible acting independently. The idea of a social immune system has been later  
142 expanded to include immune services targeting one or more recipients not only in social insects but  
143 also in other animal family structures, in social microbes or in the context of herd immunity, i.e. the  
144 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.

161

### 162 **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



171 phospholipids), hyaluronidases (which degrade the matrix component hyaluronic acid), acid  
172 phosphatases (acting on organic phosphates) and sphingomyelinases (involved in sphingolipid  
173 metabolism reactions). The second group of intermediate molecular weight (around and lower than  
174 10 kDa) is represented by a peptide fraction, including several cytolytic and neurotoxic compounds.  
175 A third group is composed of low molecular-mass substances such as ions, free amino acids,  
176 biogenic amines (commonly histamine, serotonin, dopamine and noradrenaline), neurotransmitters,  
177 polyamines, heterocyclic compounds and alkaloids. Understanding why venoms are such complex  
178 mixtures of compounds requires a clear understanding of what is the evolutionary history of venom  
179 and what functions it holds in living species.

180

#### 181 - *The evolutionary history of venom in Hymenoptera*

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

196 attractant-recruitment, and recognition pheromones, as repellents, and even as toxic agents for prey  
197 capture ([Piek 1986](#)).

198

#### 199 - *Venom use in solitary and parasitic Hymenoptera*

200 Besides the well-studied venomous functions of prey capture and defense, the antimicrobial  
201 properties of hymenopteran venoms have often been considered of secondary importance although  
202 they constitute a function broadly distributed among distant hymenopteran species ([Moreau 2013](#)).

203 A hypothesis that could explain the antimicrobial activity in hymenopteran venom is that it serves  
204 to prevent the contamination of the venom apparatus by opportunistic pathogens, contracted at the  
205 occasion of stinging events. Data in support of this hypothesis are however completely lacking  
206 except for a recent survey of bacteria, fungi and viruses associated with the venom apparatus of  
207 Hymenoptera. This survey revealed that the venom apparatus of Hymenoptera is a suitable organ  
208 for the development of viruses only and not for other microbes ([Moreau 2013](#)). An alternative  
209 hypothesis to explain the adaptive significance of antimicrobial venom in solitary and parasitic  
210 Hymenoptera is its use to control infection by opportunistic pathogens in stung prey. This makes  
211 intuitive sense, especially for parasitoid and solitary species, which need to keep the paralyzed prey  
212 alive or from decomposing during the development of their offspring. Furthermore, protection of  
213 stored food has been outlined as a likely selective pressure for the evolution of external immune  
214 defense traits such as antimicrobial active venom ([Otti et al. 2014](#)). Indeed, evidence points to the  
215 fact that Hymenoptera, especially parasitoids, appear to have evolved venom-based strategies that  
216 limit the opportunity for microorganisms to establish a secondary infection in their host (reviewed  
217 in [Asgari and Rivers 2011](#); [Moreau 2013](#)). These include the injection of venom antimicrobial  
218 proteins and peptides, but also the selective manipulation of the host's immune reactions to the  
219 benefit of the parasitoid's offspring. For example, the venom components of the endoparasitic  
220 hymenopteran *Leptopilina boulardi* specifically target their dipteran host's encapsulation and  
221 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 boulandi* 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.

235

#### 236     - *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,

248 notably mammals, birds and various other arthropods but also several microorganisms to take  
249 advantage of it. The high number of, often closely related, individuals living in high densities with  
250 frequent physical contact and the shared use of space is predicted to significantly increase the  
251 vulnerability of societies to the establishment and spread of infectious diseases. This hypothesis is  
252 generally supported by the observation across many different species that the prevalence of  
253 pathogens and parasites increases with the size of host social groups ([Côté and Poulin 1995](#); [Rifkin](#)  
254 [et al. 2012](#)) and that numerous parasites and pathogens exist in social insect societies ([Schmid-](#)  
255 [Hempel 1998](#)).

256

#### 257 **4 Venom as externalized immune defense in social Hymenoptera**

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

273

- ***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).

299 The behavioral mechanisms responsible for the presence of venom compounds on the cuticle of  
300 bees and wasps are still not completely clear. The most likely explanation is the use of cleaning  
301 movements during **grooming** to smear venom on the body. Self-grooming observations in  
302 Stenogastrinae wasps suggest the possibility that little drops of venom released from the sting can  
303 be collected with the legs by the wasps and applied all over the body surface ([Baracchi et al.](#)  
304 [2012b](#)). The importance of grooming for the spread of antimicrobial active substances derived from  
305 the venom gland has recently also been shown in the ant *Lasius neglectus* ([Tragust et al. 2013a](#)). In  
306 this species, adults continuously apply antimicrobial venom onto their pupae. While direct spraying  
307 of their venom onto the pupae can be occasionally observed, the predominant mode of application is  
308 indirect. Venom is first taken up orally during a behavior called “**acidopore grooming**” and  
309 subsequently applied to pupae during **grooming**.

310

311 Although it is likely that antimicrobial venom components on the cuticle of adults and **brood** of  
312 social bees, wasps, and ants serve as a protection against microorganisms, direct evidence for this  
313 hypothesis exists only for ants. Blockage of the **venom gland** opening in the weaver ants  
314 *Polyrhachis dives*, in the fungus growing ant *Acromyrmex echinator* and in the garden ant *Lasius*  
315 *neglectus* all resulted in a reduced survival of adults and of pupae cared by them when challenged  
316 with the entomopathogen *Metarhizium anisopliae* ([Graystock and Hughes 2011](#); [Tragust et al.](#)  
317 [2013a](#); [Tranter et al. 2014](#)), (Figure 6).

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

322 Although, so far, brood care in the ant *Lasius neglectus* is the only example of **therapeutic** use of the  
323 venom in response to pathogens reported in all Hymenoptera, it is likely that future work will reveal

324 that other species of social insects are also capable to therapeutically defend themselves and related  
325 group members from a wide array of pathogens using their antimicrobial secretions.

326

327 - ***Venom on the nest surface***

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

341

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

343 Recently, venom components on the nest surface and on the cuticle of several species belonging to  
344 the genus *Apis* (*A. mellifera*, *A. dorsata*, *A. cerana* and *A. andreniformis*) have been investigated  
345 with respect to their nesting ecology and environmental constraints ([Baracchi et al. 2011](#)).  
346 According to their nesting habits, the species can be divided into two groups: cavity dwelling  
347 species (*Apis cerana* and *Apis mellifera*) and open nesting species (dwarf honey bees *Apis*  
348 *andreniformis* and giant honey bees *Apis dorsata*). Using an analytical survey of medium weight  
349 polar venom compounds it was found that the major difference between these *Apis* species

350 corresponds to nesting habit, i.e. between the cavity dwelling and the open nesting species. While  
351 the former have venom compounds on the **cuticle**, venom peptides are almost absent on those of *A.*  
352 *dorsata* and *A. andreniformis*. Similarly, the antimicrobial venom compound melittin is present on  
353 the **nest surface** of both the cavity dwelling species but not evident on the nest surface of the open  
354 nesting giant honeybee and dwarf honeybee. This result is exactly what would be expected for the  
355 conditions favoring the evolution of external immune defense such as the use of externalized  
356 antimicrobial active venom suggested by [Otti et al. \(2014\)](#): i.e., a highly stable and confined  
357 environment with constant or high microbe pressure. In this context, it is interesting to note that  
358 extracts from the cuticle of social wasp species with paper nests, show a higher antimicrobial  
359 activity than those of solitary species which excavate burrows, while extracts of solitary mud  
360 nesting species show no antimicrobial activity at all ([Hoggard et al. 2011](#)) (Table 1). It might be  
361 argued that the environmental conditions found in excavated burrows and mud are much more  
362 variable than the conditions found in paper nests, thus not favoring the evolution of external  
363 immune defense. On the other hand, factors such as the relative contribution of **social lifestyle** and  
364 of phylogenetic relationships to the evolution of **external immune defense** clearly need to be  
365 considered and disentangled. For example, the primitive social hover wasps Stenogastrinae lack  
366 venom compounds on the nest surface, despite the fact that not a single species excavates burrows  
367 ([Baracchi et al. 2012b](#)). The following section of this chapter will explore whether life history traits  
368 of social insects, namely the high number of often closely related individuals living in high densities  
369 with frequent physical contacts, have indeed favored the use of antimicrobial active venom as  
370 external immune defense.

371  
372 - *Social lifestyle and the evolution of venom as external immune defense*

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



376 [2013](#)). Instead, researchers have started to shed light on the evolutionary significance of **antiseptic**  
377 **venoms** in social insects. Stow and co-workers ([Stow et al. 2007](#)) explored whether the evolution of  
378 sociality required the synchronous evolution of increased chemical defenses against pathogens in  
379 social bees. They found that the **strength of antimicrobial compounds** on the cuticle of bees was  
380 positively correlated to group size and genetic relatedness along a gradient of sociality ranging from  
381 solitary (*Amegilla bombiformi* and *Amegilla asserta*) and semi-social (*Exoneura robusta* and  
382 *Exoneura nigrescens*) to eusocial (*Exoneurella tridentate* and *Trigona carbonaria*). This indicates  
383 that the evolution of sociality was accompanied by the evolution of stronger antimicrobial  
384 compounds. The link between the levels of antimicrobial compounds on the cuticle and the levels of  
385 social complexity was also revealed by Hoggard and co-workers ([Hoggard et al. 2011](#)) in wasps.  
386 Besides trends of increasing antimicrobial activity along social complexity, within a single species,  
387 correlations between antimicrobial activity on the cuticle and both colony size and the level of  
388 within-colony genetic variation were also found ([Hoggard et al. 2013](#)). More precisely, in the paper  
389 wasp *Polistes humilis*, the effectiveness of antimicrobial activity on the cuticle increases with  
390 genetic diversity and decreases with colony size (i.e. the number of wasps forming the colony). It is  
391 most likely the venom that is responsible for the antimicrobial activity found on the **cuticle**, as  
392 venom components of bees and wasps are commonly found on the cuticle (see previous sections).  
393 Since the increase in **antimicrobial strength** on the cuticle found in the study of Stow and co-  
394 workers ([Stow et al. 2007](#)) was not linear, with the greatest increment being between smaller group  
395 sizes, it was suggested that selection pressure from microbial pathogens is so intense that even  
396 minimal sociality requires substantially stronger antimicrobials. Support for this hypothesis comes  
397 from the fact that even minimal societies such as those of the hover wasps *Metischnogaster*  
398 *drewseni*, whose colonies count a maximum of 2-3 females, have strong antimicrobial venoms  
399 ([Baracchi et al. 2012b](#)).

400 We have seen that the same link between the strength of antimicrobial compounds and level of  
401 sociality has been established in both **wasps** ([Hoggard et al. 2011](#)) and **bees** ([Stow et al. 2007](#)). The

402 same information is lacking for ants. However it is known that in fungus-growing ants there is a  
403 positive correlation between the size of metapleural gland reservoirs, an important source of  
404 antimicrobial compounds on the cuticle of [ants](#) ([Yek et al. 2013](#)), and social complexity. The  
405 relationship between antimicrobials compounds and the level of sociality might thus hold  
406 throughout the social Hymenoptera.

407

## 408 **5 Conclusion and future directions**

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

421 Yet, many facets of the ecological immunology of the venom remain insufficiently understood.  
422 External immune defenses come at a cost and are often tightly linked to the physiology of an  
423 organism and its internal immune system. Elucidating the costs related to the use of venom as  
424 external immune defense is thus required to clarify potential trade-offs in a more precise way. For  
425 example, it is known that the use of environment derived antimicrobials as external immune defense  
426 in ants and bees reduces the expression of the internal immune response ([Castella et al. 2008](#);  
427 [Simone et al. 2009](#)). Pros and cons of relying more on external rather than internal immunity clearly

428 depend on different ecological and environmental factors, but this needs to be evaluated in more  
429 detail. Potential trade-offs between different external immune defense traits will also have to be  
430 taken into consideration, while recent advances in many technologies and analytical techniques will  
431 undoubtedly help researchers in this endeavor. However, insights from the fields of ecological  
432 immunology, chemical ecology, biochemistry and molecular biology clearly need to be combined in  
433 order to complete our understanding of hymenopteran venom compounds and functions.

434

## 435 **6 References**

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551 Keywords: antimicrobial peptides, social insects, ecological immunology, social immunity.

552

553

554 Figure and table legends

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

561

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

569

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

576

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



581

582 Figure 5 (Line 302): Comparison of venom alkaloid gas chromatogram profiles: a) worker, b)  
583 queen, c) hexane rinse of eggs. QA= queen-specific piperidine alkaloid; WA = worker-specific  
584 alkaloids. Chromatograms (a) and (b) are from worker and queen venom sac extracts, respectively,  
585 and are very concentrated compared to chromatogram (c). Reprinted from [Vander Meer and Morel](#)  
586 [\(1995\)](#) with permission of Springer.

587

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

595

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

605

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

611

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