

External immune defence in ant societies (Hymenoptera: Formicidae): the role of antimicrobial venom and metapleural gland secretion

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Abstract

Recently, antimicrobial secretions acting in the environment of an organism have been described as external immune defence. Here, I review evidence that antimicrobial secretions from two exocrine glands of ants, the venom gland and the metapleural glands, indeed function as external immune defence in order to increase the livelihood and hygiene within the colony. I will argue that the evolution of external immune defence has likely been favoured in social insects due to their lifestyle, i.e., due to their often long-lived and large societies, with permanent nests and the potential storage of food. Although external immune defence is widely documented for social insects, we still lack a better understanding of how external immune defence integrates into other parasite defence traits of social insects and general host physiology. Therefore, I will point to potential limitations and shortcomings of our current knowledge on external immune defence in insect societies and highlight potential new avenues for future research.

Key words: Review, ecological immunology, life history, trade-off, immunity, social insects.

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Introduction

Living in a group has many benefits, as the fitness of each individual in a group is thought to increase by decreasing the costs associated with important life history activities, e.g., foraging, dispersal and competitive abilities, and cooperative brood care (KRAUSE & RUXTON 2002). However, group living has also costs, especially when it comes to the risk of parasite infection and the spread of diseases. Gregarious and social behaviour, e.g., living at high densities with frequent physical contact, often in combination with a high degree of relatedness, is predicted to increase parasite pressure and the susceptibility to the establishment and the spread of parasites within a group (ALEXANDER 1974, ANDERSON & MAY 1979). Several meta-analyses support this prediction (CÔTÉ & POULIN 1995, RIFKIN & al. 2012, PATTERSON & RUCKSTUHL 2013, NUNN & al. 2015). Especially in insect societies, the high amount of valuable resources in permanent nests, such as food and immobile developmental stages, is threatened by parasites and pathogens due to their predictability in space and time.

The ecological success of social insects suggests that they either have fewer parasites and pathogens – which is unlikely as social insects are host to numerous parasites (SCHMID-HEMPFL 1998, BOOMSMA & al. 2005) – or that they have evolved highly effective parasite defence strategies to mitigate the costs associated with their social lifestyle (BOOMSMA & al. 2005, CREMER & al. 2007, MASRI & CREMER 2014, STROEYMEYER & al. 2014, KAPPELER & al. 2015, see also WILSON & COTTER 2008). Recently, parasite defence traits acting in the environment of an or-

ganism improving its protection from parasites and pathogens, or manipulating the composition of its microbial community in its favour, have been described as external immune defence (OTTI & al. 2014). The evolution of external immune defence traits is predicted to depend on the ecological niche and the life history of an organism. An increased use of external immune defence is likely associated with an increase in parasite pressure and a low spatial and temporal variation in the microbial environment of an organism. In social insects the nest needs to be kept clean, valuable resources such as stored food need to be preserved and group members, including developing offspring, need to be protected from becoming sick. These ecological and life history characteristics of insect societies will likely increase the selective pressure to evolve external immune defences (Fig. 1).

In this review, I will explore whether the evolution of external immune defence has been favoured in social insects due to their lifestyle, i.e., social structure, foraging and nesting strategy. I will explicitly concentrate on antimicrobials produced from the exocrine venom gland and metapleural glands (MGs) with the potential to serve as external immune defence in different ant species, even though any heritable defence trait acting outside an organism might be defined as external immune defence. First, I will summarize current knowledge on the presence of antimicrobials in both glands and then identify their general function and involvement in external immune defence. Second, I will explore whether the evolution of external immune de-

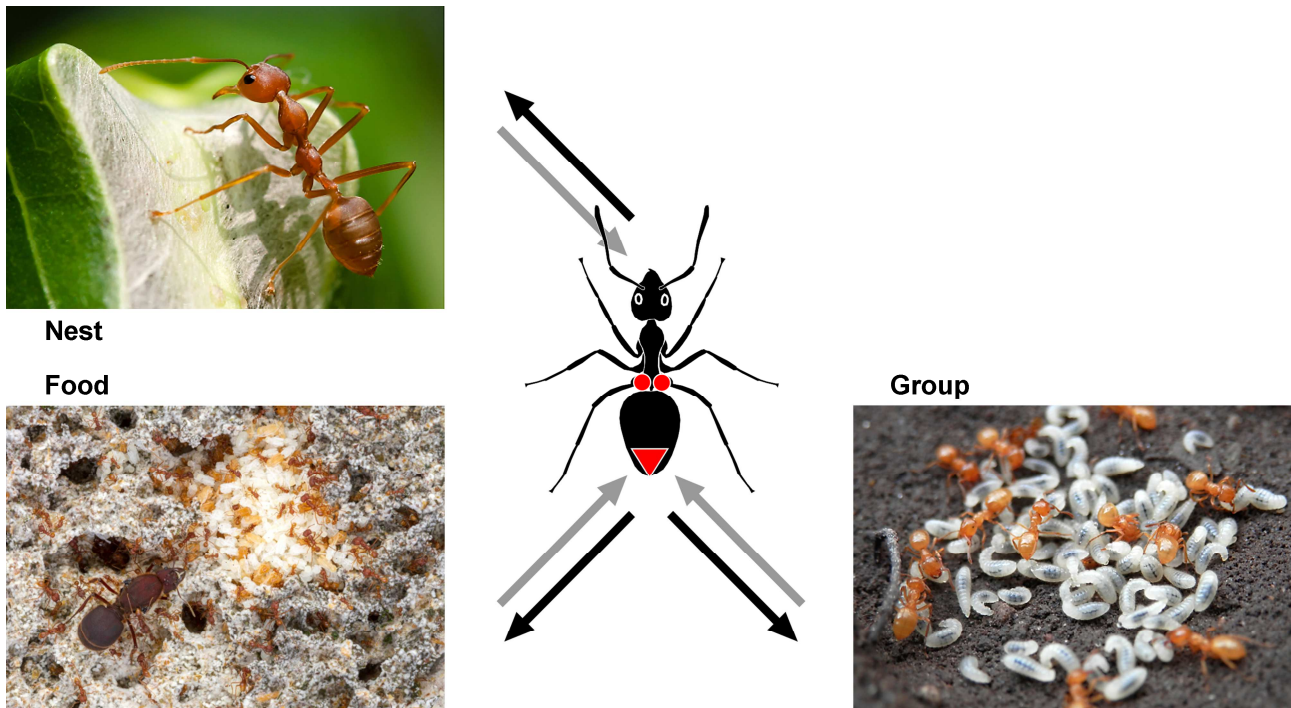


Fig. 1: Ecological and life history characteristics of insect societies can potentially increase parasite pressure and are thus selective forces (grey arrows) that have favoured the evolution of external immune defence via antimicrobial active secretions from the MGs (red dots) and venom gland (red triangle) of ants. For example, in the weaver ant *Oecophylla smaragdina* the nest is kept clean by applying venom gland secretions to the nest material (upper left picture, picture credit: Rushen, Thai National Parks), in fungus-growing ants valuable resources such as the cultivated fungal crop is protected via application of MG secretion (e.g., in *Atta texana*, lower left picture, picture credit: Alex Wild, Insects Unlocked) and in garden ants group members including developing offspring are protected from becoming sick by application of secretions from the venom gland (e.g., *Lasius* sp., lower right picture, picture credit: Ryan Hodnett). In turn external immune defence via exocrine secretions provides not only parasite defence but likely also acts as a selective pressure (black arrows) for the microbial community surrounding social insects.

fence in the form of antimicrobials from the MGs has been favoured in the fungus-growing ants (tribe Attini). In this context, I will outline general characteristics of a social lifestyle that likely favoured the evolution of external immune defence. Finally, I will point to limitations and shortcomings of our current understanding of external immune defence in insect societies and highlight potential new avenues of future research. Although the focus of this review is on ants and their use of venom gland and MG secretion as external immune defence, I will also mention other social insect systems to highlight potential generalities. Therefore, the insights into external immunity presented in this review are broadly applicable to group living animals and thus of importance to a range of organisms.

Ant venom and MG secretion as source for antimicrobial active substances

Social insects often deploy antimicrobials to their immediate environment. These antimicrobials can be derived from the environment itself, e.g., antimicrobial active plant resins that are incorporated into the nest structures of ants and honeybees (CHRISTE & al. 2003, CHAPUISAT & al. 2007, SIMONE & al. 2009), from defensive microbial symbionts that produce bioactive compounds, e.g., Actinobacteria in fungus-growing ants (MUELLER & al. 2008, KALTENPOTH & ENGL 2014), Actinobacteria and gut associated protozoa / bacteria in termites (CHOUVENC & al. 2013, UM & al.

2013, ROSENGAUS & al. 2014), and lactic acid bacteria in bees (VÁSQUEZ & al. 2012, KALTENPOTH & ENGL 2014), or from own sources, e.g., exocrine glands that contain antimicrobial compounds in ants (TRAGUST & al. 2013, BARACCHI & TRAGUST 2015), bees (MOREAU 2013), wasps (BARACCHI & al. 2012), and termites (BULMER & al. 2009, HAMILTON & al. 2011, HAMILTON & BULMER 2012). Across ants a huge variety of exocrine glands has evolved (BILLEN 2009) and several of those produce antimicrobial active substances (MORGAN 2008, VANDER MEER 2012). For most of these glands, the antimicrobial activity was only measured in vitro, which is insufficient to provide evidence for a biological function of antimicrobial exocrine secretions. Two exceptions exist for which the antimicrobial activity of gland contents or gland components of various ant species have been shown in vitro and in vivo, i.e., the venom gland and the MGs (Tab. 1). Therefore, I will focus on the secretions of these two glands.

Both, the venom gland and the MGs of ants contain a secretion that serves a range of biological functions. Typically, ant venoms are used as defensive agents that are injectable or topically applied and in some cases are used as toxic agents for prey capture. Further, venoms are used as trail, alarm, sex, recruitment, and recognition pheromones and as repellents (SCHMIDT 1986). Secretions from the MGs are used for colony or species recognition, territory, or nest entrance marking, and chemical defence against pre-

Tab. 1: MG and venom gland secretion of different ant subfamilies and species tested for their antimicrobial activity (in vivo, in vitro, or both) together with their function as external immune defence, if available. The table represents a non-exhaustive list and does not include references that only tested identified compounds of glands (isolated or synthetic), but not the whole secretion, except otherwise noted with superscript asterisks.

Exocrine gland	Subfamily	Species	Evidence	External immune defence function	References
Metapleural glands (MGs)	Dolichoderinae	<i>Dolichoderus quadripunctatus</i>	in vitro		MASCHWITZ (1974)
		<i>Liometopum microcephalum</i>	in vitro		MASCHWITZ (1974)
	Myrmeciinae	<i>Myrmecia forficata</i>	in vitro		MASCHWITZ (1974)
		<i>Myrmecia gulosa</i>	in vitro		VEAL & al. (1992), MACKINTOSH & al. (1995)
		<i>Myrmecia nigriscapa</i>	in vitro		BEATTIE & al. (1985), BEATTIE & al. (1986)
	Myrmicinae	<i>Acromyrmex echinator</i>	in vivo	protection of self	TRANTER & al. (2015)
		<i>Acromyrmex octospinosus</i>	in vitro, in vivo	protection of self	BOT & al. (2002), POULSEN & al. (2002)
		<i>Acromyrmex subterraneus</i>	in vivo	protection of food and offspring	TRANTER & al. (2014)
		<i>Atta cephalotes</i>	in vivo	protection of self	FERNÁNDEZ-MARÍN & al. (2015)
		<i>Atta columbica</i>	in vivo	protection of self	TRANTER & al. (2015)
		<i>Atta sexdens</i>	in vitro		MASCHWITZ (1970), SCHILDKNECHT & KOOB (1971)*, IZUKA & al. (1979)*, DO NASCIMENTO & al. (1996)
		<i>Crematogaster scutellaris</i>	in vitro		MASCHWITZ (1974)
		<i>Crematogaster difformis</i>	in vitro		MASCHWITZ (1974)
		<i>Myrmica rubida</i>	in vitro		MASCHWITZ (1970)
		<i>Myrmica laevinodis</i>	in vitro		MASCHWITZ (1970), SCHILDKNECHT & KOOB (1971)*
		<i>Sericomyrmex amabilis</i>	in vivo	protection of self	TRANTER & al. (2015)
	Ponerinae	<i>Amblyopone australis</i>	in vitro		MASCHWITZ (1974)
		<i>Leptogenys ocellifera</i>	in vitro		MASCHWITZ (1974)
		<i>Odontomachus haematodes</i>	in vitro		MASCHWITZ (1974)
		<i>Rhytidoponera metallica</i>	in vitro		MASCHWITZ (1974)
Pseudomyrmecinae	<i>Tetraoponera</i> sp.	in vitro		MASCHWITZ (1974)	
Venom gland	Formicinae	<i>Lasius neglectus</i>	in vivo	protection of offspring	TRAGUST & al. (2013)
		<i>Oecophylla smaragdina</i>	in vivo	protection of self	TRANTER & HUGHES (2015)
		<i>Polyrhachis dives</i>	in vitro, in vivo	protection of self, offspring, and nest	GRAYSTOCK & HUGHES (2011), TRANTER & al. (2014)
	Myrmicinae	<i>Crematogaster pygmaea</i>	in vitro		QUINET & al. (2012)
		<i>Solenopsis invicta</i>	in vitro		BLUM & al. (1958), JOUVENAZ & al. (1972)*, STOREY & al. (1991)
		<i>Tetramorium bicarinatum</i>	in vitro		RIFFLET & al. (2012)
	Ponerinae	<i>Dinoponera quadriceps</i>	in vitro		COLOGNA & al. (2013)*, LIMA & al. (2014)
		<i>Pachycondyla goeldii</i>	in vitro		ORIVEL & al. (2001)

dators (YEK & MUELLER 2011). A characteristic of both ant MG and ant venom secretion is the antimicrobial activity they display (KUHN-NENTWIG 2003, YEK & MUELLER 2011, AILI & al. 2014, BARACCHI & TRAGUST 2015, WANANDY & al. 2015).

Ant venoms may contain different antimicrobial compounds depending on species (KUHN-NENTWIG 2003, MORÉAU 2013, TOUCHARD & al. 2016). Notable components are antimicrobial peptides, e.g., pilosulins in the Australian jumper ant (INAGAKI & al. 2004, WANANDY & al. 2015) and ponerocins in the Ponerinae (ORIVEL & al. 2001, PLUZHNIKOV & al. 2014), different alkaloids in the Myrmicinae (for an overview see MORGAN 2008) and formic acid in the Formicinae (GRAYSTOCK & HUGHES 2011, TRAGUST & al. 2013). Products from the MGs have generally acidic characteristics, expressed in the form of carboxylic acid or phenol moieties (YEK & MUELLER 2011: tab. S4, VANDER MEER 2012: tab. 1). Antimicrobial activity of the MG secretion has repeatedly been shown in several ant species from different ant subfamilies (Tab. 1). Although the antimicrobial activity of both venom and MG secretion is fairly well established, the biological role and in vivo function of antimicrobial secretions from both glands as external immune defence has only recently started to be elucidated and our knowledge is still limited (Tab. 1).

Roles of antimicrobial venom and MG secretion as external immune defence

For both the venom gland and the MGs the best studied biological function as external immune defence is the protection of self, group members and developing offspring (Tab. 1). Experimentally blocking the venom gland and / or the MGs significantly reduces the survival of workers when challenged with a fungal parasite in weaver ants and fungus-growing ants (POULSEN & al. 2002, GRAYSTOCK & HUGHES 2011, TRANTER & al. 2015, TRANTER & HUGHES 2015). Blocking of venom gland and MGs in adult workers also significantly lowers the survival of brood they care for (TRAGUST & al. 2013, TRANTER & al. 2014). Indirect evidence that antimicrobial secretions from the venom gland are involved in the protection of developing offspring, has also been obtained in the red imported fire ant *Solenopsis invicta* BUREN, 1972 as venom gland secretion is dispensed onto larvae by workers and onto eggs by the queen (OBIN & VANDER MEER 1985, VANDER MEER 1995). Therefore, one important function of external immune defence comprises the protection of individuals in insect societies through the application of antimicrobial secretions on the cuticle.

Antimicrobial venom gland and MG secretion is not only used to provide a direct protective effect; the secretions are also used to manage microbes in nest material. In the weaver ant *Polyrhachis dives* SMITH, 1857 blocking the venom gland and in the leaf-cutting ant *Acromyrmex subterraneus* (FOREL, 1893) blocking the MGs, results in an increased probability of fungal growth on nest material, compared with nest material that is attended by workers with a functioning gland (TRANTER & al. 2014). In ants of the subfamily Formicinae, application of formic acid from the venom gland to the nest also leads to highly acidic nest substrates with the potential to reduce viability of entomopathogenic fungi (TRANTER & HUGHES 2015). An involvement of venom secretion for the protection of the

nest is also supported by a report that the wood ant *Formica polyctena* FOERSTER, 1850 deposits formic acid from its venom gland in their nest without the presence of an enemy (SAUERLÄNDER 1961) and by a report of increased levels of venom alkaloids in the nest soil of the fire ant *Solenopsis invicta* when the soil contains spores of a generalist fungal pathogen (STOREY 1990).

The application of antimicrobial secretions might further serve to protect stored food in the nest of social insects. Fungus-growing ants apply MG secretion to the fungus garden they cultivate as a food source in their colony (FERNÁNDEZ-MARÍN & al. 2006, 2015). Experimentally blocking the MGs, venom gland or both glands in fungus-growing ants, results in an increased hazard of the fungus garden to be overgrown by opportunistic fungi (TRANTER & al. 2014), indicating that food resources within the colony are protected via antimicrobial secretions. Alternatively, antimicrobial secretions from exocrine glands might also be used to regulate beneficial microbes such as defensive symbionts. Fungus-growing ants actively regulate defensive symbionts on the cuticle with MG secretion (POULSEN & al. 2003).

As illustrated above, antimicrobial secretions from the venom gland and the MGs of different ant species serve as external immune defences. Ants seem to change the conditions for microbial growth in the colony by using their antimicrobial gland secretions, thereby increasing the livelihood and hygiene of their nests, food sources, and colony members, probably also minimising infection risk for everyone. Although in this review I focus on antimicrobial secretions from the venom gland and the MGs of ants, it is interesting to note that a majority of defensive symbionts from social and non-social animal groups are externally located on the body surface of the host, externally on food provisions or the nesting environment (FLÓREZ & al. 2015), suggesting a similar function to antimicrobial secretions from exocrine glands as external immune defence. In the following section I am going to explore whether the social structure, foraging and nesting strategy of an ant colony might have favoured the evolution of external immune defence via antimicrobial secretions.

MG secretion in Attini: a case study on factors favouring the evolution of external immune defence in insect societies

As outlined in the introduction, the increased parasite pressure associated with life history characteristics of social insects, i.e., increased need to protect self and kin in big groups with relatively complex social organisation, as well as the need to protect a permanent nest, and stored food sources, represent different selection pressures favouring the evolution of external immune defence (Fig. 1). Indeed, in bees, wasps, and thrips, the strength of antimicrobials on the cuticle increases along a gradient from solitary to social species (STOW & al. 2007, HOGGARD & al. 2011, TURNBULL & al. 2011), indicating that the transition from solitary to a social lifestyle was accompanied by the evolution of a stronger external immune defence. For ants we lack a gradient from solitary to social species because all known living groups of ants are eusocial (HÖLLDOBLER & WILSON 1990). However, within the fungus-growing ants (tribe Attini) several lines of evidence indicate that, the transition to larger group sizes with more complex social

organization and the farming of a genetically homogeneous crop as food source in long-lived nests, were accompanied by the evolution of increased or more elaborate external immune defences in the form of antimicrobial active MG secretion.

Social structure: Large group sizes with complex social organisation: The phylogenetically most derived attine ants have a larger mature colony size and a more complex social organization, i.e., the morphological differentiation of worker castes, compared to lower attine ants (HÖLLDOBLER & WILSON 1990, MEHDIABADI & SCHULTZ 2010). From this, it can be hypothesised that the derived Attini needed to evolve a higher or a more efficient protection of developing brood and adult individuals, and one way to achieve this might have been by increasing MG size together with a more potent and more efficient antimicrobial activity. The necessary prerequisite for natural selection to have acted in such a way on external immune defence via MG secretion, namely heritable genetic variation, has been shown for MG size in the leaf-cutting ant species *Acromyrmex echinator* (FOREL, 1899) and *Acromyrmex octospinosus* (REICH, 1793) (see HUGHES & al. 2010). Although a genetic basis and heritable variation for self-produced antimicrobials can be assumed, so far, this is one of the few examples showing that variation for external immune defence traits exists.

Comparative studies across the phylogeny of fungus-growing ants have indeed shown that MG sizes in the most derived higher attine ant species, the leaf-cutting genera *Atta* FABRICIUS, 1804 and *Acromyrmex* MAYR, 1865, are significantly larger than in lower attine ant species (HUGHES & al. 2008, VIEIRA & al. 2012). In addition, derived attine ants have a higher antimicrobial activity in their MG secretion compared to lower attine ant species, when the antimicrobial activity of MG secretion is emulated with six chemical compounds identified in fungus-growing ants and tested against one entomopathogenic fungus (TRAN-TER & al. 2015). Both the significantly enlarged MGs and the higher antimicrobial activity of MG secretion, indicate that external immune defence via MGs has been favoured in the derived Attini. Intriguingly, the derived attine ant species *Acromyrmex octospinosus* is able to qualitatively and quantitatively adjust its MG secretion to specific fungal infection threads (YEK & al. 2012). Whether this is a unique feature of higher attine ants MG secretion or whether this ability can be found throughout the phylogeny of fungus-growing ants remains to be established.

Division of labour is likely to invoke differences in investment or expression of external immune defence between castes of social insects, as the risk of exposure to parasites will likely differ between tasks performed in an insect society. In the derived leaf-cutting ants smaller sub-castes tend the brood and the fungus garden, while larger sub-castes forage and cut vegetation (MEHDIABADI & SCHULTZ 2010). It might be hypothesized that castes in social insects performing tasks within the nest should invest more in external immune defence in order to protect group-members and valuable resources than castes working outside. In accordance with this prediction, the MGs of small workers of fungus-growing ants were found to be disproportionately large compared to the MGs of large workers (WILSON 1980, BOT & BOOMSMA 1996, HUGHES & al. 2010). This difference in MG size between

castes translates into a higher susceptibility and a lower inhibitory potential against fungal parasites of large workers compared to small workers (POULSEN & al. 2006, FERNÁNDEZ-MARÍN & al. 2015). Studies on the abundance of defensive microbial symbionts on the cuticle of fungus-growing ants also support the prediction that investment or expression of external immune defence depends on the task performed. In these studies, large workers within garden chambers of the leaf-cutting ant *Acromyrmex octospinosus* were found to have a greater abundance of defensive microbial symbionts compared to large workers foraging for leaf material (CURRIE & al. 2003). Interestingly, the MGs of gynes of the leaf-cutting ant *Acromyrmex echinator* are distinctly larger relative to body size in comparison to the MGs of both worker castes (HUGHES & al. 2010). Again this result is mirrored by the abundance of defensive microbial symbionts on the cuticle of *Acromyrmex octospinosus* gynes, which is higher than that of workers (CURRIE & al. 2003). One explanation for this might be that during the colony founding stage gynes need to invest heavily into external immune defence to protect the first brood until workers emerge.

Foraging strategy: Farming of a fungal crop as stored food source: Another line of evidence that the transition from small to large group sizes and more complex social organization together with the farming of a crop as food source in the nest was accompanied by the evolution of increased or more elaborate external immune defences is founded on the actual use of MG secretion in the tribe Attini. Phylogenetically less derived fungus-growing ants appear to use MG secretion mainly for self-protection, while the derived leaf-cutting ants also protect the brood, queen, nest-mates and the fungus garden with MG secretion (FERNÁNDEZ-MARÍN & al. 2006). This suggests that the use of MG secretion has expanded its function in the derived higher attines. The large-scale farming of a genetically homogeneous crop as a permanent food source in the derived leaf-cutting ants might have itself selected for a high investment into external immune defences. In contrast to lower attine genera, the fungal cultivars of leaf-cutting ants seem not capable of growing without their ant hosts (SCHULTZ & BRADY 2008) and are maintained on fresh vegetation with considerable microbial load (CURRIE & al. 1999, GRIFFITHS & HUGHES 2010). The need for protection of the fungal cultivars might thus have favoured the evolution of more potent or efficient external immune defences in the derived leaf-cutting ants compared to lower attine ants. Currently, we have only limited additional evidence that the need to protect a stored food source within the colony has generally favoured the evolution of external immune defence in social insects. However, all known social insects farming fungi as a crop, specifically fungus-growing ants, termites, and bark and ambrosia beetles, do show behavioural adaptations to protect their crop against fungal competitors and pathogens and also engage in defensive symbiosis (MUELLER & al. 2005, FLÓREZ & al. 2015). Similarly, bees protect their stored food with antimicrobial peptides produced by the hypopharyngeal gland and by antimicrobials derived from symbiont relationships (BÍLIKOVÁ & al. 2009, VÁSQUEZ & al. 2012). In relation, harvester ants might be interesting to study, as for example *Pogonomyrmex* MAYR, 1868 ants have different seed storage strategies (MACKAY & MACKAY 1984, JOHNSON

2001), which might select for differential investment into external immune defence or differential use of external immune defence depending on the amount and time seeds are stored.

Nesting strategy: Long-lived societies with permanent nests: The use of MG secretion as external immune defence in attine ants was certainly also affected by the transition from comparably short-lived societies of lower attine ant genera to the long-lived societies of leaf-cutting ant genera (MEHDIABADI & SCHULTZ 2010). Next to living in a group with complex social organisation and the storage of food, the permanent use, size and structure of a nesting site are potential factors favouring the evolution of external immune defence.

So far, comparative studies on ants and termites were not able to establish whether assumed lower parasite pressure in the nesting environment translates into a lower investment into disease resistance of the individual organisms (CALLERI & al. 2010, WALKER & HUGHES 2011). However, these studies neglect that disease resistance might not scale one to one with parasite pressure, but that variable parasite pressure in the environment necessitates a differential investment into immune defence components such as external immune defence traits (see also optimal immunity in SADD & SCHMID-HEMPEL 2009). It might be hypothesised that especially antimicrobial secretions are more effective in a confined environment such as the nest, while the constant application of antimicrobial secretions to a large, open space might incur high costs. Unfortunately, we currently lack data to support this hypothesis in ants.

In bees, relatively good evidence exists that nesting ecology has favoured the use of venom as external immune defence for the protection of self and to sanitise the nest. While in cavity dwelling species venom compounds can be detected on the cuticle and on the nest surface, venom compounds are almost absent on the cuticle and the nest surface of open nesting species (BARACCHI & al. 2011, BARACCHI & TRAGUST 2015). One tentative explanation for this pattern is that the confined environment of cavity nesting species has favoured the evolution of external immune defence via antimicrobial active venom, while the more variable environment in open nesting species has not. For ants, an interesting tribe to study would be Camponotini. It might be hypothesised that arboreal nest-weaving species in the tribe Camponotini (JOHNSON & al. 2003) might rely more on venom as external immune defence to sanitise the nest than their soil nesting congeners, as their nests represent stable and confined environments in contrast to the arguably more variable environment in the soil. Many social insects, e.g., wasps (TURILLAZZI & al. 2006, BARACCHI & al. 2012), bees (BARACCHI & TURILLAZZI 2010, BARACCHI & al. 2011), and termites (BULMER & al. 2009, HAMILTON & al. 2011) distribute antimicrobial secretions from the venom gland (bees and wasps) and the salivary gland (termites) not only on the cuticle but also in their nests. This indicates that sanitation of the nest via external immune defence is a frequent strategy in social insects, presumably to reduce parasite pressure or to manipulate the microbial community surrounding them in their favour.

Although in fungus-growing ants we have some indications that the social lifestyle has favoured the evolution of external immune defence in the form of MG secretion,

we lack comparative studies in other ant genera and social insects in general. More comparative studies are needed to disentangle the relative contributions of social structure, foraging and nesting strategy as well as of phylogenetic relationships to the evolution of immune defences and to firmly establish whether the evolution of external immune defence has been favoured in social insects. A promising system are termites, as a variety of different lifestyles exists in termites (EGGLETON 2011) and as external immune defence traits are shared between social termites and their closest sub-social woodroach relative (BULMER & al. 2012).

Potential avenues of research on antimicrobial secretions as external immune defence

Antimicrobial secretions as part of an external immune defence provide us with measurable immune traits that can be scrutinised to address a series of questions concerning the costs and benefits of using external immune defence and the reaction of the microbial environment to those defences. One of the most important future tasks will be to integrate external immune defence with other parasite defence levels such as internal immunity and immunity mediated through social interactions alongside the investigation of trade-offs between for example external and internal immune defence. Currently, we have only partial answers to these questions.

While the benefits of manipulating the microbial community in the surrounding of organisms through external immune defence is becoming increasingly clear, we lack an understanding of the costs imposed by maintaining and using external immune defence. In the fungus-growing ant *Acromyrmex octospinosus* the synthesis of antimicrobial MG secretion accounts for more than 15% of the basal metabolic energy (POULSEN & al. 2002). Interestingly, the MGs of ants parasitizing fungus-growing ants are significantly smaller than the MGs of their host counterparts (SUMNER & al. 2003, DE SOUZA & al. 2007). This suggests that these social parasitic ants are able to invest less into a costly antimicrobial MG secretion as their hosts are constantly providing them, a hypothesis supported also by the peculiar absence or significant reduction of MGs in many social parasitic ants (BROWN 1968, see also YEK & MUELLER 2011: appendix S3).

A costly external immune defence will likely be traded-off with internal immune defence traits and / or other life history traits (ROFF 1992). For example, in the honeybee *Apis mellifera* LINNAEUS, 1758 and the wood ant *Formica paralugubris* SEIFERT, 1996, the experimental enrichment of nests with plant resin – an antimicrobial derived from the environment, used by honeybees and ants to sanitise their nests – results in a significantly lower expression of internal immune defence genes and the systemic antimicrobial activity, respectively (CASTELLA & al. 2008, SIMONE & al. 2009). Evidence for a potential trade-off between external and internal immune defence exists also for MG size and internal immune gene expression in the leaf-cutting ant genera *Atta* and *Acromyrmex*. The expression of genes involved in internal immune system pathways is higher in large workers of *Atta vollenweideri* FOREL, 1893 compared to small workers (KOCH & al. 2013), while MG size relative to body size is smaller in large workers of *Acromyrmex echinatio*r compared to small workers (HUGHES & al. 2010). This evidence for potential trade-

offs between external and internal immune defences needs further experimental tests to get a good understanding of how external immune defence is integrated into the whole suite of defence traits. A negative relationship between MG grooming frequency and the abundance of defensive symbionts on the cuticle has repeatedly been found in fungus-growing ants (FERNÁNDEZ-MARÍN & al. 2009, 2013), suggesting that antimicrobial active MG secretion and symbiont-derived antimicrobials represent alternative defence strategies. Additionally, in social insects and other group living animals, social interactions are known to potentially facilitate immunity and thus to provide parasite defence at the group-level (ROSENGAUS & TRANIELLO 2001, TRANIELLO & al. 2002, CREMER & al. 2007, UGELVIG & CREMER 2007, WILSON-RICH & al. 2009, COTTER & KILNER 2010, EVANS & SPIVAK 2010, KONRAD & al. 2012, KAPPELER & al. 2015, MEUNIER 2015). However, to date, the role and contribution of social interactions to external immune defence and vice versa, are still unexplored.

Another important aspect is the microbial or parasite perspective. We completely lack an understanding of the selection pressures on the microbial environment imposed by external immune defences. Recently, FERNÁNDEZ-MARÍN & al. (2015) showed that a component of MG secretion in the leaf-cutting ant *Atta cephalotes* (LINNAEUS, 1758), phenylacetic acid, inhibits the growth of the specialist fungus garden parasite *Escovopsis* MUCHOVEJ AND DELLA LUCIA, 1990 (Ascomycota). Interestingly, the inhibition depended on the phylogenetic level of the attine taxon from which morphotypes of the fungal parasite were collected. Morphotypes of the fungal parasite obtained from phylogenetically less derived attine species were more sensitive to phenylacetic acid than morphotypes from higher taxonomic levels (FERNÁNDEZ-MARÍN & al. 2015). This can be taken as evidence that the antimicrobial component in gland secretions can exert selection pressure on the fungal parasite, suggesting that a co-evolutionary relationship between external immune defence and parasites that attack the food fungus is possible (Fig. 1). A potential co-evolutionary relationship between fungus-growing ants and the fungus garden parasite *Escovopsis* is also supported by the fact that the prevalence of this parasite is higher within gardens of more derived genera of fungus-growing ants (CURRIE & al. 1999).

Conclusion

In this review, I have outlined evidence that antimicrobial secretions from the venom gland and the MGs of ants are used as external immune defence for the protection of self and kin, the nest, and stored food. Although the evidence as whole is compelling, we still need more comparative investigations for a wider range of ant taxa to decide whether the evolution of external immune defence has actually been favoured in social insects due to characteristics of their lifestyle. Given the large diversity of lifestyles present in social insects, they represent important study systems for the investigation of evolutionary and ecological factors promoting the use of external immune defence. The antimicrobial activity of many exocrine secretions in social insects forms a discrete immune trait with which external immune defence can be measured and used to investigate a series of questions such as for exam-

ple trade-off relationships with internal immunity. From these investigations we will be able to draw a more complete picture of the evolutionary ecology of parasite defence not only in social insects, but also other animals. In addition, the ecological and economical importance of many social insect taxa depends on a good understanding of their parasite defence and resistance.

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