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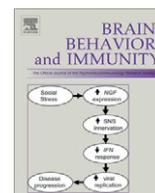
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Invited Review

Chemical and genetic defenses against disease in insect societies

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ABSTRACT

The colonies of ants, bees, wasps and termites, the social insects, consist of large numbers of closely related individuals; circumstances ideal for contagious diseases. Antimicrobial assays of these animals have demonstrated a wide variety of chemical defenses against both bacteria and fungi that can be broadly classified as either external antiseptic compounds or internal immune molecules. Reducing the disease risks inherent in colonies of social insects is also achieved by behaviors, such as multiple mating or dispersal, that lower genetic relatedness both within- and among colonies. The interactions between social insects and their pathogens are complex, as illustrated by some ants that require antimicrobial and behavioral defenses against highly specialized fungi, such as those in the genus *Cordyceps* that attack larvae and adults and species in the genus *Escovopsis* that attack their food supplies. Studies of these defenses, especially in ants, have revealed remarkably sophisticated immune systems, including peptides induced by, and specific to, individual bacterial strains. The latter may be the result of the recruitment by the ants of antibiotic-producing bacteria but the extent of such three-way interactions remains unknown. There is strong experimental evidence that the evolution of sociality required dramatic increases in antimicrobial defenses and that microbes have been powerful selective agents. The antimicrobial chemicals and the insect-killing fungi may be useful in medicine and agriculture, respectively.

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1. Introduction

Insect societies—ants, bees, wasps and termites—are important to the public and scientists alike. To many people they are pests such as wood-eating termites, crop-eating leaf-cutter ants and stinging wasps, but to others, social insects such as bees have enormous economic benefits, especially as honey makers and crop pollinators. They are important and conspicuous animals in most terrestrial ecosystems, sometimes rivaling the vertebrates in biomass. Insect societies have also received the attention of scientists comparing the social organization of insects and humans. Some such comparisons are ancient, although not necessarily scientific, for example, the Aesop Fable that contrasted the industry of ants with the apparent idleness of grasshoppers, the moral being that it is best to prepare in advance for the days when conditions get tough; as relevant to the modern world of global warming as it was to the 6th century BC. In the last few years, however, such comparisons have focused on the diseases of insect and human societies, providing insights into fundamental evolutionary biology and, potentially, for the pharmaceutical industry.

Consider a colony of social insects consisting of a million individuals jammed together in a nest, metabolic heat maintaining a comfortable ambient temperature and most of them sisters,

three-quarters related to the ruling queen. It's hard to imagine better conditions for contagious diseases. In fact, the high degree of genetic relatedness creates conditions far more conducive to disease than even the most disadvantaged human populations. We have enquired into the hypothesis that the evolution of sociality required the evolution of specialized mechanisms to fight disease, most notably antimicrobial compounds. We have also explored the possibility of applying our results to the human predicament, specifically the growing global crisis of antibiotic resistance to human pathogens and the search for novel sources of therapeutic agents.

2. Ant colonies, disease and chemical defenses

Ants are a very convenient place to start this discussion because recent research has shown that they possess two biochemical defense systems, one external and one internal, which immediately suggests that microbial diseases have been important selective agents during the evolution of insect societies. Antimicrobial compounds are secreted externally by a pair of metapleural glands, with openings located just above the point where the third pair of legs articulate with the rear of the thorax (Beattie et al., 1986). Some lipid components of the secretions have been identified (see below) but little is known about how they vary or the genes behind their production or regulation. Male ants and larvae do not possess these glands and their importance to individual hygiene was demonstrated by Macintosh et al. (1999) who

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experimentally isolated cohorts of males and larvae from the gland-bearing workers. In the absence of grooming with metapleural secretions, male and larval microbial isolates were 27 and 126 times greater than in controls. A few genera such as *Camponotus* do not possess metapleural glands but another, *Calomyrmex*, secretes antimicrobials in its mandibular glands. There is considerable variation in the size of the glands and, in pursuit of an explanation, Angus et al. (1993) demonstrated a very strong correlation ($r^2 = 0.92$; $P = 0.001$) between the number of cells in the gland and the length of the animal, suggesting that as the surface area increased, so did the size of the glands. Poulsen et al. (2002) demonstrated that when the openings to the metapleural glands of the ant *Acromyrmex octospinosus* were experimentally closed, the attacks of the fungus *Metarhizium anisopliae* were fatal within a few days. By contrast, individuals with open glands but exposed to the same fungus, lived on. Bot et al. (2002) exposed a variety of microorganisms to different fractions of the metapleural secretions of the same ant species: acetic acid, short, medium and long-chain fatty acids, indolacetic acid, γ -lactones and γ -ketoacids. There was widespread but differential inhibition of microbial growth.

The mode of action of the secretions from the bull-ant, *Myrmecia gulosa*, so called because of its size and fearsome appearance, was reported by Veal et al. (1992) and Macintosh et al. (1995). The secretion is a broad-spectrum antibiotic active against both Gram-negative and Gram-positive bacteria, and the yeast *Candida*. Only the endospores of *Bacillus cereus* were resistant. It is stable at 100 °C, resistant to proteolytic enzymes and active over a wide pH range. Ultrastructural studies show that it attacks the cell membrane, aggregates the cytoplasm and degrades some membrane-bound organelles. Overall, the principal mode of action is the disruption of the structure and function of the phospholipid bilayer.

Internally, ants have a remarkably sophisticated immune system. Workers of *Myrmecia gulosa*, when challenged with strains of *Escherichia coli*, induced antibacterial peptides, rich in proline with *N*-acetylgalactosamine O-linked to a conserved threonine. The response was highly specific as the peptides were active only against growing *E. coli* of the challenge strain and not stationary-phase cells, other bacteria, *Candida albicans*, two types of mammalian cells or bovine pestivirus. Further, the synthetic non-glycosylated form was ineffective, showing that glycosylation was necessary for maximum antibacterial activity (Macintosh et al., 1998). These biochemical properties were of both evolutionary and commercial interest (see below). A defensin gene has been identified from *Formica aquilonia* and compared with the defensin genes of the Hymenoptera *Apis mellifera* and *Bombus ignitus* and with three other insect species. It differs from each of them with respect to the number and length of the introns and exons, providing insights into the role of selection in the evolution of defensins in ants (Viljakainen and Pamilo, 2005).

Metapleural glands are unique to the ants and their secretions may have had major evolutionary effects in a very unexpected direction: Their close relatives, the bees and wasps, are the major pollinators in most ecosystems worldwide. Hundreds of thousands of flowering plant species, including many crops, rely on them for seed set. By contrast, only about two dozen species are known to be pollinated by ants. Our research has shown that pollen grains are sufficiently like microbes to be susceptible to metapleural secretions, once again, cell membranes being the weak link (Beattie, 2006). Now, co-evolution between the early flowering plants and beneficial insects such as pollinators is widely thought to have been one of the keys to the success of this plant group. One can only speculate how much more successful they may have been had they also co-evolved with ants as pollinators.

3. The special case of leaf-cutter ants

Leaf-cutting ants are notorious crop pests in central and South America and have been made famous by television natural history documentaries showing lines of workers, each carrying a leaf fragment, forming a procession of tiny, wobbling green sails along the forest floor. The subterranean, room-sized nests that contain millions of workers that clear highways to favored trees are marvels of engineering and organization. They are also remarkable for their antimicrobials, not merely for colony hygiene but also because the leaves they harvest, rather than being eaten fresh, are taken below ground, sliced into tiny pieces and inoculated with a highly specialized fungus. The fungi are tended by specialist workers below ground in vast, moist, warm 'gardens' and produce swollen, glycogen-rich structures that appear to have no function other than as ant food. The relationship between the fungus and the ants began approximately 50 million years ago and is now obligate so that each newly fertilized queen carries a pellet of fungus in her mouth to start a new garden should she get the chance (Currie et al., 2006). In effect the ants, lacking the enzymes required to digest leaves, employ the fungi to perform the task for them and thus have found a way to utilize a virtually inexhaustible food supply. However, the question will have arisen in the reader's mind—how do the ants grow only the desired fungus and why are their 'gardens' not quickly overwhelmed by a horde of unwanted microbial species?

Currie et al. (2006) showed that the leaf-cutter *A. octospinosus* harbors a mutualistic, antibiotic-producing filamentous bacterium, *Pseudonocardia* on the cuticle. These antibiotics are especially effective against the main competitor for the sliced-leaf substrate, a highly specialized parasitic fungus of the genus *Escovopsis*. These bacterially derived antibiotics, together with mandibular and metapleural secretions, appear to maintain the reasonably pure monoculture the ants require. These remarkable interactions prompt two questions of general importance: First, to what extent are antibiotics of bacterial origin? Other insects utilize symbiotic bacteria in the same way, for example, the larvae of the hunting wasp, *Philaenus triangulum* (Kaltenpoth et al., 2005), and bacteria are involved in a vast range of symbioses with eukaryotic organisms (Sapp, 1994). Thus, although we have looked for, but never found, bacteria in metapleural glands (unpublished data), there may be many novel antibiotics and many novel bacterial sources of antibiotics to be discovered in these kinds of natural interactions. Second, the intruder, *Escovopsis*, in the leaf-cutter ant/food fungus/*Pseudonocardia* interaction apparently has not evolved antibiotic resistance even over millions of years. This is in stark contrast to the many human pathogens that have become immune to our pharmaceutical defenses within 60 years. Is there some secret to maintaining vulnerability to be found here (Currie et al., 2006)?

Acromyrmex echinator also has an immune response to the fungal pathogen *Metarhizium anisopliae* significantly greater in workers than males, as measured by encapsulation rates (Baer et al., 2005). It appears that males, which do not possess metapleural glands (see above), also do not invest heavily in immune molecules, possibly because their tenure within the colony is chiefly as juvenile forms easily protected by gland-bearing workers and, upon maturing, leave the nest and are excluded from re-entry.

4. Chemical defenses in other social insects

A substantial literature demonstrates that antimicrobial compounds are well-known in many other social insects. The termites possess a varied arsenal of defenses including the secretion of antimicrobials from sternal and head glands, the external cuticle and in

faecal matter, containing a wide range of compounds including *n*-Hexanoic acid, terpenoids, naphthalene and peptides (Rosengaus et al., 1998, 2000, 2004). However, termites never evolved an analogue of the ant metapleural gland, possibly because they are dependent on microbial symbionts in their guts. Honeybees have been intensively studied and its genome has now been sequenced. This genus is a little puzzling because although it is attacked by Gram-positive and Gram-negative bacteria, viruses, microsporidia and amoebae, (as well as mites and other invertebrates), its immune system shows “diminished capacities” relative to other insects (Evans et al., 2006). These authors suggest that honeybees possess a wide array of preventative behaviors such as grooming (Roy et al., 2006) and the sequestration of each larva in a separate wax cell and feeding larvae with royal jelly that has antibiotic properties that may complement the immune system. Social wasps produce a variety of antimicrobial compounds, especially peptides, from salivary and venom glands (eg Gambino, 1993; Hancock and Chapple, 1999; Turillazzi et al., 2004).

5. Genetic diversity: an alternative defense

The ubiquitous pressure of microbial attack requires that defenses be continually updated. The generation of genetic variation underpins this ‘arms race’ by providing the raw material for evolution so that, for example, low variation heightens disease risk. Thus, microbial attack is important among the various selective pressures driving responses that maintain genetic variation, which include multiple mating, mate choice and dispersal.

Social insects seemingly provide ideal conditions for infectious disease, including observations of relatively low genetic variation within colonies (Fig. 1a), a finding which has frequently been explained in terms of kin selection (Hamilton, 1964). With the recent

development of cost and time efficient genetic assays it is now emerging that multiple mating is reasonably commonplace in some social insects, including ants, bees and wasps (Boomsma and Ratnieks, 1996; Strassmann, 2001). Multiple mating serves to increase levels of within-colony genetic variation which, in turn, has been demonstrated to enhance resistance to infectious disease in ants (Hughes and Boomsma, 2004, 2006), honey bees (Tarpy, 2003; Tarpy and Seeley, 2006; Seeley and Tarpy, 2007), bumble bees (Baer and Schmid-Hempel, 1999) and termites (Calleri et al., 2006). In many of these studies, enhanced resistance is simply attributed to genetic diversity lowering the variability in disease prevalence and mortality within colonies (see Schmid-Hempel, 2003). Indeed, it is reasonable to suppose that particular genotypes may confer resistance, for example, in the caterpillar *Spodoptera littoralis* cellular immune response varied between genotypes (Cotter and Wilson, 2002). Further, a moderate degree of heritability in immune traits has been demonstrated e.g. in honey bees (Decanini et al., 2007).

While polygyny appears a wonderful preventative of disease it is also worth noting that sexually transmitted pathogens represent a cost (Boomsma and Ratnieks, 1996) and that monogamy would reduce disease transmission between mates. Sexually transmitted pathogens of insects include mites, nematodes, fungi, protists and viruses. In comparison, vertebrates usually only sexually transmit bacterial and viral pathogens (Knell and Webberley, 2004) and some ectoparasites such as pubic lice (Kenwood, 1999). Nevertheless the benefit of multiple mating obviously outweighs the costs in some cases. In addition, polygamy may not necessarily be entirely driven by disease risk other fitness benefits of polygamy include a faster rate of colony formation and more efficient foraging rates, food storage and population growth (Mattila and Seeley, 2007).

Given the benefits apparent with polygamy in respect to disease, it follows that in the absence of multiple mating, disease risk may increase thereby selecting for alternative defenses. Evidence for the evolution of stronger cuticular antimicrobials is supplied in Stow et al. (2007) where the strongest antimicrobials were isolated from the Australian *Trigona* bees where large colonies are formed via monoandry and monogyny, resulting in high pairwise relatedness within colonies (mean = 0.695). It is also conceivable that immune response is ramped up under risky conditions of low genetic relatedness. For example, immune activities have been seen to increase in several insect species under crowded conditions, where disease risk is heightened (Schmid-Hempel, 2003 and references therein).

As an alternative, or adjunct to polygamy, kin-avoidance in mate choice can work towards maintaining levels of genetic variation. While kin recognition and avoidance is a relatively common strategy in vertebrates (e.g. Pusey and Wolf, 1996), relatively little work is available on invertebrates. While a lack of inbreeding avoidance was concluded for the Argentine ant (Keller and Fournier, 2002) there is some evidence for kin recognition/avoidance in mate choice (e.g. sweat bee; Smith and Ayasse, 1987; Field Cricket; Bretman et al., 2003). Further work is required to establish the extent of kin recognition in insects, though it seems reasonable to suppose that the development of kin recognition (either pre- or post-copulation) is not necessary when colonies are widely dispersed, especially if dispersal is sex-biased (Pusey and Wolf, 1996; Gyllenstrand and Seppä, 2003).

Scales beyond that of the individual or colony are also relevant to the risk of disease transmission, but difficult to predict. On one hand, genetic heterogeneity among colonies may be expected to minimize the level and rate of infection within a population. On the other hand, maintenance of genetic variation requires outbreeding. In this context, dispersal events may act to transmit infection, for e.g. sexually transmitted disease. Levels of genetic

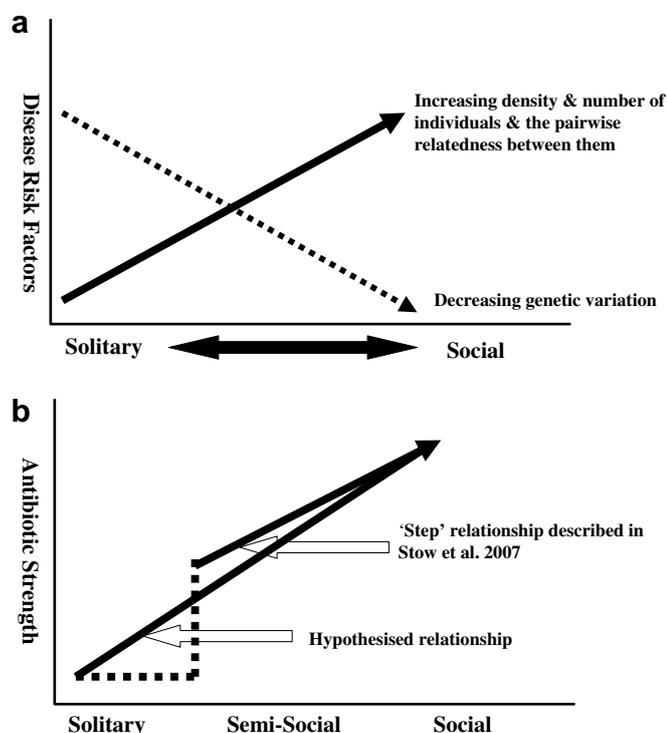


Fig. 1. (a) Illustrates a concomitant increase in disease risk with sociality owing to increased densities and numbers of individuals sharing high levels of relatedness. A trend towards lower genetic variation within and among social insect colonies further heightens the risk of disease epidemic. (b) Depicts the large increase in strength of cuticular antimicrobials in social bees compared with solitary bees.

variation and partitioning reported for populations of social insects vary enormously both within- and among species (e.g. Gyllenstrand and Seppä, 2003; Stow et al., 2006). The haplodiploid nature of many social insects suggests a lower effective population size and therefore increased loss of allelic variation being eroded by genetic drift (e.g. Hedrick and Parker, 1997), although this is influenced by sex ratio (e.g. Crozier, 1976) and comparison with diploid–diploid insects is not easy to assess statistically (Packer and Owen, 2001). The significance of this is difficult to evaluate with high genetic diversities still apparent in many social haplodiploid species (e.g. Stow et al., 2006) and the absence of many studies of disease incidence in wild populations of varying genetic diversities.

6. Antimicrobials, genetic diversity and the evolution of sociality

The idea that the evolution of sociality required the synchronous evolution of increased defenses against microbial attack has only recently been addressed in detail. Pursuing the hypothesis that increased crowding and decreased genetic diversity within colonies provided ideal conditions for microbial pathogens (Fig. 1a), Stow et al. (2007) demonstrated that the strength of antimicrobial compounds increased along a gradient of solitary, semi-social and social native Australian bee species. Using a novel, highly sensitive assay (Smith et al., 2008), we showed that such increased defenses appear to be critical to the evolution of sociality because the most primitively semi-social bee species showed an increase in antimicrobial strength an order of magnitude greater than solitary species (Fig. 1b). Whether or not a similar evolutionary pathway occurred in the other social insects remains unknown.

Our results encapsulated in Fig. 1b resulted from assays using the standard organism *Staphylococcus aureus* but we were able to isolate a native bee pathogen from the fungal genus *Cordyceps* from the bee species we assayed. The semi-social bee species showed the strongest response to the pathogen, possibly because these were the species from which the fungus had been isolated and thus resistance might have been under active selection. The solitary bee response was weak, as anticipated. The response of the fully social species was intermediate, possibly because in such a complex society there are alternative antimicrobial defenses generated by the presence of large number of workers. These include mutual grooming and raising the temperature in the colony (Starks et al., 2000). Antimicrobials were more effective at suppressing spore germination than hyphal growth and this suggested a possible role for fungal pathogens in social evolution according to the following scenario: If antimicrobial quantities were limited, for example by metabolic constraints in individual bees, then some spores might 'escape', germinate and produce destructive hyphae. One consequence may have been intense selection, not only for increasing the strength of the antimicrobials, which Stow et al. (2007) demonstrated, but also for increasing the numbers of individuals contributing to the medicine cupboard, inevitably resulting in larger colonies having some advantage in the arms race. This research is in progress.

7. Commercial possibilities

7.1. Pharmaceuticals

Ecologically driven bioprospecting provides many commercial possibilities, not least among ants (Beattie, 1995). The field relies on a deceptively simple question: Where has the desired product already evolved? Thus, in our case, where have antibiotics evolved? Historically, whether they have consciously recognized

it or not, the major pharmaceutical companies have answered this question by focusing on bacteria. However, novelty is at a premium given the widespread of cross-resistance, and various groups are looking elsewhere for it. Among insects, as Stow et al. (2007) have shown, strong antimicrobials have evolved especially in social species, suggesting that commercial bioprospecting should be profitably focused on these insects which form an interesting and, to some degree, evidence-based group to focus on. Ants have yielded two patents, one based on metapleural secretions, the other from immune peptides (Macintosh et al., 1998). Bioprospecting for biologically active molecules in ants and other social insects is still in its infancy but may reveal the chemical novelty that is so much in demand. In this context, we may ask about the role of combinatorial chemistry. We are no experts in this field but note the appeals for a return to natural products and bioprospecting that have appeared in the front-rank journals, for example, Chapman (2004) who noted: "Natural products offer unsurpassed chemical diversity and novelty, often with better affinity characteristics and evolutionary selection for biological activity. "The big advantage is you don't meet this kind of high number attrition you meet with small-molecule chemistry, you end up much earlier with a good lead that might even be the natural product itself." Similarly, Paterson and Anderson (2005) pointed out: "...evolutionary selection – nature's own high-throughput screening process for the optimization of biologically active compounds. Also: "...the continual isolation of an increasing range of novel bioactive secondary metabolites suggests that we have barely scratched the surface of nature's vast library of small-molecule ligands".

We recently assayed the strongest bee antimicrobial secretions against equine herpesvirus cultured in rabbit kidney cells. At certain concentrations, the antimicrobial extract killed 99% of the virus without harm to the mammalian host cells. This research is on-going and serves to emphasize the points we have made concerning the promise of natural products.

7.2. Pest control

Among the agencies and corporations interested in crop protection, there is serious interest in the fungal pathogens of insects and pest control agents. Many of the most serious agricultural pests are insects. Fungi that attack and kill insects—entomopathogenic fungi—have evolved a truly astonishing variety of mechanisms to find and infect their hosts (Roy et al., 2006) and these are being exploited to attack crop pests, and patents have been taken out on specific, highly virulent fungal strains (Shah and Pell, 2003). Ants, like all social insects, are attacked by many different kinds of microbes, including microsporidia and these too are being screened as pest control agents (Briano et al., 2002). This is not the place to review this fascinating applied science but this essay serves as a reminder that detailed knowledge of the natural history, ecology and evolution of insect/social insect–microbial interactions are a necessary precursor to the investment in, and development of, this crop protection biotechnology.

In summary, social insect researchers are revealing the interactions between social, behavioral and chemical (including immunological) factors that constitute the complex of defenses against microbial disease. The research suggests some interesting parallels between social insect and human defense systems, in the first instance demonstrating that the organization of individuals into societies incurs immediate threats of invasion by contagious pathogenic microorganisms, whether the society is insect or human. Further, the evolution of societies diversifies the defensive repertoire, most notably in the development of behavioral responses finely tuned both to the disease organisms and to the other, complementary defensive mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2008.03.008.

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