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Antimicrobial defences increase with sociality in bees

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Evidence for the antiquity and importance of microbial pathogens as selective agents is found in the proliferation of antimicrobial defences throughout the animal kingdom. Social insects, typified by crowding and often by low genetic variation, have high probabilities of disease transmission and eusocial Hymenoptera may be particularly vulnerable because of haplodiploidy. Mechanisms they employ to reduce the risk of disease include antimicrobial secretions which are particularly important primary barriers to infection. However, until now, whether or not there is selection for stronger antimicrobial secretions when the risk of disease increases because of sociality has not been tested. Here, we present evidence that the production of progressively stronger antimicrobial compounds was critical to the evolution of sociality in bees. We found that increases in group size and genetic relatedness were strongly correlated with increasing antimicrobial strength. The antimicrobials of even the most primitive semi-social species were an order of magnitude stronger that those of solitary species, suggesting a point of no return, beyond which disease control was essential. Our results suggest that selection by microbial pathogens was critical to the evolution of sociality and required the production of strong, front-line antimicrobial defences.

Keywords: bee; antimicrobials; disease; relatedness; sociality

1. INTRODUCTION

Two well-documented trends accompany the evolution of sociality in Hymenoptera, an increase in the density of individuals living in close proximity and, frequently, a reduction in the genetic diversity among those individuals. Both trends are conducive to contagious disease epidemics (e.g. Zasloff 2002; Lawniczak *et al.* 2007). A variety of antimicrobial mechanisms have been described for arthropods in general and social insects in particular (Sadd & Schmid-Hempel 2006) and resistance to disease has most probably been a major contribution to the evolutionary success of insects (Otvos 2000). The methods social insects use to reduce the risk of disease include antimicrobial secretions which are particularly important primary barriers to infection (Zasloff 2002). Other defences include immune systems, both humoral and cellular, allogrooming (Hughes *et al.* 2002) and multiple mating to enhance genetic variation (Hughes & Boomsma 2004). For example, multiple mating and subsequent reductions in colony relatedness significantly reduce disease risk in social hymenopteran species including *Apis mellifera* (Tarpy 2003) and *Bombus terrestris* (Baer & Schmid-Hempel 2001).

Ants secrete antimicrobial compounds onto the integument from paired thoracic glands highly specialized for the purpose (Beattie *et al.* 1986; Mackintosh *et al.* 1995). Termites secrete strong antimicrobial compounds, for example, from their faecal pellets or soldier defensive secretions (Chen *et al.* 1998; Rosengaus *et al.* 1998) as do social wasps although, in this group, they appear to be secreted principally by the salivary or the venom glands (Turillazzi *et al.* 2004).

These studies have established that cuticular antimicrobial compounds, or compounds which are secreted onto the surface of the insect, are important primary barriers to invasion by microbial pathogens. However, much less is known about the possible role of microbial attack as a selective agent in the evolution of aggregative behaviour, particularly sociality. Insect social systems are considered to exhibit three basic characteristics: overlap in generations between parents and offspring; cooperative care of the brood; and specialized castes of non-reproductive individuals (Crozier & Pamilo 1996; Wilson & Hölldobler 2005). We suggest that there may be a fourth characteristic—specialized antimicrobial defences—especially front-line barriers to the microbial invasion of colonies.

To test this hypothesis, we assayed cuticular antimicrobial secretions from bees along a gradient of sociality, selected from two sub-families and three tribes, to include species that are solitary (*Amegilla bombiformis*, *Amegilla asserta*), semi-social (*Exoneura nigrescens*, *Exoneura robusta*) and eusocial (*Exoneurella tridentata*, *Trigona carbonaria*) against *Staphylococcus aureus*, a standard bacterial assay species.

2. MATERIAL AND METHODS

(a) *Antimicrobial assays*

Whole bees were washed in 70% ethanol (24 h) to remove putative antimicrobials from the cuticle surface, the solvent then removed by vacuum evaporation at 25°C, and the residual extract resuspended in LB broth. The assay used opposing gradients of residual antimicrobial extract concentration and *S. aureus* cell number across a row of 12 wells in flat bottom 96-well microtitre plates to produce concentration–growth response curves for each species. Test rows contained 1 : 2 serial dilutions of *S. aureus* cells (ranging from 10 colony forming units, CFUs, to 2.0×10^4 CFUs) to which opposing 3 : 4 serial dilutions of antimicrobial extract had been added. In this way, the most concentrated antimicrobial was tested against the fewest number of *S. aureus* cells and this ratio varied systematically across the plate. After preliminary assay optimization, tests were standardized to a maximum concentration equivalent to the extract from two individual bees. Each 96-well plate supported three rows of bacterial control wells containing 1 : 2 serial dilutions of *S. aureus* cells only; a sterility control row containing growth medium only and a sterility control row with the serially diluted antimicrobial extract only.

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Table 1. Minimum inhibitory concentrations (50 and 100% kill) values for six bee species showing a sequence from solitary to eusocial organization. (Group size was the mean from 20 nests for each species. Within-group relatedness (*R*) was estimated using microsatellite genotypes. Standard errors are given in parentheses.)

status	species	MIC(50)	MIC(100)	group size	*R*
solitary	*Amegilla asserta*	201.1 (±17.0)	362.0 (±28.9)	1	—
solitary	*Amegilla bombiformis*	220.8 (±42.5)	280.2 (±59.4)	1	—
semi-social	*Exoneura robusta*	29.3 (±2.8)	38.2 (±3.4)	6.4 (±1.2)	0.312
semi-social	*Exoneura nigrescens*	15.6 (±0.2)	17.3 (±0.1)	8.2 (±1.83)	0.468
eusocial	*Exoneurella tridentate*	50.4	68.3	14.1 (±2.4)	0.664
eusocial	*Trigona carbonaria*	0.7 (±0.2)	2.2 (±0.3)	>1000	0.695

Following incubation for 22 h, growth in the presence of each of the 12 antimicrobial extract concentrations was measured as optical density (OD) and expressed as (OD of test well/mean OD of *S. aureus* control wells)×100. Growth data were fitted to a modified Gompertz function (Gooding *et al.* 2000 and shown below) using SPSS software (Windows v. 13.0).

$$G = 100 \exp[-\exp(-k\ (\log 10(\text{conc.}) - m))].$$

Sample sizes for the assays were as follows: for solitary species, 20 individuals were consolidated for each assay as preliminary tests showed the antimicrobial strength to be weak. We performed five assays for each species, for a total of 200 individuals. For each of the semi-social and eusocial species, five colonies were sampled five times, with the exception of *E. tridentata* where the samples were limited and comprised two colonies. Growth values predicted by the Gompertz model were plotted for each bee species. From these concentration–response curves, the strength of the bee extract was quantified following the method employed by du Toit & Rautenbach (2000). The antimicrobial strength of each bee species was considered in terms of the MIC_{50} (the concentration of test extract that resulted in 50% inhibition of growth) and the MIC_{100} (the lowest concentration of test extract that resulted in 100% inhibition of growth).

To account for different sized bees, MIC values were expressed in terms of the surface area (mm^2) required to yield the MIC. We considered various techniques of calculating the surface area of each bee species, having confronted a similar problem for ants (Angus *et al.* 1993), and concluded that the best was to treat the bee body as a cylinder. Thus, we obtained the mean length and mean diameter of 50 adult individuals from each species, selected randomly from vials of preserved specimens, to calculate surface area.

(b) *Relatedness estimates*

Pairwise relatedness estimates were calculated using allele frequency data generated from independently segregating microsatellite loci with the software KINSHIP v. 1.2 (Goodnight *et al.* 1998). Microsatellite loci were amplified by polymerase chain reaction (PCR) as described for *E. robusta* (Repaci *et al.* 2006), *E. nigrescens* (Stow *et al.* 2006) and *T. carbonaria* (Green *et al.* 2002). Genotyping *E. tridentata* used four novel microsatellite loci, isolated using an enrichment protocol (Gardner *et al.* 1999). For each microsatellite locus, primer sequences and conditions for PCR are given in the electronic supplementary material.

3. RESULTS

Our analyses (table 1) show that as group size and within-nest genetic relatedness increased monotonically (*E. tridentata* excepted), so did antimicrobial strength (Spearman's rank correlation $p<0.05$ for MIC_{50} and MIC_{100}). The surface area of bees that inhibited 50 or 100% of growth in *S. aureus* was substantially less in social than solitary bees. The data also reveal a large increase in antimicrobial strength in the steps from solitary to semi-social and MIC values decreasing by an order of magnitude from even the least social species (*E. robusta*).

4. DISCUSSION

These results strongly suggest that the evolution of sociality in these bees was accompanied by the evolution of stronger antimicrobial compounds. Further, our data suggest that selection pressure from microbial pathogens was so intense that even minimal sociality required substantially stronger antimicrobials. This result is consistent with the 'point of no return' hypothesis (Wilson & Hölldobler 2005) which suggests that major functional traits associated with sociality appeared early in the transition from solitary to social organization. As the evolution of eusociality in bees may have occurred more than once (Cameron 1993), our data suggest that microbial pathogens have exerted strong selection on any joint increase in group size and genetic relatedness, necessitating the evolution of stronger antimicrobials. With the exception of *E. tridentata*, cuticular antimicrobials progressively increased in potency as group size and within-colony relatedness increased. The MIC values for *E. tridentata* may reflect its primitive eusociality. While not conforming precisely to the otherwise linear relationship generated by all the other species examined, they fell, as expected, between the semi-social *E. robusta* and *E. nigrescens* and the fully eusocial *T. carbonaria*. Another possible explanation for its MIC values is its arid habitat where microbial challenge may be less than in the mesic habitats in which all the other bee species were found. We were not able to make the most relevant comparative measures such as moisture levels in the twig nests of arid versus mesic species; however, there is compelling evidence that aridity limits both general and pathogenic fungal abundance and richness (Talley *et al.* 2002) and some experimental data to that effect (e.g. Entry *et al.* 2004), although there are many variables that determine the microbial communities (Atlas 1984).

While group living may have increased survivorship (Traniello *et al.* 2002), our results strongly suggest a requirement for superior, front-line, antimicrobial chemical defences. At this point, we are uncertain of the potential trade-offs between the various mechanisms of antimicrobial defences including, for example, between physical and chemical barriers in nest materials and structure (Cruse 1998), genetic diversity, antimicrobial production and group size. We are currently exploring these trade-offs among social arthropods such as wasps and thrips that present opportunities to partition the relative strengths of these effects.

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