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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

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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	
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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

145 immune individuals ([Cotter and Kilner 2010](#)). With the focus on immune defense of organisms in
146 general, it was recently proposed to view any heritable trait acting outside an organism and
147 improving the protection from pathogens, or manipulating the composition of the microbial
148 community in favor of an organism, as **external immune defense** ([Otti et al. 2014](#)). This broad
149 definition of immune defense integrates ideas on **social immunity** and proposes that the expression
150 of internal or external immune defenses will depend on the ecological niche or life history of an
151 organism. Furthermore it provides a framework in which costs and benefits of immune defense
152 traits can be evaluated from an evolutionary and ecological perspective. In particular the framework
153 proposes that variation in the level of microbe pressure present in a given environment and the
154 temporal or spatial variation of the environment itself represent the two most important factors in
155 the evolution of external immune defense and its effectiveness ([Otti et al. 2014](#)), (Figure 1).
156 Focusing on antimicrobial active venoms, the following sections of this chapter will explore
157 whether the evolution of external immune defense has indeed been favored due to **life history traits**
158 found in solitary and social Hymenoptera, i.e. the storage of food, the use of a stable and confined
159 **nest** and group living. However, first, the antimicrobial active venom of Hymenoptera and its
160 biological role and function as external immune defense will be described.

161

162 **3 Hymenoptera venoms: a complex multifunctional secretion**

163 The majority of Hymenoptera have a **venom gland** associated with the ovipositor or the sting ([Piek](#)
164 [1986](#)), (Figure 2). Details on the function and composition of the secretions of these glands are
165 known for only a part of the over 150.000 hymenopteran species, and for the sawflies (Symphyta)
166 such knowledge is almost completely lacking. Hymenoptera venom glands produce extremely
167 complex cocktails of diverse bioactive compounds. It is possible to distinguish at least three
168 different groups of chemical substances according to their molecular weight ([Kuhn-Nentwig 2003](#);
169 [Piek 1986](#)). The first group of heavy compounds (higher than 10 kDa) consists of proteins,
170 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

222 antifungal peptides ([Moreau 2013](#)). Another example is the venom of the Jewel Wasp *Ampulex*
223 *compressa*, which induces excessive **grooming** behavior in the stung prey ([Libersat and Gal 2014](#)).
224 Both venom-based strategies presumably function to counteract the increased risk of infection,
225 resulting from a complete suppression of the host's immune responses in the case of *Leptopilina*
226 *boulardi* or from pathogens on the host's cuticle in the case of *Ampulex compressa*, which may be
227 harmful for the wasp's egg or developing larva. Similar to parasitic Hymenoptera, several
228 **antimicrobial peptides** in the venoms of solitary predatory Hymenoptera are known ([Moreau 2013](#)).
229 Although the potential to regulate infections in animals they sting can be envisaged, the exact
230 biological roles are still unclear.

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

235

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

237 In the escalation of parental care, we pass from species in which the females of **parasitoid**
238 **Hymenoptera** lay their eggs on paralyzed prey, to species in which a **solitary female** builds a shelter
239 before capturing a prey on which to lay an egg, and then to species in which the growing larvae are
240 kept in a nest and progressively furnished with prey in **social Hymenoptera**. The nest provides
241 social insects with an element of control over the environment, improving colony capacities for
242 rearing the immature brood through storage of food reserves. Apart from cooperative brood care,
243 living in a society has many other benefits. The fitness of each individual in a group is thought to
244 increase by decreasing the costs associated with important life-history activities such as foraging
245 efficiency, colonizing and competitive abilities, and the ability to adaptively modify the
246 environment. In turn, the social life style requires highly developed defense abilities. The amount of
247 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
et al. 2012](#)) and that numerous parasites and pathogens exist in social insect societies ([Schmid-
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*, *Vespula*, *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

274 - *Venom on the cuticle*

275 Interestingly, venom components can be found on the **cuticle** of social bees, wasps and ants. The
276 primary function of the epicuticle, the most external layer of the insect cuticle, and the complex
277 mixtures of lipids on it, is thought to protect against dehydration and to provide a mechanical
278 barrier against invasion of foreign matter. The presence of venom compounds with strong
279 antimicrobial activity on insect surfaces suggests that the venom acts also as a chemical barrier
280 providing a first line of protection against microorganisms. Besides *Polistes* paper wasps ([Turillazzi](#)
281 [2006](#); [Turillazzi et al. 2006](#)) the presence of venom components with strong antimicrobial activity
282 on the epicuticle has been recently documented in Stenogastrinae wasps ([Baracchi et al. 2010](#);
283 [Baracchi et al. 2012b](#)). Stenogastrinae wasps are a sub-family of tropical facultative eusocial wasps,
284 closely related to Polistinae and Vespinae, forming simple societies that are very small in size. The
285 medium molecular weight polar substances found on the wasp epicuticle (roughly from 900 to 4000
286 Da) were identical to those found in the venom of all the ten studied species from four different
287 genera, suggesting the venom reservoir as the primary source of cuticular polar substances. Support
288 for the idea that the venom reservoir is the source of antimicrobial compounds on the cuticle comes
289 also from the study of different social bees of the genus *Apis* ([Baracchi et al. 2011](#); [Baracchi and](#)
290 [Turillazzi 2010](#)). While venom peptides are present on the cuticle of females, irrespective of their
291 colony duties, they can be found only in traces on the cuticle of drones, which lack the sting
292 apparatus (Figure 3). The fact that newly emerged bees lack venom antimicrobial peptides both in
293 the venom reservoir and on the cuticle further confirms this hypothesis. The presence of
294 antimicrobial venom components on the cuticle of ants is known only for the fire ant *Solenopsis*
295 *invicta*. In this ant species, small quantities of venom are dispensed on the **brood** surface during a
296 behavior called “**gaster flagging**” ([Obin and Vander Meer 1985](#)), (Figure 4) and venom components
297 are also deposited on eggs by queens during the egg-laying process ([Vander Meer and Morel 1995](#)),
298 (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**

436

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550

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 Apoidea (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 *tamaritii* (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 (mm²) 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.