# THE EVOLUTION OF SOCIALITY AND ANTIMICROBIAL DEFENCES IN WASPS

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## DECLARATION

The work described in this thesis was carried out in the Conservation Genetic Laboratory at Macquarie University. All work described in this thesis is original and has not been submitted, in any other form, for a higher degree at any other institution.

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## ABSTRACT

Cuticular antimicrobial compounds are a front-line defence against disease that restrict the growth and transmission of pathogens within groups of social insects. I explore the hypothesis that these compounds were integral to both the evolution of early social aggregations and the development of more complex social systems. The strength of cuticular antimicrobials was assayed from wasp species representing a range of group sizes and life history traits. Wasps were chosen because they are a basal group within social Hymenoptera and present species with a range of social structures. Across species, nesting substrate type and levels of social complexity were strongly correlated with the strength of antimicrobial compounds. Phylogenetic analysis suggested that these compounds arose first in solitary, ground-dwelling species. Across colonies of a social species, traits varied with respect to how they influenced the strength of antimicrobial defences; group size and genetic diversity had strong but opposing correlations with antimicrobial strength. Conversely, microbial load was low across colonies and did not appear to vary with antimicrobial strength. These data suggest antimicrobial defences in wasps first evolved as a defence against soil-borne pathogens. As group sizes increased the pressure of disease risk selected for increasingly strong antimicrobial compounds. Low microbial associations indicate that these compounds are highly effective in social species however correlations with group size and genetic diversity suggest that disease risk remains a major selective pressure on maximum attainable group sizes. I propose a model of the relationship between group size, genetic diversity and the risk of disease that may be used to predict the largest stable group sizes. These findings illuminate both the historical and contemporary

selective pressure that disease risk has placed on cuticular antimicrobial compounds. Further, these data provide unique insights and research avenues for both bio-prospecting and social insects in general.

## CHAPTER DESCRIPTION

Chapter One: Disease risk and the evolution of social insects

I performed the review of literature and writing of this paper. My supervisor, Dr Adam Stow, and Prof Andrew Beattie provided constructive feedback.

Chapter Two: Methodology

I performed the review of literature and writing of this paper. My supervisor Dr Adam Stow provided constructive feedback and Dr Peter Wilson (Macquarie University) provided advice on statistical modelling approaches.

Chapter Three: Social complexity and nesting habits are factors in the evolution of antimicrobial defences in wasps

I performed all aspects of field work and laboratory work myself including study design, selection of sampling sites, sampling of wasps (with assistance from Dr Christine Turnbull, Macquarie University), DNA and antimicrobial extractions, PCR, bioassays and sequence analysis. The statistical modelling package for bioassay data was designed in conjunction with, and coded by, Dr Peter Wilson (Macquarie University). I carried out all analyses and wrote the manuscript with feedback provided by my supervisor, Dr Adam Stow, and Prof Andrew Beattie. This chapter has been published in *PLoS ONE* under the same title and is presented as formatted as required for submission to said journal.

Chapter Four: The effectiveness of cuticular antimicrobials in a social wasp declines with group size and genetic similarity

I performed all aspects of field work and laboratory work myself including study design, selection of sampling sites, sampling of wasps, DNA and antimicrobial extractions, optimisation of microsatellite markers, PCR, genotyping and bioassays. The statistical modelling package for bioassay data was designed in conjunction with, and coded by, Dr Peter Wilson (Macquarie University). Statistical analyses were performed with the assistance of Dr Peter Wilson (Macquarie University). I wrote the manuscript with feedback provided by Dr Adam Stow and Prof Andrew Beattie. This chapter is formatted in the style required for submission to *Journal of Zoology*.

Chapter Five: Microbial load in a social insect and the implications for the role of antimicrobial defences

I performed all aspects of field work and laboratory work myself including study design, selection of sampling sites, sampling of wasps, microbe washes, microbe culturing and DNA extraction, PCR and fragment analyses. My co-supervisor Prof Michael Gillings advised me on microbial wash techniques and Liette Waldron (Macquarie University) assisted with optimisation of genetic techniques for bacterial analysis. I wrote the manuscript with critical feedback provided by Dr Adam Stow and Prof Andrew Beattie.

Chapter Six: Conclusions

I summarised the major findings of my thesis and outlined the implications for both current theories on social insect evolution and future research applications. I wrote the manuscript with feedback provided by Dr Adam Stow and Prof Andrew Beattie.

Appendix A: Antimicrobial strength increases with group size: implications for social evolution

I performed analysed data and constructed the figures used in the manuscript. I also provided assistance with laboratory work and provided feedback on the manuscript. This study has been published in *Biology Letters* under the same title and is presented as formatted as required for submission to said journal.

Appendix B: Mating system and genetic structure in the paper wasp (*Polistes humilis*)

I performed all aspects of field work and laboratory work myself including, selection of sampling sites, sampling of wasps, DNA extractions, PCR, microsatellite optimisation and sequence analysis. Dr Adam Stow assisted with initial study design and analyses. The sampling and laboratory component of this study was performed during the preparation for my BSc (Hons) thesis, however these data were reanalysed, composed and published during the preparation of this thesis. I wrote the manuscript with critical feedback provided by Dr Adam Stow, Prof Andrew Beattie and Prof Michael Gillings. This study has been published in *Australian Journal of Zoology* under the same title and is presented as formatted as required for submission to said journal.

Appendix C:  R Script for Gompertz curve calculations

This is the complete code for the R script used to generate Gompertz curves as described in Chapters Three and Four.  This script was designed in conjunction with, and coded by, Dr Peter Wilson (Macquarie University).

Appendix D:  Biosafety Approval

This is the letter from the Macquarie University Biosafety Committee granting final approval for this project.

## PERSONAL ACKNOWLEDGEMENTS

*I have always had a somewhat determined impatience when it comes to learning. The progression from one academic tier to the next has felt so natural over the years that at no point have I ever considered resting prior to the completion of this thesis. Although my impatient attitude towards education served me well throughout my more structured undertakings, when I entered the wider world of scientific discovery it became more of a double-edged sword. At first when given the freedom of my own research, I became overwhelmed by the possibilities; my logical flows became fractured as I enthusiastically (but admittedly, haphazardly) synthesised new theories and hypotheses from the old. Without proper training, I fear I may never have learnt to calmly and logically formulate new ideas which I could clearly and effectively communicate with others. Therefore, I must first acknowledge and thank with all sincerity, my supervisors and mentors – Dr Adam Stow, Prof. Michael Gillings and Prof. Andy Beattie. Thank you for your expertise, guidance and, perhaps most of all, patience. Over the last few years these have transformed me from the fledgling research student who attempted to think and write in at least three directions at once, into (tentatively) a scientist.*

*During my PhD, I was exceptionally fortunate to work within a friendly, cohesive and supportive environment. I would like to thank the members of the Conservation Genetics Lab at Macquarie University for their friendship, good humour and support over the years; particularly Vincenzo Repaci, Christine Turnbull, Shannon Smith, Paul 'Ted' Duckett and Siobhan Dennison. I would also like to give special thanks to my long-time friend and colleague Ashlie Hartigan (University of Sydney)*

*for her support, advice and friendship throughout both my undergraduate and postgraduate studies.*

*To my family, I am indebted for everything you have given me over the years. In addition to the support, emotional and financial, which you have provided, you have each instilled and nurtured within me what I have come to find to be some of my better traits. From my parents thank you for my sense of independence and strength of character; from Mum, my appreciation of the natural world; from Dad, my technical aptitude. And to my sister Amy, thank you for keeping me closer to Earth than I may have managed myself, and for being a constant reminder of things which are truly important in life.*

*And finally, to my partner Roshana Vander Wall, whose radiance and support has kept me connected to my humanity throughout even the most difficult periods of this undertaking, I offer you my most sincere love and thanks. Of all the classes, in all the universities, in all the world; I am forever grateful that you walked in to mine.*

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# CHAPTER ONE: DISEASE RISK AND THE EVOLUTION OF SOCIAL INSECTS

- "*Natura non facit saltum*" (Darwin 1859)

## Introduction

'Social insects' is an umbrella term for various species, found in five insect orders, which are characterised by complex behavioural interactions and co-operative behaviour (Wilson 1971). These five insects orders which house examples of social species are Hymenoptera (bees, wasps and ants), Isoptera (termites, however recent debate suggests they should be reclassified as part of Blattodea; Inward *et al*. 2007), Thysanoptera (thrips; Crespi 1992), Homoptera (gall-forming aphids) and Coleoptera (a single species of ambrosia beetles; Smith *et al*. 2009). Collectively, social insects have a global distribution, found on every continent with the exception of Antarctica (Hoggard *et al*. 2011). They vary greatly in group size ranging from the tens to millions (Giraud *et al*. 2002; Michener 1974), constructing nests (bees and wasps), mounds (termites and ants), galls (thrips and aphids) or galleries (ambrosia beetles) and some which develop morphologically distinct castes (Michener 1974).

## Terminology

Historically there has been debate regarding terminology of social hierarchy, and more recently debate as to the virtues, if any, of applying these terminologies (Costa & Fitzgerald 2005; Lacey & Sherman 2005; Crespi & Yanega 1995). The driving force behind these arguments is the observed variation in social complexity across

different social insect taxa, and manifests in most debates as attempts to qualify the decreasing levels of sociality (Lacey & Sherman 2005; Michener 1969). Eusociality is the accepted label for the pinnacle of social organisation and complexity, and is strictly defined by three characteristics; overlapping generations, co-operative brood care and division of labour (predominantly reproductive labour; Michener 1969). Difficulty arises when attempting to classify those species which only partially meet these requirements; some species may only partially qualify for a characteristic (e.g. worker castes may reproduce; *Polistes* wasps, Reeve 1991) or lack one or two characteristics entirely (e.g. no overlapping generations of adults in *Augochloropsis* bees; Michener 1969). Past authors have attempted to assigned labels for combinations of different traits (e.g. parasocial, pseudosocial, quasisocial, subsocial; Michener 1969) but a convention for these categories has never been established and as such they are often transient in the literature (Costa & Fitzgerald 2005). Recently, in an attempt to circumvent these difficulties, there have been two alternative terminological schemes proposed, each focusing on a different aspect of sociality to differentiate between species. The 'eusociality continuum' was first discussed by Sherman *et al*. (1995) and then revisited by Lacey & Sherman (2005) and was proposed to remove any ambiguity regarding 'division of labour' instead replacing it with 'reproductive skew' as the third defining state for sociality. Specifically, this assesses the degree to which individuals reproductive output is affected by their social standing (Sherman *et al*. 1995). While this measure is difficult to quantify, conceptually it allows all co-operatively breeding species (vertebrates and invertebrates) to be defined and ranked on a single scale (Lacey & Sherman 2005). Alternatively, Costa & Fitzgerald (2005) argued that pre-occupation with defining categories distracts from their overall purpose; that is,

highlighting traits of biological relevance. As such, they proposed that all hierarchal terminologies be collectively replaced by the term 'social' and that sociality should be examined in the light of ecological and evolutionary relevant traits. In addition to the three traditional categories, traits such as nest construction, defence and foraging efficiencies were suggested useful additions to identify factors driving convergent evolution in social species (Costa & Fitzgerald 2005). In this thesis, which examines factors influencing the evolution of social complexity, this latter view is more useful. As such, within the context of this thesis, following Costa & Fitzgerald (2005), 'social' will refer to any point on the spectrum for social complexity, with individual species distinguished by ecological and evolutionary relevant traits. Further, 'solitary' will refer only to species which do not co-operate or associate with with their conspecifics, except during mating periods.

**Natural selection and the evolution of altruism**

The interest in social insects (particularly eusocial) gained prominence when Darwin remarked upon eusocial organisms as a "special difficulty" that was "fatal" to his Theory of Evolution by Means of Natural Selection (Darwin 1859). To Darwin eusocial insects behaved in a manner seemingly paradoxical to his theory; housed within eusocial colonies were individuals who increased only the fitness of other individuals because they did not themselves reproduce. Under Darwin's theory, traits that improve the fitness (i.e. the ability to produce viable offspring in the next generation) of the organism possessing said trait will become more prevalent in a population over time, while traits that decrease fitness will become less prevalent over time (Darwin 1859). Therefore, a caste of non-reproductive individuals should not exist for more than one generation as none of its members

can pass on the trait for being non-reproductive. Darwin suggested that the group as a whole may maintain beneficial traits which were not directly passed on to the individuals possessing the traits, but could not suggest a mechanism for how these traits had been slowly accrued over time (Darwin 1859).

**Inclusive fitness theory**

In 1964, Hamilton proposed the theory of inclusive fitness – a mathematical model attempting to explain altruistic behaviour as a function of genetic similarity (Hamilton 1964a). Although this model was not specifically designed to model social insects, the social hymenoptera were one of the first groups to which Hamilton's theory was applied (Hamilton 1964b). His inclusive fitness model holds that fitness can be increased by aiding and increasing the fitness of individuals to whom they are genetically similar. Although this behaviour does not result in an individual directly passing on its genetic material to subsequent generations, any shared genes will still be in the resultant generation and thus, there is an indirect fitness benefit (Hamilton 1964a). Using Hamilton's model, it became possible to calculate theoretical thresholds at which direct fitness costs become outweighed by indirect fitness benefits, thus allowing altruistic behaviour, such as that seen in eusocial insects, to be favoured by natural selection (Figure 1.1; Hamilton 1964b).

**Figure 1.1: Calculating theoretical altruism thresholds with Hamilton's rule**

$b\,r_s > r_o\,c$
(altruism favoured)

$b\,r_s < r_o\,c$
(selfishness favoured)

Altruism is selected when the product of the inclusive fitness benefits ($b$) and relatedness between siblings ($r_s$) is greater than the product of the costs of reproducing individually ($c$) and the degree to which they are related to their own offspring ($r_o$). These formulae are simplified from those which appear in Hamilton's original papers (Boomsma 2009; Hamilton 1964a; Hamilton 1964b).

In Hamilton's model the degree to which two members of the same species are related (i.e. the amount of genetic material they have in common) is (often) seen as the key variable; as genetic similarity increases, so does the indirect fitness benefit conferred by altruistic behaviour as more shared genes being passed onto the next generation. Conversely, as genetic similarity decreases, the indirect fitness benefits will eventually outweigh the direct fitness costs and natural selection will once more favour selfish behaviours.

**Early challenges to inclusive fitness theory**

Although Hamilton's theory was a mathematically plausible explanation for the maintenance of altruism in social insects, reviews of inclusive fitness were critical the underlying evolutionary assumptions. The earliest review presented arguments against inclusive fitness theory on the basis that it was not applicable to all social species (Lin & Michener 1972). While accepting that inclusive fitness was plausible in explaining behaviour in eusocial insects that exist in closely-related family groups, the authors were critical of its application to all social insects as they felt it placed too much emphasis on altruism as the main evolutionary pathway (Lin & Michener 1972). Their argument followed that if altruism was required for the evolution of social insects, then incidence of individuals with few shared genes transferring between colonies would be selected against, and the production of workers and reproductive individuals within a single generation would be rare (Lin & Michener 1972). This is because in both cases, the inclusive fitness benefit presumed to be driving their altruism is removed; in the case of the former, neither party shares significant amounts of genetic material to confer a fitness benefit and, in the case of the latter, the sterile caste develops at the same time as their

reproductive siblings and thus cannot assist them in order to gain an indirect fitness benefit. Presenting examples of non-eusocial species with such traits, which were assumed to be the evolutionary precursor to eusocial species, Lin & Michener (1972) instead proposed that mutual colony defence promoting tolerance between individuals was a more likely evolutionary pressure. This was later referred to as the communal aggregation hypothesis (Ito 1993). Despite this criticism, Hamilton's theory remained popular as a conceptual model and was demonstrated to be useful in making predictions with regard to sex-ratios in some eusocial species (Trivers & Hare 1976).

**The haplodiploidy hypothesis**

Another early prediction arising from inclusive fitness theory pertained to the evolution of eusociality itself. Hamilton commented that greater fitness accrues when full-sisters of haplodiploid species help to rear full-sisters arising in subsequent generations, rather than their own offspring ($r_s = 0.75$, $r_o = 0.5$: see Figure 1.1; Hamilton 1964b). This asymmetry in relatedness among haplodiploid individuals was presented as the mechanisms which drove the evolution of sterile castes in eusocial organisms (Wilson 2005), and while initially well supported it has encountered difficulties. The first and perhaps most obvious issue with this theory however is the existence of termites: diploid eusocial organisms. Hamilton (1972) initially proposed that termites were exceptional as they represented a distinct social lineage (Isoptera), and that transference of their symbiotic protozoan between generations promoted social interaction instead. This rationale later ran into difficulty however with the discovery of more diploid organisms with sterile castes; ambrosia beetles (Smith *et al*. 2009), snapping shrimps (Duffy 1996) and naked

mole-rats (Sherman *et al*. 1991). Further, Trivers & Hare (1976) noted that the haplodiploidy hypothesis overlooks the relatedness of brothers to which their full-sisters are far less closely related ($r_s$ = 0.25, $r_o$ = 0.5: see Figure 1.1), potentially causing conflict within colonies with regards to gender-biased reproductive output. It was not until the development of molecular biology that these potential virtues and criticisms of inclusive fitness theory could be properly explored and addressed.

**Social insects: a molecular approach**

The development of high-resolution molecular markers (microsatellites) in the late 1980s (Ellegren 2004) and their subsequent use in the study of social insects beginning in the early 1990s (Queller *et al*. 1993) allowed inclusive fitness theory to be directly tested for the first time. The development of microsatellites allowed for the quantification of relatedness between individuals (r), as well as greatly increasing the accuracy of parentage analyses which had previously relied on allozyme markers (require large numbers of markers to confidently exclude potential parents; Queller *et al*. 1993). The combination of these technologies paved the way for a series of studies which culminated in a theory which could clearly demonstrate the role of inclusive fitness in the evolution of eusociality. Beginning in 2001, a review of the available data on female mating frequency in social Hymenoptera demonstrated that multiple matings were relatively rare across all taxa (Strassmann 2001). Additionally, all species which were found to be polyandrous were highly-evolved eusocial species (honeybees, *Vespula* wasps, advanced fungal-growing ants and harvester ants; Strassmann 2001). From this, it was hypothesised that monandry in females was the ancestral state for all social Hymenoptera (Strassmann 2001). While not discussed in the review, this finding

was inline with the inclusive fitness theory; mating only once maximises relatedness amongst daughters and should therefore have promoted altruism in early evolution of sociality given the right ecological conditions. Strong support for this hypothesis arose following a review examining mating frequency data in the context of an extensive phylogenetic reconstruction of social insect taxa (Hughes *et al*. 2008). Using data from 267 species, monandry was demonstrated to be the ancestral state across eight independent eusocial lineages within Hymenoptera (no mating frequency data were available for the ninth lineage, Allodapine bees) (Hughes *et al*. 2008). All cases of polyandry were found to be derived states, suggesting that it was derived following an irreversible transition to obligate altruism, thus increasing relatedness within the broods (potentially lowering disease transmission; Baer & Schmid-Hempel 1999) without reducing altruistic behaviour between individuals.

**The evolution of eusociality and the role of inclusive fitness**

Utilising the increasing body of evidence suggesting that high relatedness and monogamous pairings were integral to the evolution of altruistic behaviour and eusociality as a whole, Boomsma (2009) developed a theory which offers a causal framework for these states. The theory, referred to as the 'monogamy window' deals primarily with the conditions required for a species to transition to eusociality and explores the implications of the inclusive fitness theory for species across different levels of social complexity. The theory holds that under lifetime monogamous pairings, average relatedness between siblings ($r = 0.5$) is the same as the degree to which an individual is related to its own offspring, and when input into Hamilton's rule, the terms cancel each other out reducing the equation to $b > c$ (alternatively $b/c > 1$) (Boomsma 2009). At this point any consistent increase

(regardless of how small) to the benefits of rearing siblings relative to the costs of reproducing oneself should promote altruism and begin the transition towards eusociality (Boomsma 2009). It is stressed that both monogamy and altruistic benefits must remain constant; if monogamy is not constant then overlapping generations of siblings will have a relatedness of less than 0.5, or if altruistic benefits are not constant then there is no incentive for long-term adoption of altruism over selfish behaviour (i.e. $b = c$). It is noted that the monogamy window can extend from r = 0.4 – 0.5, however, this lower limit requires $b/c > 1.25$ and theoretical likelihood of this event is greatly diminished (Boomsma 2009). While a relatively simple model, the conditions required to pass through the 'monogamy window' are either rare or unstable in nature. Lifetime genetic monogamy is generally rare in the animal world. However, when it arises in social insects it is often elegantly enforced: Females of social species are generally singly-mated (both historically and currently; Hughes *et al*. 2008; Strassmann *et al*. 2001) and males often die before colonies are founded (with the exception termites, Matsuura *et al*. 2009) and are therefore unable to re-mate, instead existing vicariously as sperm within their mated female. Similarly, ecological conditions promoting group benefits (or discouraging selfish behaviour) must remain stable throughout the lifespan of a cohort in order to maintain the required benefit-cost ratio (i.e. $b/c > 1$) required to promote altruistic behaviour (Boomsma 2009). Further, both of these conditions must be maintained over a long evolutionary timescale before worker castes develop and the transition to eusociality is completed (see Figure 1.2; Boomsma 2009), highlighting why eusocial species are so rare despite being such an ecologically successful strategy (Wilson & Hölldobler 2005). Finally, under the monogamy window theory, those species for which the cost-benefit ratio does not

remain stable develop co-operative breeding strategies (i.e. sociality) instead of eusociality, and thus do not develop truly sterile castes and may exhibit opportunistic selfish behaviour (see Figure 1.2; Boomsma 2009).

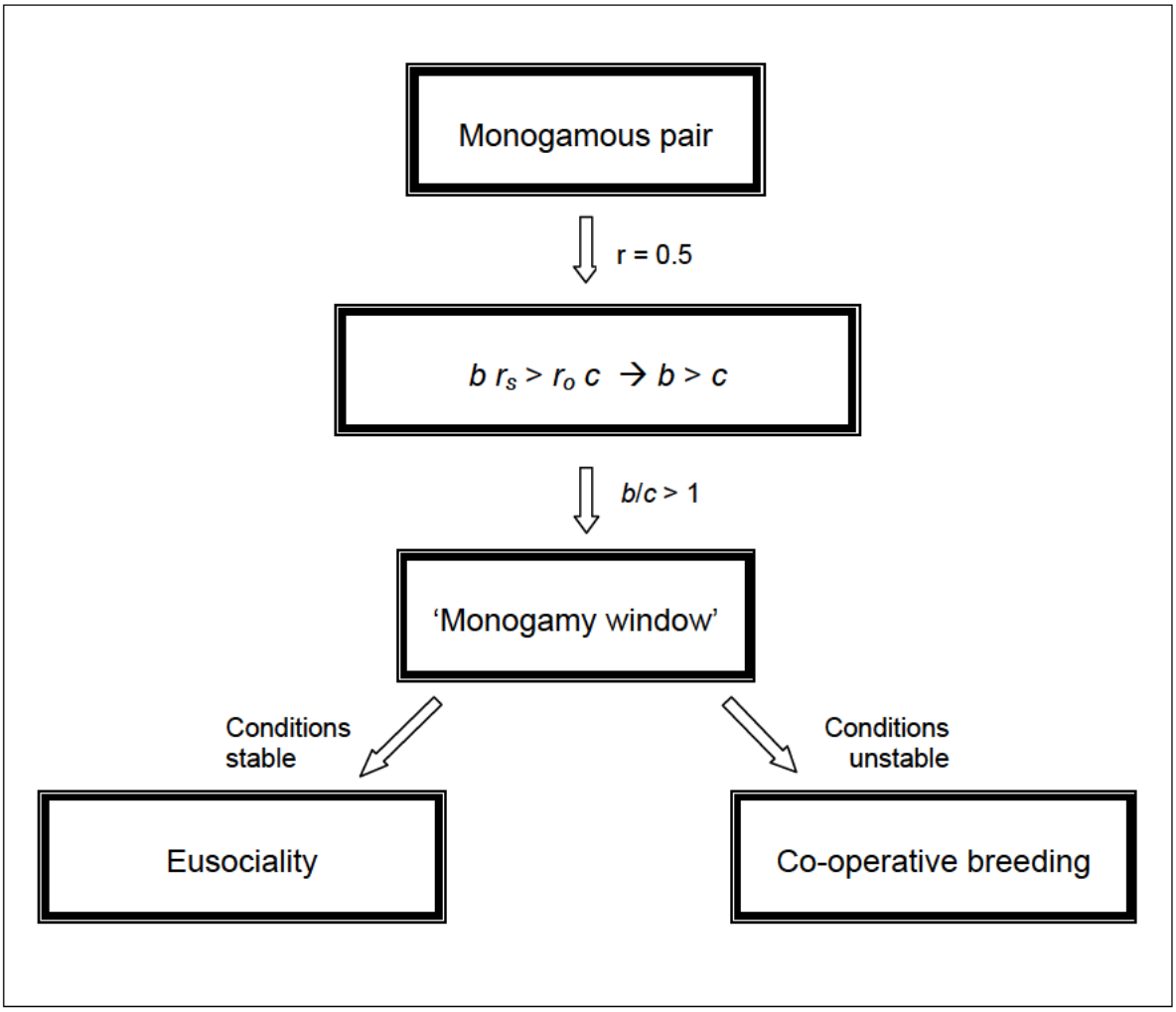


**Figure 1.2: Simplified flow-diagram of the 'monogamy window'**

Monogamous pair generates offspring with high average relatedness (r = 0.5), leading to the removal of the relatedness terms on both sides of the adapted Hamilton's rule. When altruistic benefits exceed selfish reproductive costs ($b/c > 1$), this creates the 'monogamy window'. Stability of these conditions leads to the evolution of eusociality, while instability creates opportunistic mating strategies, providing a pathway to co-operative breeding strategies instead (Boomsma 2009).

**Modern challenges to inclusive fitness theory**

While the 'monogamy window' theory provides a parsimonious model for the convergent evolution of eusociality, a similar model for explaining pathways for co-operative breeding strategies (the 'monogamy window' represents a single potential pathway) is still largely elusive. The highly varied co-operatively breeding species provide direct tests of Hamilton's rule, but the variables are often very difficult to quantify and subsequently model. The rigid nature of eusocial society makes assessing costs, benefits and relatedness values relatively easy to predict, whereas these variables in more primitively social species may be more plastic, fluctuating with ecological conditions (Boomsma 2009). Similarly, as eusociality is the only well defined social condition, developing a model of convergent evolution to a nebulous condition is equally troublesome, particularly if one is attempting to incorporate a theorem such as Hamilton's rule. It is this issue that lead to a recent controversy within the scientific community in which the limitations of Hamilton's rule were explored. In 2010, Nowak *et al*. published a review challenging both the longevity and utility of Hamilton's rule in the study of social evolution and offered an extensive mathematical model as an alternative (Nowak *et al*. 2010). Central to their argument was the demonstration that Hamilton's rule was mathematically flawed as it could not take into account selective pressure or synergistic benefits to fitness and relied upon a series of stringent conditions (Nowak *et al*. 2010). Additionally it was asserted that when Hamilton's rule could be applied appropriately, it behaved exactly as the alternative model presented by the authors based on the theory of natural selection (Nowak *et al*. 2010). Further, the authors claimed previous empirical studies which support inclusive fitness theory were

superficial, often only correlating measures of relatedness with social behaviour instead of quantifying each variable in the equation (Nowak *et al*. 2010).

Perhaps owing partially to the high-profile nature of both the authors and journal in which it was published, and that it was seen to be disparaging four-and-a-half decades of social insect research, Nowak *et al*. (2010) received a swift and overwhelming rebuke from the research community; five separate responses were published in a following issue, including one with 103 co-authors. Broadly, there were four main counter-points to arguments proposed by Nowak *et al*. (2010). Firstly, responding authors asserted that the generalised theory of inclusive fitness has inspired volumes of studies into social insect research and continues to do so (Strassmann *et al*. 2011) and in their response Nowak *et al*. (2011) did not deny this. Further, it was noted that there have been many hypotheses tested using specific models based on Hamilton's rule (Abbot *et al*. 2011; Ferriere & Michod 2011; Strassmann *et al*. 2011) and that relatedness is often examined because it is a significant biological variable in social insects (Abbot *et al*. 2011). Secondly, Nowak *et al*. were accused of creating a false dichotomy between theories of natural selection and inclusive fitness by implying they were mutually exclusive (Ferriere & Michod 2011; Herre & Wcislo 2011). Nowak *et al*. (2011) responded by clarifying their position that inclusive fitness is a derivative of natural selection theory and thus cannot be used to make predictions independent of the proposed alternative hypothesis. Thirdly, it was suggested that the alternative model proposed by Nowak et al. (2010) relied on stringent conditions itself and ultimately contributed no new insight into the evolution of sociality (Abbot *et al*. 2011; Boomsma *et al*. 2011). Finally, and perhaps fatally, Nowak *et al*. (2010) were

accused of ignoring studies which directly contradicted their model (Boomsma et al. 2011; Herre & Wcislo 2011), notably asserting that high relatedness can arise after the evolution of sociality without making reference to Hughes *et al*. (2008) which demonstrated all eusocial lineages are ancestrally monogamous (and thus have high relatedness). Nowak *et al*. (2011) did not respond directly to either of these two criticisms and instead reasserted that there had yet to be any other theories which could explain why inclusive fitness would be maximised and which processes drive this effect.

**An alternative approach**

Overall, most of the assertions made by Nowak *et al*. (2010) were challenged or dismissed, the success of which can only be fully judged by the results of future studies. If it accomplished nothing else, it highlighted the ongoing interest and controversial nature of the evolution of sociality. It can also be seen that for the last four-and-a-half decades the majority of research into the evolution of sociality in insects has focused on the evolution and maintenance of altruistic behaviour, with the main antagonist to this process being selfish reproduction. While this may be the most obvious opposition to the formation of altruistic groups there are other factors, such as disease risk, that may select against the evolution of social insects and until recently, these have gone largely unstudied (Viljakainen *et al*. 2009). Pathogenic outbreaks become more frequent when a population's genetic diversity is low (alternatively, when within-group relatedness is high) and there is a high density of individuals (Stow *et al*. 2007). While this scenario is rarely (if ever) encountered by individuals of a solitary species it a significant threat to those species which form large social groups. Given that the ancestral state of all social

insects was likely monogamous pairs (i.e. high relatedness) (Hughes *et al*. 2008) forming increasing group sizes, it is likely that either evolving prior to, or along with social complexity must have been some manner of disease resistance strategy which allowed group sizes to increase while mitigating the associated increased disease risks. It is this hypothesis that my thesis, as part of a multi-discipline collaboration, is aiming to examine.

**The importance of cuticular antimicrobial compounds**

While there is a large body of research into the internal immunological defences of social insects (e.g. Schmid-Hempel 2003) we focus on external cuticular defences because these are the front-line barriers to infection. In evolutionary terms, this makes these compounds the equivalent of prevention rather than cure (Stow *et al*. 2010). Highlighting the importance of these compounds, Poulsen *et al*. (2002) showed that the entomopathogen *Metarhizium anisopliae* was rapidly lethal when the antibiotic-producing metapleural glands of *Acromyrmex octospinosus* were blocked. Further, Baer *et al*. (2005) showed that the immune response to the same pathogen rapidly declined after only 96 hours, post-infection, suggesting that it is both costly and limited, placing an evolutionary premium on front-line defences. The first evidence suggesting that these compounds were of evolutionary importance, however, arose when our research group demonstrated that bees possessed cuticular antimicrobial compounds and that the strength of these compounds increased with social complexity (Stow *et al.* 2007). Further, and perhaps more interestingly, this relationship was not linear. There was a dramatic increase in antimicrobial activity between solitary and social species with very small group sizes (6+ individuals) which suggested that a disease risk threshold

exists above which group sizes cannot increase without possessing some manner of disease resistance mechanism (Stow *et al.* 2007). While interesting, this result was still only obtained using a single taxon (bees) and examining a single factor (social complexity). Thus, to examine whether this was a ubiquitous phenomenon across social insect taxa (and therefore likely integral in their evolution) and what factors drove the evolution of these compounds, the scope of the study was expanded to examine several different social insect taxa and a large variety of potentially evolutionarily important traits found across these species. For my role in this research I chose to investigate the cuticular antimicrobial compounds in wasps, examining the effects of social complexity, phylogenetic relationships and ecological constraints, both across and within species.

**The utility of wasps as a model**

For examining our hypothesis, wasps are potentially the most informative taxa in terms of research opportunities. Foremost, examples of every grade of social complexity can be found within wasp taxa (O'Donnell 1998) as well as group sizes across species ranging from solitary up to thousands (O'Donnell 1998; Jeanne & Nordheim 1996). Secondly, wasps provide great insight into the conditions which led to the evolution of other social taxa as both ants and bees arose from wasp lineages (Brothers 1999). Finally, there is already an extensive body of literature relating to wasps across many different fields. In terms of ecology, wasps are well studied as many species act as natural predators of many pest species (Barlow *et al*. 1996) or are pest species themselves (Johnson & Starks 2004). Wasp venom is well studied by both biochemical and medical disciplines as it is known to have allergenic effects in humans (Erdmann *et al*, 2004), as well as unique neurological

effects on prey species (Haspel & Libersat 2003). Finally, wasps are one of the iconic social taxa and thus house many model species are well studied in terms of genetics and social behaviour (e.g. paper wasp genus *Polistes*; Reeve 1991). When combined, this provides an enormous knowledge base from which hypotheses can be based, and exceptionally diversity of taxa allows for specific traits to be examined for their evolutionary significance.

**Thesis overview**

In this thesis I examine factors influencing the evolution and production of antimicrobial compounds in wasps using a combination of genetic, microbial and statistical methods. This will be broken primarily into two main approaches, one focusing on trends across species, and the other examining trends within a single species. By examining trends across species, I intend to replicate the initial experiment performed using bees (Stow *et al.* 2007) and build upon it by investigating the effects, if any, of additional traits I hypothesise represent a factor in the disease risk of a species and thus provoke an evolutionary response in terms of disease resistance strategies, such as group size and nest substrates. Following this, I examine trends across colonies of a social species and investigate whether variation in group size, genetic variation and colony composition across colonies affects the production of antimicrobial compounds. I also investigated the microbial loads of wasps to assess to what degree the production of antimicrobial compounds affects the presence of different microbes, with the aim of leading into identifying specific pathogens for future studies. In addition to potentially identifying those colony traits which most influence disease risk, this approach was intended to highlight traits which arise from within a species that might confound any

conclusion drawn about patterns of disease risk across species. In addition, the phylogenetic relationships between the species examined in this study were investigated so as to provide an evolutionary context to both levels of social complexity and traits determined to influence the strength of antimicrobial compounds. By adding the dimension of evolutionary time, it became possible to gain insight as to the order in which traits arose and which traits, if any, were either prior to, or co-evolved with socially complex insect taxa.

**Summary**

In summary, in this thesis I examined the evolution of disease resistance and social complexity in wasps, focusing on cuticular antimicrobial compounds. Trends both across and within species with respect to these traits were examined in contemporary and evolutionary contexts using genetic, microbiological and statistical methods. The overall goal was to determine which traits across wasp species have the greatest influence on the strength of antimicrobial compounds and use this information to increase our overall knowledge of the conditions which lead to the evolution of all social insects.

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# CHAPTER TWO: METHODOLOGY

## Preface

These methodologies have been synthesised from published materials which form the latter sections of this thesis. As such, there is some degree of repetition between the methods presented here and those presented in Chapters Three, Four and Five.

## Introduction

In this thesis I explore sociality and antimicrobial defences in wasps with the aim to identify both historical and contemporary evolutionary pressures on these traits in order to identify the roles which disease and disease defence have played the evolution of social insects as a whole. In the course of my research, I propose and test five main hypotheses, listed below:

- Different nesting substrates and levels of social complexity carry different inherent disease risks; this should be reflected in the relative strength of a species' cuticular antimicrobial compounds (Chapter Three).
- Disease risk was an important factor during the evolution of sociality. This was examined by phylogenetic reconstruction of species assayed for antimicrobial defences. These species represent a range of different social complexities and nesting substrates and their position in the phylogeny should reveal which (if any) of these traits were prerequisites or co-requisites to the evolution of sociality (Chapter Three).
- Low genetic variation is associated with stronger antimicrobials. Low genetic diversity is predicted to increase the risk of disease epidemic.

Consequently colonies with low genetic variation may have stronger antimicrobials – if there is facultative control over antimicrobial production. (Chapter Four).

- Large colony size is associated with stronger antimicrobial defences. Group size is known to affect disease risk and varies among colonies within a species, and changes throughout the life of a colony. Colonial antimicrobial defences may increase with colony size – if there is an additive effect from individuals producing their own antimicrobial (Chapter Four).
- Colonies with weaker antimicrobial compounds are at greater risk of disease outbreaks; this should be reflected in individuals' microbial load (Chapter Five).

Key to all of these hypotheses is quantifying the relative strength of cuticular antimicrobial defences and accurately comparing these within and among species. As a part of my multi-disciplined approach to exploring these hypotheses, phylogenetic, microbiological, statistical and genetic methods are used in conjunction with these data where appropriate.

This chapter explores and justifies the methodologies and analyses which were employed to explore the above hypotheses forming the experimental basis of this thesis. Complete details of methodologies are presented in the relevant later chapters.

**Field sites and collection methods**

*Field sites*

Wasps were collected from six locations across Australia; Macquarie University North Ryde Campus (NSW; -33.77317, 151.11424), Hornsby (NSW; -33.70143, 151.09665), South Turramurra (NSW; -33.76111, 151.10537), Mortlake (NSW; -33.83879, 151.10817), Berowra Heights (NSW; -33.60245, 151.13751) and Alice Springs (NT; -23.70722, 133.83639). All sampling was performed on public land or on private property with the permission of the owners.

*Collection methods*

One of two methods of collection was used depending on the species of wasp being collected. For solitary and communally aggregating wasps, a net was used to collect wasps which were then placed in individual collection tubes. For social wasps, a container with dry ice was placed under the nest to subdue the colony before dislodging the nest and sealing it inside a suitable container. Following both methods, individuals are stored for a short time at low temperatures (~4ºC) in order to incapacitate them prior to preparation for analysis.

*Species identification*

Species were identified by morphology and sequencing of 28S nrDNA and COI mtDNA fragments. In four wasp species, identification could only be resolved to genus-level due to lack of identification keys for many Australian wasp genera. In one instance, identification could only be resolved to the level of sub-family (designated as 'Pepsinae Sp1'). As part of the cross-species analyses species were then categorised by social complexity (social, communal aggregator or solitary) and

by nest type (paper nest, mud nest or burrow; Table 2.1), representing broad categories of group size and environmental exposure. Paper nests were defined as clusters of cells constructed from pulp and attached to the substrate by a petiole (Hunt 2007). Mud nests were defined as sealed and provisioned cells constructed from mud and above-ground (Brockmann 1980; Cooper 1957). Burrows were defined as either provisioned terrestrial burrows (Evans & O'Neil 2007; Evans & Matthews 1973), or as those species which burrowed for prey which was subsequently paralysed and ectoparasitised (Inoue & Endo 2008).

*Sampling summary*

In total, 1268 individual wasps comprising members of nine different species were collected (Table 2.1). All wasps were collected at NSW locations (see 'Field Sites' above), with the exception of *Abispa ephippium* which was collected at in Alice Springs (NT).

| **Species (Family)** | ***n*** | **Sociality** | **Nest type** |
|---|---|---|---|
| *Polistes humilis* (Vespidae) | 1077 (10) | Soc. | Paper |
| *Ropalidia plebeiana* (Vespidae) | 49 (2) | Soc. | Paper |
| *Bembix sp.* (Crabronidae) | 83 | Com | Burrow |
| *Austroscolia sp.* (Scoliidae) | 47 | Sol. | Burrow |
| *Cryptocheilus sp.* (Pompilidae) | 4 | Sol. | Burrow |
| Pepsinae Sp1 (Pompilidae) | 1 | Sol. | Burrow |
| *Abispa ephippium* (Vespidae) | 1 | Sol. | Mud |
| *Sceliphron laetum* (Sphecidae) | 5 | Sol. | Mud |
| *Delta sp.* (Vespidae) | 1 | Sol. | Mud |

**Table 2.1. Characterisation of wasp species**

*n*: number of individuals (number of colonies for social species); Sociality: social (Soc.), communal aggregator (Com.), solitary (Sol.)

**Assessing antimicrobial defences**

*Bioassay*

Putative antimicrobial compounds were extracted from wasps using ethanol as a solvent, which was removed prior to assaying using vacuum evaporation at room temperature. As no mass spectrometry or gas chromotography has been previously performed to identify compounds examined in these studies, ethanol was selected as the most appropriate solvent for extraction of antimicrobial compounds, providing the best compromise of practicality and efficiency. Although multiple solvents could be used to ensure maximum extraction of compounds (alkanes, nitriles: Turillazzi *et al*. 2006; water, alcohols, ethers: Cowan 1999) this would have prohibitively increased the sample size required to maintain reliable results. Ethanol has previously been demonstrated to be an effective solvent when assaying antimicrobial compounds in thrips and bees (Stow *et al*. 2010; Turnbull *et al*. 2010; Stow *et al.* 2007).

Over the last decade, there has been a trend towards miniaturisation of antimicrobial assays. Micro-broth culture techniques have greatly reduced time and resource costs compared to older, plate-culture methods such as disc diffusion (Casey *et al*. 2004). Further, these miniaturised assays have been demonstrated to be far more accurate than their predecessors (Patton *et al*. 2006). The main drawback of all these techniques, however, is a lack of sensitivity. Until a recent innovation, all antimicrobial assays used serial dilutions of extracts against a fixed concentration of target cells (Smith *et al*. 2008). Smith *et al*. (2008) developed an enhanced serial dilution assay technique which overcame this issue whilst maintaining accuracy and efficiency of results. By using opposing serial dilutions of antimicrobial

compounds and microbial culture, a growth-response curve is generated whereby false negatives are greatly reduced as the highest concentration of extract is assayed against the weakest concentration of microbial cells (Smith *et al*. 2008). Further, exceptionally effective extracts are still accurately assayed, as at the opposing end of the serial dilution they are assayed in their weakest concentration against the highest concentration of microbial cells (Smith *et al*. 2008). Growth-response curves are generated by measuring optical density of experimental wells, expressed as the percentage growth compared to control wells (i.e. untreated microbial culture) (Smith *et al*. 2008; Patton *et al*. 2006). Combined these provided an accurate, relative measure of antimicrobial strength, highly appropriate to investigate my hypotheses.

Although use of species-specific pathogens in this assay may identify specific selective pressures, there are two main problems with this approach. Firstly, all species assayed would require a microbial assay prior to an antimicrobial assay, and even if identified, the species-specific pathogen may not be culturable in a laboratory (Schloss & Handelsman 2005) for use in this assay. Secondly, selective pressure driving extreme responses to specific pathogens may bias or confound cross-species comparisons (as seen with *Cordyceps* and bees; Stow *et al*. 2010). The use of an evolutionarily novel pathogen (i.e. no prior exposure resulting in a specific response) bypasses both of these issues. *Staphylococcus aureus* was chosen as the most appropriate microbe for use in this assay, as it is only found in mammals and birds (Kloos 1980) and should therefore be novel to all species examined in this study. Further, *S. aureus* has been previously demonstrated to be technically

appropriate for this assay in terms of growth times, susceptibility to antibiotics (as a control) and optical density (Stow *et al*. 2007)

*Normalisation of growth-response curves*

Following Smith *et al.* (2008), a modified Gompetz function was fitted to the data using R (version 2.5.1; R Development Core Team 2010) and used to calculate the methods the concentration of extract (expressed as a equivalent number of wasps) required to kill or inhibit 50% of microbial growth (herein referred to as IC50) for each species (see Appendix C). A modified Gompertz function have been previously used in similar biological applications (Smith *et al*. 2008; Stow *et al*. 2007; Gooding *et al*. 2000) and was deemed appropriate for this study given the reverse-sigmoid nature of generated growth-response curves. The initial modification of the Gompertz function was used to model decline of percentage green leaf area with increasing time (Goodling *et al*. 2000) however when applied to the above assay this was modified to instead model percentage microbial growth with increasing antimicrobial concentration (Smith *et al*. 2008).

In this study, data from assays was handled in one of four ways depending on what was required to fit the modified Gompertz function (see below and Figure 2.1). For three of these four IC50 values could be calculated.

(i) Curves with a gradient approaching vertical (preventing calculation of the 'shoulders' of the curve) were replaced with a linear line joining the two asymptotes.

(ii) A lower asymptote was estimated from data points for assays where data points were trending downwards towards zero but lacked a lower asymptote (likely because it exists outside the examined concentration range). The modified Gompetz curve was fitted using both the actual and estimated data points.

(iii) Data where there was a clear upper and lower asymptote with a curve gradient not approaching vertical were fitted with no further modification or estimation.

(iv) Data with no lower asymptote and no points trending towards zero were recording as having no activity over the concentrations being tested.

Both methods of estimation were tested for appropriateness and developed using the complete data set. Mean IC50 was calculated for each species and each category of social complexity and nest type.

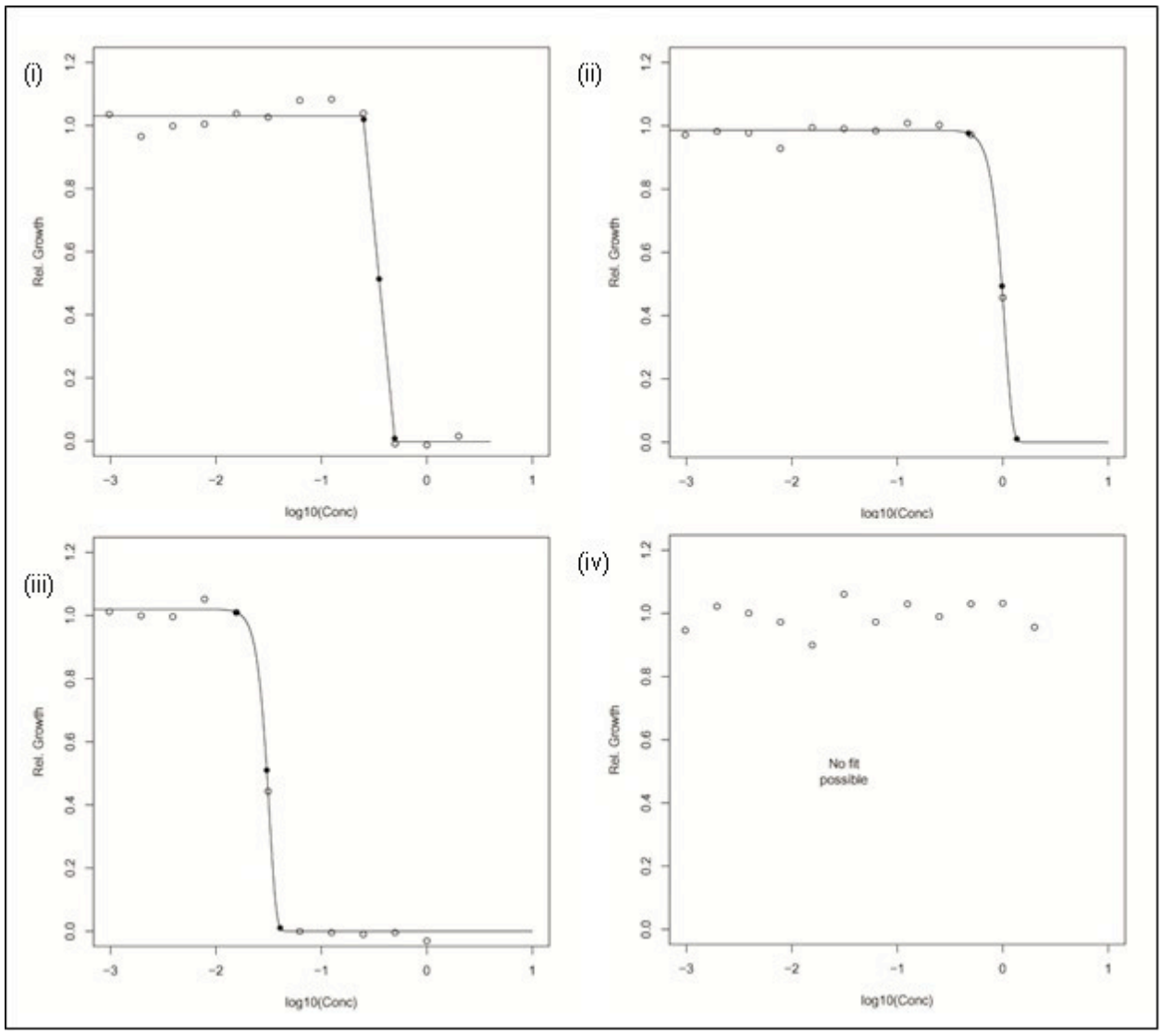


**Figure 2.1: Methods for fitting Gompertz functions to raw data**

Gompertz functions were fitted in one of four ways depending on the state of data: (i) gradient of the curve approaches vertical; (ii) data lacks a lower asymptote but is trending downwards; (iii) curve has both an upper and lower asymptote and gradient is not approaching vertical; (iv) curve lacks lower asymptote but does not trend downwards.

*Standardisation of data across species*

To standardise measurements across different species, concentrations of wasp equivalents were converted to concentrations of equivalent surface area. Adapting methods previously applied to bees (Stow *et al*. 2007), mean surface area for each species was estimated by generating elliptical cylinders using measurements from up to ten individuals per species. Although regular cylinders would have been appropriate for most species, some of the larger species exhibited up to ~1.5x difference between height and width, rendering regular cylinders an imprecise measure.

**Providing an evolutionary context**

*Phylogenetic reconstruction*

An evolutionary context (i.e. a phylogeny) is required to make inferences regarding the emergence of natural history traits and their correlation with antimicrobial defences. This can be accomplished with a traditional approach using morphological data, however given the overall lack of systematic data available for Australian wasp species, a molecular approach was deemed more appropriate.

*Selection of genetic markers*

Initially three genetic markers were selected for use in a phylogenetic reconstruction; cytochrome oxidase I (COI; mitochondrial DNA; Folmer *et al*. 1994), 16S (mitochondrial ribosomal DNA; Dowton & Austin 1998) and 28S (nuclear ribosomal DNA; Dowton & Austin 1998). These were selected as they had previously been used in phylogenetic studies of parasitic wasp species (Dowton & Austin 2001; Dowton & Austin 1998). Following amplification and sequencing of

these fragments (Table 2.2), initial data exploration revealed that only 28S and COI gene fragments could be successfully concatenated for use in a phylogenetic reconstruction. Both a partition homogeneity test performed in PAUP* v4.0b10 (Swofford 2003) and comparison of topologies of trees generated using the makers individually revealed significant incongruencies between 16S and the other two makers; thus the 16S gene fragment was removed from the analysis. Two species, representing each sister taxa to Vespoidea were chosen outgroups for these analyses (Apoidea: *Apis mellifera*; Chrysidoidea: *Chrysis cembricola*). As two species in this study are housed outside Vespoidea (Families Sphecidae and Crabronidae are part of Apoidea), the second outgroup from Chrysidoidea was required to test whether results could be confounded by the use of *A. mellifera*. The COI and 28S gene fragments for these species were obtained from GenBank (Table 2.2).

| Species name | 28S | COI |
|---|---|---|
| *Polistes humilis* | JF510015 | JF510006 |
| *Ropalidia plebeiana* | JF510016 | JF510007 |
| *Bembix sp.* | JF510020 | JF510011 |
| *Austroscolia sp.* | JF510021 | JF510012 |
| *Cryptocheilus sp.* | JF510022 | JF510013 |
| Pepsinae Sp1 | JF510023 | JF510014 |
| *Abispa ephippium* | JF510017 | JF510008 |
| *Sceliphron laetum* | JF510019 | JF510010 |
| *Delta sp.* | JF510018 | JF510009 |
| *Apis melifera* (outgroup) | AJ302936.1 | FJ582092.1 |
| *Chrysis cembricola* (alt. outgroup) | GQ374718.1 | GQ374633.1 |

**Table 2.2. GenBank accession numbers by species**

28S: GenBank accession number for 28S nrDNA fragment sequence; COI: GenBank accession number for COI mtDNA fragment sequence.

*Selection of phylogenetic models*

Phylogenetic reconstruction was performed using both distance-based (bootstrap) and Bayesian approaches. Comparative studies suggest Bayesian approaches are more likely to recreate trees with correct topology, higher support and using fewer characters than distance-based approaches (Alfaro *et al*. 2003). However as a counter-point, there are also data which suggests Bayesian approaches inflate statistical confidence, potentially leading to higher incidence of type I errors (Misawa & Nei 2003). Therefore, to minimise the risk of drawing incorrect conclusions, both methods of analyses were performed. As it was expected that the confidence in posterior probabilities would vary between the two approaches, integrity of results was determined by consistency of topology in generated trees between both approaches.

**Assessing intrinsic colony traits**

Investigating within-colony processes is important for a more complete understanding of factors influencing antimicrobial defences in wasps. For individual species, this may demonstrate specific selective pressures affecting the species, as well as highlight any potential confounding effects when examining selective pressures across species.

*Selection of microsatellite markers*

Microsatellites are genetic markers consisting of short, tandem repeats of nucleotides (1-6 bp) and are found throughout all eukaryote genomes (Tóth *et al*. 2000). These markers are non-coding, mutate rapidly and are assumed to be selectively neutral (Ellegren 2004). These traits make them exceptionally useful for analysing relatedness and gene flow among and within populations (Jarnea & Lagoda 1996). Further, as these loci are often highly polymorphic, they greatly improve the accuracy of parentage analyses compared to older allozyme-based methods (Queller *et al*. 1993). Microsatellites have been used in the study of social insects since the early 1990s (Queller *et al*. 1993), however prior to this study no markers had been developed for this study's model species, *Polistes humilis*. For detailed life-history data on *P. humilis* refer to Appendix B. Given that the *Polistes* genus is widely studied across the world, cross-amplification of markers developed for closely related species was deemed preferable to development of new markers in terms of both time and resource cost. In total, 21 microsatellite markers were trialled which originated from four species of *Polistes*; *P. dominulus* (9 loci, Henshaw 2000), *P. chinensis* (5 loci, Tsuchida *et al.* 2003), *P. bellicosus* (5 loci,

Strassmann *et al.* 1997) and *P. annularis* (2 loci, Strassmann *et al.* 1997). Of these, only six loci were successfully amplified and found to be polymorphic (Table 2.3).

| Locus | Origin | Reference |
|---|---|---|
| Pdom7 | *Polistes dominulus* | Henshaw (2000) |
| Pdom93 | *Polistes dominulus* | Henshaw (2000) |
| Pc68 | *Polistes chinensis* | Tsuchida *et al.* (2003) |
| Pc80 | *Polistes chinensis* | Tsuchida *et al.* (2003) |
| Pbe203AAG | *Polistes bellicosus* | Strassmann *et al.* (1997) |
| Pbe205AAG | *Polistes bellicosus* | Strassmann *et al.* (1997) |

**Table 2.3.** Microsatellite markers successfully cross-amplified with *Polistes humilis*

Microsatellite data were analysed to determine minimum number of contributing parents, effective number of alleles per colony (Nielsen *et al*. 2003) and mean within-colony pairwise relatedness (Queller & Goodnight 1989). As genetic diversity of a colony (reflected in the above measures) is known to affect colonial disease resistance (Baer & Schmid-Hempel 1999), I hypothesised that these data along with colony size would correlate with, and therefore explain, any variation in cuticular antimicrobial strength observed among colonies. As these measures represent different (but related) aspects of colony processes, some degree of correlation between variables was expected. Although had the potential to generate redundancy in our data set, the precise levels of correlation could not be known prior to performing analyses. Therefore, data for all variables was generated and during subsequent exploratory data analyses, correlations between these variables were analyses and redundant variables were removed as appropriate.

*Selection of statistical model*

This study is of nested design as multiple measurements were taken from independent samples (i.e. separate colonies of wasps), creating clusters of uniquely correlated measurements. Two classes of statistical models are normally used to assess nested data: generalised linear and generalised additive mixed models (Zuur *et al.* 2009; Faraway 2006). From these, a generalised estimating equation (GEE) was selected as the most appropriate model as it easily accommodates nested sampling designs with repeated measures, such as those found within this study (Zuur *et al.* 2009; Faraway 2006). As all variables being tested were either measures of genetic diversity of population size, to avoid issues of collinearity (Faraway 2005) models were tested using pairs of genetic and population measures. Quality of models was assessed using quasi-likelihood information criterion (QIC) scores (Pan 2001). This is similar to the more common Akaike's information criterion (AIC) score however the QIC is more appropriate in this case as it takes into account the complexity of the fitted model (Pan 2001).

**Assessing microbial load**

Although investigations into antimicrobial defences may reveal disease-related evolutionary pressures on a species, their practical role is to defend against disease outbreaks. Therefore, I expected colonies with comparatively weak antimicrobial defences to be at greater risk of disease, reflected in a heavier microbial load. To investigate this, microbial richness of individuals whose colonies had previously been assayed for antimicrobial strength were compared and correlated to investigate what effect, if any, variation in antimicrobial strength has on microbial associations.

*Extraction of microbes*

With respect to cuticular washing, both the process of obtaining cuticular compounds and cuticular microbes are effectively destructive sampling techniques, precluding the use of same individual in both studies. As antimicrobial compounds are the focus of this thesis and require a large sample size for accurate results, the numbers of individuals available for this assay was limited.

Following Stow *et al*. (2010), microbes were washed from the cuticle of wasps using KCl solution. Washes were then centrifuged and the supernatant was removed. The microbial pellet was resuspended in KCl w/v 20% glycerol solution and stored at -20°C. This approach preserved viable cells for later culturing without introducing chemicals which may inhibit PCR, allowing for both genetic and traditional microbiological techniques to be performed, maximising available data. As the vast majority of microbes are unculturable (Schloss & Handelsman 2005), it is likely that traditional culture techniques would underestimate the numbers of microbes present. Therefore, microbial species richness was assessed by extracting DNA from microbial washes which was then amplified by two genetic markers (one each for fungi and bacteria). Traditional culturing of microbes served as a technical control whereby the genetic technique would be deemed to lack sufficient sensitivity if it detected less species than could be successfully cultured.

*Choice of genetic markers*

Microbial richness was assessed using two genetic markers; 16S ribosomal RNA (bacteria; Heuer *et al*. 1997) and ribosomal internal transcribed spacer 2 (ITS2) (fungi; Turenne *et al*. 1999). These are standard markers used to assess the presence of microbes with length polymorphisms in these markers serving as unique

identifiers for individual species. This approach does not allow for identification of individual species (no sequence data) or quantification of species abundance; however it does provide a rapid and accurate measure of microbial richness, which can be easily compared among colonies. Homoplasy of amplicon length polymorphisms may underestimate overall species richness using this approach, however this is of more concern for methods which us multiple length polymorphism markers (Caballero *et al*. 2008). Further, as this method does not generate sequence data, nucleotide substitutions among fragments will not be detected. Again while this may underestimate species richness, it has been demonstrated that species within genera can differ by 1–173 bp in fragment lengths (ITS2) and therefore this will more likely result in non-detection of species strains rather than species themselves (Turenne *et al*. 1999).

*Analyses of microbial richness data*

Due to the limited sample size normality of the data could not be accurately assessed and therefore could not be assumed to be normally distributed. As such, the significance of any correlations between microbial load and the strength of antimicrobial compounds within colonies was assessed using a rank-order correlation test. Separate correlations were performed for fungal and bacterial richness data.

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# CHAPTER THREE: SOCIAL COMPLEXITY AND NESTING HABITS ARE FACTORS IN THE EVOLUTION OF ANTIMICROBIAL DEFENCES IN WASPS

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**Abstract**

Microbial diseases are important selective agents in social insects and one major defence mechanism is the secretion of cuticular antimicrobial compounds. We hypothesised that given differences in group size, social complexity, and nest type the secretions of these antimicrobials will be under different selective pressures. To test this we extracted secretions from nine wasp species of varying social complexity and nesting habits and assayed their antimicrobial compounds against cultures of *Staphylococcus aureus*. These data were then combined with phylogenetic data to provide an evolutionary context. Social species showed significantly higher (18x) antimicrobial activity than solitary species and species with paper nests showed significantly higher (11x) antimicrobial activity than those which excavated burrows. Mud-nest species showed no antimicrobial activity. Solitary, burrow-provisioning wasps diverged at more basal nodes of the phylogenetic trees, while social wasps diverged from the most recent nodes. These data suggest that antimicrobial defences may have evolved in response to ground-dwelling pathogens but the most important variable leading to increased antimicrobial strength was increase in group size and social complexity.

**Introduction**

Disease risk in social insects can be influenced by a variety of intrinsic and extrinsic factors. Large group sizes and limited genetic diversity commonly associated with social species are known to be factors [1] and different nest substrates and climates present arrays of pathogens which may compromise a colony. Despite this, social Hymenoptera have successfully established on every continent (except Antarctica) suggesting that they have found one or more strategies to successfully combat

disease. Indeed, multiple disease resistance strategies have been observed in various hymenopteran species including behavioural (e.g. allogrooming, nest cleaning), genetic (e.g. increased diversity) or biochemical (e.g. immune response, antibiotic secretions) [2,3].

Cuticular antimicrobial compounds [1,4,5] are important because they target pathogens before they can infect an individual. Previous studies have shown that antimicrobial activity scales positively with group size in both bees and thrips [1,4] supporting the theory that larger group sizes lead to greater risk of disease. However, in order to establish the generality of these findings, more insect lineages should be examined.

Within the Hymenoptera, wasps are of particular interest as both bees and ants arose from wasp lineages (Apoidea and Vespoidea superfamilies respectively [6]) and examples of all major nesting habits and levels social complexity are found within extant wasp taxa [7]. Additionally, antibacterial peptides have been isolated from the cuticle and venom of social wasp species [5]. Therefore, wasps provide the opportunity to examine the factors affecting disease resistance from some of the most primitive states up until the most recent and complex derivatives.

Given the diversity of wasp taxa we predicted that we would observe a large amount of variation in the relative strength of antimicrobial defences among species, based on life history traits. For this study, we extracted putative antimicrobial compounds from the cuticle of a variety of wasp species which span a range of nesting habits and social complexity. Using an established bioassay [8], the activity of these

compounds was measured and compared. Based on previous studies we expected those species with the greatest group sizes (i.e. the social species) to possess the strongest antimicrobial compounds.

According to published phylogenies [6,9] solitary wasp species are ancestral to social wasp lineages. Individuals of solitary species do not cooperate with one another and aggregations, if any, result from the availability of limited nesting sites [10,11]. While not subjected to the evolutionary pressures of disease arising from sociality, as the ancestral lineage of social species, we predicted that solitary wasps would possess weak cuticular antimicrobial compounds. In contrast, for social species we predicted they would possess strong cuticular antimicrobial compounds used to protect themselves from disease risks associated with large, high-density group sizes. To test this hypothesis, we performed a phylogenetic reconstruction of the examined species as there are no comprehensive phylogenies for Australian vespid or apoid wasps. These data provided our results with an evolutionary context, allowing for inferences to be made regarding the order in which traits evolved or co-evolved.

## Materials and methods

### *Ethics statement*

No animal ethics approval was required for this study however wasps were incapacitated with carbon dioxide during sampling and prior to extraction of cuticular compounds and internal tissue to minimise stress to the animals.

*Sampling locations and species identification*

1268 individual wasps comprising members of nine different species were collected from public land across Sydney (New South Wales) and Alice Springs (Northern Territory), Australia. Species were identified by morphology and sequencing of 28S nrDNA and COI mtDNA fragments (see below). In four wasp species, identification could only be resolved to genus-level due to lack of identification keys for many Australian wasp genera. In one instance, identification could only be resolved to the level of sub-family (designated as 'Pepsinae Sp1'). Species were then categorised by social complexity (social, communal aggregator or solitary) and by nest type (paper nest, mud nest or burrow; Table 3.1), representing broad categories of group size and environmental exposure. Paper nests were defined as clusters of cells constructed from pulp and attached to the substrate by a petiole [7]. Mud nests were defined as sealed and provisioned cells constructed from mud and above-ground [12,13]. Burrows were defined as either provisioned terrestrial burrows [10,14], or as those species which burrowed for prey which was subsequently paralysed and ectoparasitised [15].

| Species (Family) | *n* | Sociality | Nest type | IC50 (±95% CI) | $n_r$ |
|---|---|---|---|---|---|
| *Polistes humilis* (Vespidae) | 1077 (10) | Soc. | Paper | 6.03 (±2.26) | 28 |
| *Ropalidia plebeiana* (Vespidae) | 49 (2) | Soc. | Paper | 7.58 (±5.91) | 5 |
| *Bembix sp.* (Crabronidae) | 83 | Com | Burrow | 31.97 (±27.62) | 6 |
| *Austroscolia sp.* (Scoliidae) | 47 | Sol. | Burrow | 158.27 (±152.82) | 5 (3)* |
| *Cryptocheilus sp.* (Pompilidae) | 4 | Sol. | Burrow | 14.47 | 1 |
| Pepsinae Sp1 (Pompilidae) | 1 | Sol. | Burrow | 90.26 | 1 |
| *Abispa ephippium* (Vespidae) | 1 | Sol. | Mud | No Inhibition | 1 |
| *Sceliphron laetum* (Sphecidae) | 5 | Sol. | Mud | No Inhibition | 2 |
| *Delta sp.* (Vespidae) | 1 | Sol. | Mud | No Inhibition | 1 |

**Table 3.1. Characterisation of wasp species**

*n*: number of individuals (number of colonies for social species); Sociality: social (Soc.), communal aggregator (Com.), solitary (Sol.); IC50: mean equivalent surface area ($mm^2$) of wasp cuticle required to kill or inhibit 50% of *S. aureus* growth; $n_r$: number of replicates per species

* Only three replicates for Austroscolia sp. showed activity over the assayed concentration gradient and the IC50 value given was calculated using only these data.

*Bioassay*

Putative antimicrobial compounds were assayed by established methods to assay antimicrobial compounds obtained from thrips and bees [1,4] and were removed from the cuticle of live wasps by washing whole animals with 70% ethanol for 10 minutes, followed by two rinses to maximise extraction. Solvents were removed by vacuum evaporated at 25°C and the recovered residue was resuspended in LB broth. Extracts were assayed against *Staphylococcus aureus* using opposing gradients of extract concentration and cell numbers across rows of 12 wells in 96-well microtitre plates [8]. As *S. aureus* is exclusively found in birds and mammals [16], it is unlikely that any species of wasp has developed pathogen-specific defensive compounds that would bias our study of broad-scale antimicrobial compounds. Concentration-growth curves were generated for each species with a minimum of five replicates per species, and a minimum of three replicates per colony for social species, when sample size permitted. Three control rows were used in each assay:

LB broth, resuspended extract with LB broth and a gradient of *S. aureus* cell numbers with LB broth. Initially, the maximum concentration of extract used was equivalent to a single wasp, however, preliminary assays indicated this was too low to detect activity in many species. Where additional samples were available the maximum concentration of extract was increased to 2.0 (*Cryptocheilus sp.*, *Sceliphron laetum* and *Austroscolia sp.*) or 4.0 (*Bembix sp.*). Where only a single individual was collected (*Delta sp.*, *Abispa ephippium* and Pepsinae Sp1) the highest concentration of extract was equivalent to 0.5 wasps (i.e. one wasp equivalent which is immediately diluted to 0.5 by the serial dilution process). For social and communal aggregator species washes from between three and eight individuals were pooled and then diluted to the required wasp equivalent concentrations. All assays used a one-half serial dilution for both the *S. aureus* and extract gradients. Following incubation at 37°C for 19 h, growth in treatment and control wells was measured as an increase in optical density (OD) at 590 nm. These data were expressed as [increase in OD of treatment well] / [increase in OD of control well] and then used to determine the concentration of extract required to kill or inhibit 50% of *S. aureus* growth (herein referred to as IC50). A total of 50 assays were performed across the nine species (Table 3.1).

*Calculating relative antimicrobial strength*

Following Smith *et al.* (2008), a modified Gompertz function was fitted to the data using R (version 2.5.1 [17]) to calculate the IC50 value for each assay. Mean IC50 (±95% CI) was calculated for each species and each category of social complexity and nest type. Two-sample t-tests were performed between pairs of social complexity and nest type categories. To standardise measurements across different species, concentrations of wasp equivalents were converted to concentrations of

equivalent surface area. Adapting methods previously applied to bees [1], mean surface area for each species was estimated by generating elliptical cylinders using measurements from up to ten individuals per species. Although regular cylinders would have been appropriate for most species, some of the larger species exhibited up to ~1.5x difference between height and width, rendering regular cylinders an imprecise measure.

*DNA extraction and amplification*

Using one member of each sampled species, DNA was extracted from internal tissues in the thoracic region of wasps using proteinase-K and 'salting-out' [18]. Phylogenetic reconstruction was performed using two gene fragments; 28S nuclear rDNA and COI mitochondrial DNA. Amplification of the 28S gene fragments was performed using primers previously used in the construction of microgastrid wasp phylogenies [19] and COI gene fragments amplifications were performed using generic invertebrate primers for that region [20]. PCRs for both gene fragment were carried out in 10μL volumes containing 0.5U of GoTaq Flexi DNA polymerase (Promega), 1 μM forward primer, 1 μM reverse primer, 0.8 μM DNTPs, 1x GoTaq Buffer (Promega) and 2.0 mM $MgCl_2$. PCR amplifications had an initial denaturation at 94°C for 3 min followed by six 'touch down' cycles of 94°C denaturation for 30 s, annealing temperatures (60°C, 58°C, 56°C, 54°C, 52°C, 50°C) for 30 s and an extension step of 72°C for 45 s. On the completion of the last touchdown cycle, another 35 cycles were carried out at 50°C annealing temperature and a final extension of 10 min at 72°C. Following PCR amplicons were purified using ExoSap-IT (USB) according to the manufacturer instructions and purified products were sequenced using their corresponding forward primers with dye terminator reactions on a 3130x1 Genetic Analyser (Applied Biosystems).

*Sequence alignment*

Phylogenetic reconstruction of nine wasp species (plus *Apis mellifera* as an outgroup) was performed using a 941 bp sequence generated by concatenating the two gene fragments: 28S nrDNA (484 bp) and COI mtDNA (457 bp). Generated sequences (Table 3.2) plus corresponding sequences from *A. mellifera* acquired from GenBank (28S: AJ302936.1; COI: FJ582092.1) were aligned using the ClustalW option with default parameters in MEGA v4.0 [21]. Length polymorphisms in sequence data were removed following alignment and prior to concatenation of gene fragments. Concatenation of the two sequences was deemed appropriate as a partition homogeneity test performed in PAUP* v4.0b10 [22] revealed no significant incongruencies between the two data sets ($p = 0.124$). Phylogenies were created using both distance-based (neighbour-joining) and Bayesian methods (maximum clade credibility).

| Species name | 28S | COI |
|---|---|---|
| *Polistes humilis* | JF510015 | JF510006 |
| *Ropalidia plebeiana* | JF510016 | JF510007 |
| *Bembix sp.* | JF510020 | JF510011 |
| *Austroscolia sp.* | JF510021 | JF510012 |
| *Cryptocheilus sp.* | JF510022 | JF510013 |
| Pepsinae Sp1 | JF510023 | JF510014 |
| *Abispa ephippium* | JF510017 | JF510008 |
| *Sceliphron laetum* | JF510019 | JF510010 |
| *Delta sp.* | JF510018 | JF510009 |

**Table 3.2. GenBank accession numbers by species**

28S: GenBank accession number for the amplified 28S nrDNA fragment sequence; COI: GenBank accession number for the amplified COI mtDNA fragment sequence.

*Distance-based phylogenetic analysis*

Neighbour-joining phylogenetic reconstruction was performed in MEGA v4.0 [21] using the maximum composite likelihood model. Both transition and transversion substitutions were included, assuming homogeneous patterns among lineages and uniform rates among sites. Gaps were treated as complete deletions. Bootstrap values were obtained using 10000 replicates.

*Bayesian phylogenetic analysis*

Bayesian phylogenetic analysis was performed using BEAST v1.5.4 [23]. The input file for BEAST was generated using BEAUti v1.5.4. Trees were generated using a single MCMC chain of 20 million steps sampling every 5000 steps. Generalised time-reversible plus gamma (GTR+G) was selected as the model for nucleotide substitution using jModelTest v0.1.1 [24]. The molecular clock rate was fixed to 1.0 and the Yule process was selected as the tree prior. All other parameters were left in their default state as generated by BEAUti. Integrity of generated data was checked using Tracer v1.5 [25]. TreeAnnotator v1.5.4 (distributed with BEAST v.1.5.4) was used to generate a maximum clade credibility tree (MCC) using a burn-in period of 400 trees and a posterior probability limit of 0.5. The MCC tree was visualised using FigTree v1.3.1 [26].

**Results**

*Antimicrobial activity*

IC50 values could be calculated for 44 of the 50 assays performed (Table 3.1). No antimicrobial activity was observed from species belonging to the 'mud nest' category. Two of the five assays performed using extract obtained from *Austroscolia sp.* also showed no activity hence IC50 values were calculated using

data from the three assays for which IC50 values could be calculated. When grouped by social complexity, mean IC50 values for social species were significantly lower than those of solitary species (Two-sample t-test: social = 6.26, solitary = 115.93; p = 0.038). When grouped by nest type, mean IC50 values for paper nest species were significantly lower than those of burrow species (Two-sample t-test: paper nest = 6.26, burrow = 70.13; p = 0.015). As no species belonging to the 'mud nest' category showed any antimicrobial activity it was not possible to compare mean IC50 values with the other nest types. Similarly these species were not included when calculating differences in mean IC50 by social complexity.

*Phylogenetic reconstruction*

Following the removal of length polymorphisms and concatenation of 28S and COI gene fragments, of the 941 bp sequence, 651 bp were found to be variable of which 346 bp were parsimony informative. Both the neighbour-joining and maximum clade credibility trees were highly congruent, except when placing the clades containing the two pompilid species and *Austroscolia sp.* which were switched between the two trees. In both trees the placement of *Austroscolia sp.* was the least supported branch (bootstrap support of 0.52; credibility support of 0.7853). Additionally, our phylogenetic reconstruction showed that solitary burrowing wasps diverged at basal nodes in both trees and that social lineages arose following a single divergence event (bootstrap support of 0.82; credibility support of 1.0; Figure 3.1).

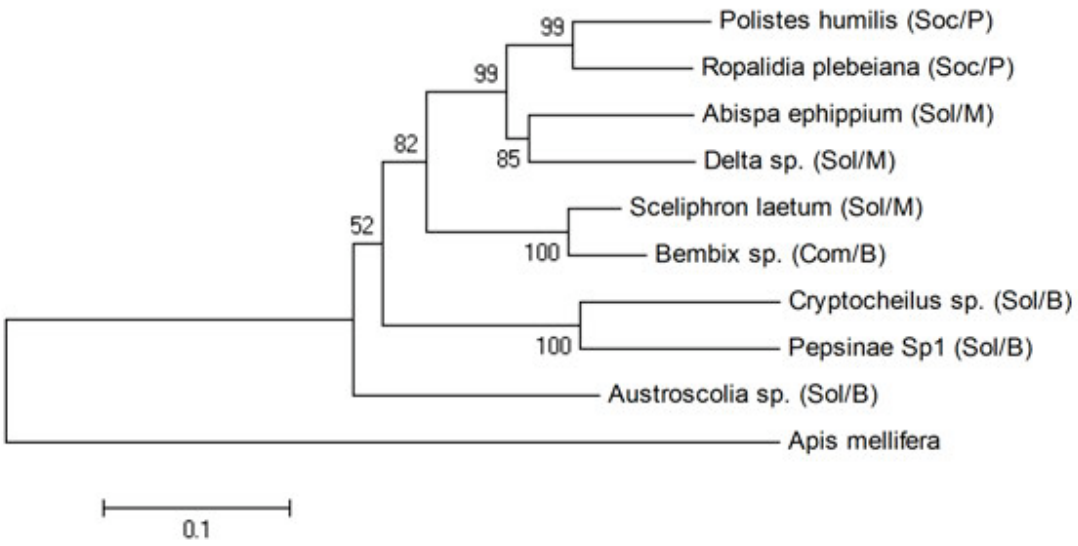


**Figure 3.1. Distance-based neighbour-joining tree**

Neighbour-joining phylogenetic reconstruction of nine wasp species using 941 bp sequence generated by concatenating the two gene fragments: 28S nrDNA (484 bp) and COI mtDNA (457 bp). Social complexity and nest type are indicated after the species names; social (Sol), communal aggregator (Com), solitary (Soc), paper nest (P), mud nest (M) and burrower (B). Bootstrap values were obtained using 10000 replicates.

## Discussion

It is clear from the data that levels of antimicrobial defences vary among different wasp taxa and that these differences are strongly linked to levels of social complexity. When examined together, the phylogenetic and antimicrobial data suggest that the production of antimicrobial compounds may have first arisen in solitary wasps as a response to environmental, probably soil-borne, pathogens.

### *Social complexity*

We observed an eighteen-fold difference in the mean strength of cuticular compounds when comparing social and solitary wasp species. This difference did

not take into account those species for which a mean value could not be calculated, thus the actual difference between the two groups could be much higher. This finding is consistent with previous studies linking increased antimicrobial defences with group size and sociality [1,4]. As wasps are an ancestral lineage in Hymenoptera [6,9] and this pattern has already been demonstrated among bees [1] the relationship may hold throughout the social Hymenoptera.

*Nest type*

Burrowing wasps, exposed to soil-based pathogens, may have developed broad-scale antimicrobial defences in response. These may have evolved into stronger compounds in the social lineages. Perhaps the lack of antimicrobial activity in solitary species that construct mud nests is because they are constructed above-ground where there is less risk of disease. Alternatively, individual species may have developed specific compounds to combat niche pathogens which are ineffective against *S. aureus*. We acknowledge that there may be confounding of results when examining comparisons of sociality and nest types as both 'social' species were also 'paper nest' species. With a sufficiently large sample size it may be possible to separate the effects of each of these traits, however time required to perform a single assay prohibits this.

*Phylogenetic considerations*

Traditionally, wasp lineages have been placed in one of three distinct superfamilies (Vespoidea, Apoidea and Chrysidoidea) [6] however evidence from recent molecular-based phylogenies [9] has cast doubt on traditional taxonomies [9] which may explain some of the incongruencies between the phylogenetic trees presented

in this paper and pre-existing, morphology-based phylogenies [6]. Our placement of species belonging to Vespidae is supported by both traditional taxonomic and more recent molecular phylogenies [6,27], however our placement of *Sceliphron* and *Bembix* (which belong to the Apoidea superfamily) within Vespoidea is more congruent with molecular phylogenies published by Pilgrim *et al.* [9]. This is unsurprising as we did not use morphological data in our phylogenetic reconstruction, however to ensure that this result was not due to outgroup choice, we replicated our phylogenetic analysis replacing *Apis mellifera* (Apoidea) with *Chrysis cembricola* (Chrysididae; 28S: GQ374718.1; COI: GQ374633.1) as an outgroup (Chrysidoidea is a sister taxa to both Apoidea and Vespoidea). This substitution did not change the placement of Apoidea species or branch support for their nodes. We similarly removed *Apis mellifera* without replacement and again, this did not alter the placement of Apoidea species within the trees relative to the other taxa. The only difference observed between any of these trees (excepting minor changes to branch support values) was the placement of *Austroscolia* and Pompilidae taxa, which as previously stated, are the least supported branches in the analysis.

This study provides evidence for the origin of antimicrobial defences in wasps and Hymenoptera as a whole, and increases our understanding of trends in disease resistance strategies in all social insects. Assaying against pathogens for which wasps have no evolutionary relationship revealed those species which have potentially evolved to cope with wide-ranging or rapidly evolving pathogenic threats. The absence of such a response in mud-nest constructing species may be indicate that they are not subject to the same pathogenic evolutionary constraints as

social and ground-dwelling wasp species. Further investigation is required to determine whether these species have lost their antimicrobial defences or evolved specific compounds to cope with a much narrower range of pathogenic threats.

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# CHAPTER FOUR: THE EFFECTIVENESS OF ANTIMICROBIAL DEFENCES DECLINES WITH INCREASING GROUP SIZE AND GENETIC SIMILARITY

The following manuscript is formatted in the style required for submission to *Journal of Zoology*, excepting tables and figures which are labelled sequentially throughout this thesis.

**Abstract**

The secretion of cuticular antimicrobial compounds is an important defensive mechanism for social insects and recent studies have demonstrated their role in the evolution of sociality. However, the factors that might affect their production and effectiveness are not well understood. For example, natural variation in colony size and genetic diversity are predicted to influence susceptibility to disease and, as a consequence, we hypothesised that colonies adjust by varying antimicrobial production. We examined the effects of colony size and genetic diversity on the antimicrobial properties of cuticular compounds in 10 colonies of the Australian paper wasp, *Polistes humilis*. The effectiveness of antimicrobial compounds produced by a colony was found to increase with genetic diversity but decrease with colony size. Further, genetic diversity appears to be the stronger factor in determining effectiveness of antimicrobial compounds and may compensate for larger group sizes. These data highlight a possible trade-off between increasing group size for work efficiency and maximising relatedness for indirect fitness benefits, with disease risk being the underlying driving factor.

**Keywords:** *Polistes*, antimicrobials, microsatellites, sociality, evolution

## Introduction

Recently the production of cuticular antimicrobial compounds has been shown to be an important strategy in social insects as a front-line defence that prevents infection (Turnbull *et al.*, 2010). Current evidence suggests that these compounds may have been present prior to, or to have co-evolved with social species, and were driven by the increasing risk of a disease epidemic with group size (Stow *et al.*, 2007;

Turnbull *et al.*, 2010; Hoggard *et al.*, 2011). Cuticular antimicrobials may counteract the risk of epidemics which might otherwise limit increases to maximum group size. However, this hypothesis has only been investigated by examining trends across species in the context of group sizes and social complexity, and thus far the impact of variation within species has gone unstudied.

In social insects, both high population density and reduced genetic diversity increase the risk of disease outbreaks (Stow *et al.*, 2007). To mitigate this risk, social insects often exhibit high levels of genetic diversity which has been shown to limit disease transmission within colonies (Hughes & Boomsma, 2004; Cremer *et al.*, 2007; Evans & Spivak, 2010). The maximum group size obtained by a species may be dictated by factors that include the degree of genetic variation and other defensive mechanisms such as cuticular antimicrobials. To better understand the evolutionary basis for the range of group sizes exhibited by social insects, we aim to identify any among-colony variation in the strength of antimicrobial compounds and we test hypotheses that might account for any variation observed. First, because cuticular compounds may be metabolically expensive to produce, a facultative relationship may exist between genetic diversity and cuticular antimicrobial compounds whereby production is down-regulated when intrinsic colony disease resistance is high (i.e. high within-colony genetic diversity) and the need for these compounds to compensate may be reduced. Alternatively, we may observe a quantitative effect arising from increasing group size, suggesting that antimicrobial strength constantly increases with group size, and therefore maximum group sizes are being limited by other factors.

Wasps of the genus *Polistes* are a particularly useful model for examining these hypotheses. The genus is defined as primitively eusocial as it exhibits co-operative

brood care and overlapping generations, but has no morphologically distinct castes or strict division of reproductive labours (Reeve, 1991). Lacking a strict social hierarchy, colonies can exhibit a wide variation in colony size, genetic relatedness and number of parents contributing to each generation (Sayama & Takahashi, 2005; Hoggard *et al.*, 2009). They are known to produce antibiotic peptides in both their saliva and venom (Turillazzi *et al.,* 2006).

To test our hypotheses, we collected 10 colonies of the Australian paper wasp (*Polistes humilis*) and examined variation in the number of individuals (colony size), genetic variation and the strength of cuticular antimicrobials. Measures of genetic variation were obtained using microsatellite markers. Cuticular antimicrobial strength was assessed using an established bioassay (Smith *et al*., 2008). These data were gathered to give us a unique opportunity to examine the relationships, if any, between colony size and genetic factors in the production of antimicrobial compounds.

## Materials and methods

*Sampling locations and DNA extraction*

Our 10 *Polistes humilis* colonies contained a total of 1077 adult wasps (Table 4.1) and were collected from two areas in New South Wales, Australia: Macquarie University (-33.7740, 151.1143) and Hornsby (-33.7013, 151.0969). Gender was determined both morphologically (Richards, 1978) and by allele counts (see below). DNA was extracted from internal thoracic tissue of females using proteinase-K and 'salting-out' (Sunnucks & Hales, 1996). Because *Polistes* are haplodiploid, only the diploid females were used for the majority of genetic analyses (see below).

| Colony | n | $n_f$ | $n_m$ | IC50 | $n_{e\ (mean)}$ | $n_{e\ (Pc80)}$ | R |
|---|---|---|---|---|---|---|---|
| *1* | 7 | 4 | 3 | 4.25 | 2.51 | 3.38 | 0.21 |
| *2* | 11 | 3 | 2 | 7.98 | 2.13 | 2.63 | 0.42 |
| *3* | 14 | 3 | 2 | 2.77 | 1.73 | 2.67 | 0.64 |
| *4* | 17 | 4 | 3 | 9.00 | 1.61 | 1.00 | 0.81 |
| *5* | 18 | 6 | 3 | 14.33 | 1.57 | 1.40 | 0.58 |
| *6* | 38 | 7 | 4 | 4.65 | 1.55 | 1.68 | 0.68 |
| *7* | 58 | 6 | 3 | 5.18 | 1.69 | 2.44 | 0.53 |
| *8* | 73 | 8 | 5 | 1.97 | 2.59 | 5.36 | 0.03 |
| *9* | 179 | 11 | 5 | 3.67 | 2.22 | 3.28 | 0.14 |
| *10* | 662 | 10 | 8 | 6.57 | 2.47 | 4.48 | 0.12 |

**Table 4.1. Description of *Polistes* colony traits**

Adult colony size (n); inferred number of mothers (nf); inferred number of fathers (nm); mean antimicrobial strength (IC50); mean effective number of alleles (ne (mean)); effective number of alleles at Pc80 (ne (Pc80)); mean within-colony pairwise relatedness (R)

*PCR amplification*

Adult females were genotyped at six microsatellite loci that were isolated from three other *Polistes* species (*P. dominulus*, *P. chinensis* and *P. bellicosus*; Table 4.2). In colonies containing equal to or less than 25 adult females, all adult females were genotyped. Colonies with more than 25 adult females were randomly sub-sampled down to 25 individuals for genotyping using List Randomizer (www.random.org). A sample of 25 individuals is estimated to contain approximately 90% of the genetic variation within a colony (Hoggard *et al.*, 2009). PCRs were performed in 10 uL volumes containing 0.5U of GoTaq Flexi DNA polymerase (Promega), 12.5 nM forward primer, 0.1 μM reverse primer, 20 nM -29M13 fluorescent tag primer, 0.8 μM DNTPs, 1x GoTaq Buffer (Promega) and 2.0 mM $MgCl_2$. PCR amplifications had an initial denaturation at 94°C for 3 min followed by six 'touch down' cycles of 94°C denaturation for 30 s, annealing temperatures (60°C, 58°C, 56°C, 54°C, 52°C, 50°C) for 30 s and an extension step of 72°C for 45 s. On the

completion of the last touchdown cycle, another 35 cycles were carried out at 50°C annealing temperature and a final extension of 10 min at 72°C. PCR products were electrophoresed using a 3130xl Genetic Analyzer (Applied Biosystems) and scored using the software GeneScan (Applied Biosystems) and checked by eye.

| Locus | Alleles | $H_O$ | $H_E$ | *P* | Reference |
|---|---|---|---|---|---|
| Pdom7 | 8 | 0.571 | 0.681 | 0.596 | Henshaw (2000) |
| Pdom93 | 5 | 0.543 | 0.544 | 1.000 | Henshaw (2000) |
| Pc68 | 6 | 0.529 | 0.533 | 0.995 | Tsuchida *et al.* (2003) |
| Pc80 | 12 | 0.557 | 0.709 | 0.5839 | Tsuchida *et al.* (2003) |
| Pbe203AAG | 4 | 0.329 | 0.281 | 1.000 | Strassmann *et al.* (1997) |
| Pbe205AAG | 7 | 0.586 | 0.567 | 0.997 | Strassmann *et al.* (1997) |

**Table 4.2. Characterisation of microsatellite markers in *Polistes humilis***
Observed heterozygosity ($H_O$); expected heterozygosity ($H_E$), probability of deviation from Hardy-Weinberg equilibrium (*P*).

*Bioassay*

The relative strength of putative antimicrobial compounds were assayed by established methods to assay antimicrobial compounds obtained from thrips and bees (Stow *et al.*, 2007; Turnbull *et al.*, 2010) and were removed from the cuticle of live female wasps by washing whole animals with 70% ethanol for 10 minutes, followed by two rinses to maximise extraction. To control for surface area only females were used (females of *P. humilis* are larger; Richards, 1978) and because males were not relevant to antimicrobial defence of these colonies being (generally) produced only late in the season (Reeve, 1991). Solvents were removed by vacuum evaporation at 25°C and the recovered residues were resuspended in LB broth. Extracts were assayed against *Staphylococcus aureus* using opposing gradients of extract concentration and cell numbers across rows of 12 wells in 96-well microtitre plates (Smith *et al.*, 2008). *S. aureus* has previously been successfully used in

similar assays of cuticular antimicrobial compounds obtained from wasps (Hoggard *et al.*, 2011), bees (Stow *et al.*, 2007) and thrips (Turnbull *et al.*, 2010). Concentration-growth curves were generated for each colony with a minimum of three replicates per colony, when sample size permitted. Three control rows were used in each assay: LB broth, resuspended extract with LB broth and a gradient of *S. aureus* cell numbers with LB broth. The maximum concentration of extract used was equivalent to a single wasp and all assays used a one-half serial dilution for both the *S. aureus* and extract gradients. Following incubation at 37°C for 19 h, growth in treatment and control wells was measured as an increase in optical density (OD) at 590 nm. These data were expressed as [increase in OD of treatment well] / [increase in OD of control well] and then used to determine the concentration of extract required to kill or inhibit 50% of *S. aureus* growth (herein referred to as IC50 – see below). A total of 29 assays were performed across the 10 colonies.

*Calculating relative antimicrobial strength*

Following Smith *et al.* (2008), a modified Gompertz function was fitted to the data using R (version 2.5.1; R Development Core Team, 2010) to calculate IC50 for each assay as a function of equivalent number of wasps. Concentrations of wasp equivalents were converted to concentrations of equivalent surface area and mean surface area was estimated by generating elliptical cylinders from morphological measurements (Hoggard *et al.*, 2011).

*Genetic composition of colonies*

Microsatellite data were analysed to determine minimum number of contributing parents, effective number of alleles per colony (Nielsen *et al*., 2003) and mean within-colony pairwise relatedness (Queller & Goodnight 1989). The minimum number of parents per colony was inferred from groups of full-siblings and half-

siblings calculated by the software COLONY 2.0.0.1 (Jones & Wang, 2010). Error rate for each marker was set at 0.02. Because *Polistes* females are singly mated (Strassmann, 2001; Hoggard *et al*., 2009), each group of full-siblings was interpreted as requiring a unique female to contribute to the colony. Full-sibling groups that were also half-siblings to another full-sibling group were interpreted as having resulted from a single male individual mating with more than one female within a colony. Individuals that which could not be assigned as siblings to any other individuals were not included when calculating numbers of siblings groups to avoid arteficially inflating numbers of parents. The effective number of alleles per locus and colony were calculated using GenAlEx 6.0 (Peakall & Smouse, 2006). Mean pairwise relatedness was calculated using the method of Queller & Goodnight in GenAlEx 6.0 (Peakall & Smouse, 2006).

*Factors influencing antimicrobial strength*

A generalised estimating equation (GEE) was used to assess how population factors (colony size, inferred numbers of male and female parents) and genetic factors (mean effective number of alleles and mean within-colony pairwise relatedness) influence IC50 values. Generalised linear mixed models or generalised additive mixed models are normally used to assess nested data (i.e. multiple measures taken from independent colonies) such as these (Faraway, 2006; Zuur *et al.*, 2009). From these classes of models the generalised estimating equation (GEE) was selected as the most appropriate as it easily accommodates nested sampling designs with repeated measures, such as those found within this study (Hardin & Hilbe 2002; Faraway 2006; Zuur *et al.*, 2009). GEE models were fitted using the *geeglm* function in the *geepack* package (Halekoh *et al.*, 2006) for the R statistical environment (R Development Core Team, 2010). To avoid issues of collinearity

(Faraway, 2005) pairs of population and genetic factors were analysed using the GEE model to determine which, if any, had a significant causal effect on IC50 values. Quality of models was assessed using quasi-likelihood information criterion (QIC) scores (Pan, 2001).

## Results

*Summary statistics of microsatellite data*

Pairs of loci were tested for linkage disequilibrium using the software FSTAT 2.9.3.2 (Goudet, 1995) and found to be segregating independently. There was no evidence for the presence of null alleles as PCR amplification was successful with all DNA samples and no particular loci exhibited significant homozygote excess. To avoid biases from differences in colony size, we tested for Hardy-Weinberg Equilibrium with genotypes from seven randomly selected individuals per colony. Tests were performed using the software GENEPOP 1.2 (Raymond & Rousset, 1995). Table 4.2 shows the numbers of alleles per locus and expected and observed heterozygosity.

*Colony composition*

The number of breeding male and female wasps contributing to a colony ranged from three to eleven and two to eight for females and males respectively. There was large variation in mean IC50 values across colonies (1.97 - 14.33) and mean within-colony pairwise relatedness (0.03 – 0.81). Mean effective number of alleles per colony was found to be strongly driven by the effective number of alleles at a single locus (Pc80) and was analysed as a separate variable (in addition to using mean effective number of alleles – see below). Colony trait data are presented in full in Table 4.1.

*Factors influencing antimicrobial strength*

Initial exploration revealed IC50 data to be log-normally distributed. Log-transformed IC50 values were used in subsequent analyses. Correlation matrices of predictor variables revealed some redundancy in our data set (Table 4.3). There was a strong relationship between the mean number of effective alleles and the effective number of alleles at the locus Pc80 (0.912) and a strong inverse relationship between each of these values and mean pairwise relatedness (-0.942 and -0.918 respectively). Because locus Pc80 was largely driving differences in the mean effective number of alleles, subsequent analysis used only the effective number of alleles at Pc80. Although there was a strong relationship between the effective number of alleles at the Pc80 locus and mean pairwise relatedness, both were used in analyses as they represented two potentially distinct causal factors; genome-wide variation (relatedness) which is known to improve general disease resistance (Cremer *et al.*, 2007) and locus-specific variation (Pc80) which may indicate linkage to functional disease-resistance genes.

| | **n** | **$n_f$** | **$n_m$** | **R** | **$n_{e\ (mean)}$** | **$n_{e\ (Pc80)}$** | **IC50** |
|---|---|---|---|---|---|---|---|
| **n** | 1.000 | | | | | | |
| **$n_f$** | 0.657 | 1.000 | | | | | |
| **$n_m$** | 0.904 | 0.849 | 1.000 | | | | |
| **R** | -0.521 | -0.649 | -0.658 | 1.000 | | | |
| **$n_{e\ (mean)}$** | 0.488 | 0.443 | 0.587 | -0.942 | 1.000 | | |
| **$n_{e\ (Pc80)}$** | 0.513 | 0.504 | 0.624 | -0.918 | 0.912 | 1.000 | |
| **IC50** | 0.035 | -0.150 | -0.070 | 0.339 | -0.311 | -0.448 | 1.000 |

**Table 4.3: Correlation matrix of colony variables**

Adult colony size (n); inferred number of mothers ($n_f$); inferred number of fathers ($n_m$); mean within-colony pairwise relatedness (R); mean effective number of alleles ($n_{e\ (mean)}$); effective number of alleles at Pc80 ($n_{e\ (Pc80)}$); mean antimicrobial strength (IC50)

Following the removal of redundant data, the GEE generated six models correlating pairs of genetic and population variables with log-transformed IC50 values. Of these, two were found to be highly competitive; colony size with effective number of alleles at the Pc80 locus, and colony size with mean pairwise relatedness. Both models revealed significant correlations between their fitted variables and IC50 values and they have equivalent QIC (and the lowest) values indicating they were equally appropriate (Table 4.4). Colony size and mean pairwise relatedness were significantly positively correlated with IC50 in these models, while number of effective alleles at the Pc80 locus was significantly negatively correlated with IC50 values (Table 4.4).

| Model | Pop. variable | Genetic variable | Fitted value (population) | Fitted value (genetic) | Intercept | QIC |
|---|---|---|---|---|---|---|
| *1* | n | $n_{e\ (Pc80)}$ | 0.000607 (***) | -0.154 (***) | 1.0067 (***) | 39.8 |
| *2* | n | R | 0.000478 (***) | 0.599 (**) | 0.3376 (***) | 39.9 |
| *3* | $n_f$ | $n_{e\ (Pc80)}$ | 0.0129 | -0.121 (**) | 0.9047 (***) | 41.8 |
| *4* | $n_f$ | R | 0.0140 | 0.503 | 0.3449 | 41.2 |
| *5* | $n_m$ | $n_{e\ (Pc80)}$ | 0.0682 (*) | -0.164 (***) | 0.8379 (***) | 40.0 |
| *6* | $n_m$ | R | 0.0501 | 0.630 (**) | 0.1848 | 40.5 |

**Table 4.4: Fitted GEE models**

Adult colony size (n); inferred number of mothers ($n_f$); inferred number of fathers ($n_m$); mean within-colony pairwise relatedness (R); effective number of alleles at Pc80 ($n_{e\ (Pc80)}$). Significance levels: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$

## Discussion

We observed significant correlations between antimicrobial defences and both colony size and levels of genetic variation within colonies. These findings give us an important insight into how natural variation in colonies affects production of antimicrobial compounds. To date, studies into antimicrobial production in social

insects have examined trends across species. Here we demonstrate for the first time, the opposing effects of two intra-colony factors on the production of antimicrobial compounds within a single species.

*Factors influencing antimicrobial strength*

While both genetic diversity and group size have significant (but opposing) effects on the effectiveness of antimicrobial compounds, it appears that genetic diversity has the greater influence of the two traits. Comparing the relevant coefficients from the two most competitive models it is clear that moderate increases to genetic diversity (i.e. the recruitment of one or two extra reproductives) may (effectively) compensate for large increases to colony size. From *Model 1* (Table 4.4) we can see that increasing the number of effective alleles at the Pc80 locus by 1.0 increases antimicrobial effectiveness by a magnitude that will be offset only by raising colony size by approximately 254 individuals. Similarly, from *Model 2*, decreasing mean within-colony relatedness from 0.75 to 0.5 would allow for colony size increase of approximately 313 individuals with no loss of antimicrobial strength.

*Maximum colony size threshold*

At first glance these results may appear counter-intuitive as they are opposite to those predicted by our hypotheses. The best explanation for this is that that the original hypotheses underestimated the complexity of the relationships among the factors we were examining. Specifically, they did not account for potential synergistic effects between group size and genetic diversity on either disease risk or defence. Greater genetic diversity is known to improve innate disease resistance in insects (lowering overall disease risk; Cremer *et al.*, 2007; Figure 4.1) but data from

this study suggest they may improve the effectiveness of cuticular antimicrobial defences (see below). This two-fold effect may explain why genetic diversity has a much greater influence on antimicrobial strength within our GEE models. Evidence that genetic diversity may improve antimicrobial compound effectiveness comes from the Pc80 locus as effective number of alleles at this locus was positively correlated with antimicrobial strength. This indicates that the Pc80 locus may be linked to functional genes which control production of antimicrobial compounds, and increased variation at these loci may improve overall effectiveness of these compounds. The observed negative correlation between antimicrobial defences and group size is best explained by shifting sex-ratios during colony expansion. *Polistes* are known to produce antimicrobial compounds in their venom and it is hypothesised that this is can be used to coat and protect other individuals (Turillazzi *et al.,* 2006; Turillazzi, 2006). As only females possess a sting, and therefore venom (Gilboa *et al*., 1977), as colony size increases more males are produced and therefore the ratio of available antimicrobial compounds to colony size decreases. By modelling the relationships between the above traits and disease risk it is possible to predict theoretical thresholds for maximum stable group size, as shown in Figure 4.1. Above these group sizes colony disease defence is exceeded by disease risk, effectively limiting maximum attainable colony size. Disease risk, although not directly tested in this study, is known to increase with increasing population density (Getz & Pickering, 1983). These thresholds can be increased by improving effectiveness of antimicrobial compounds, either through long-term selective pressure (Hoggard *et al*., 2011) or increasing colonial genetic diversity (this study; Figure 4.1).

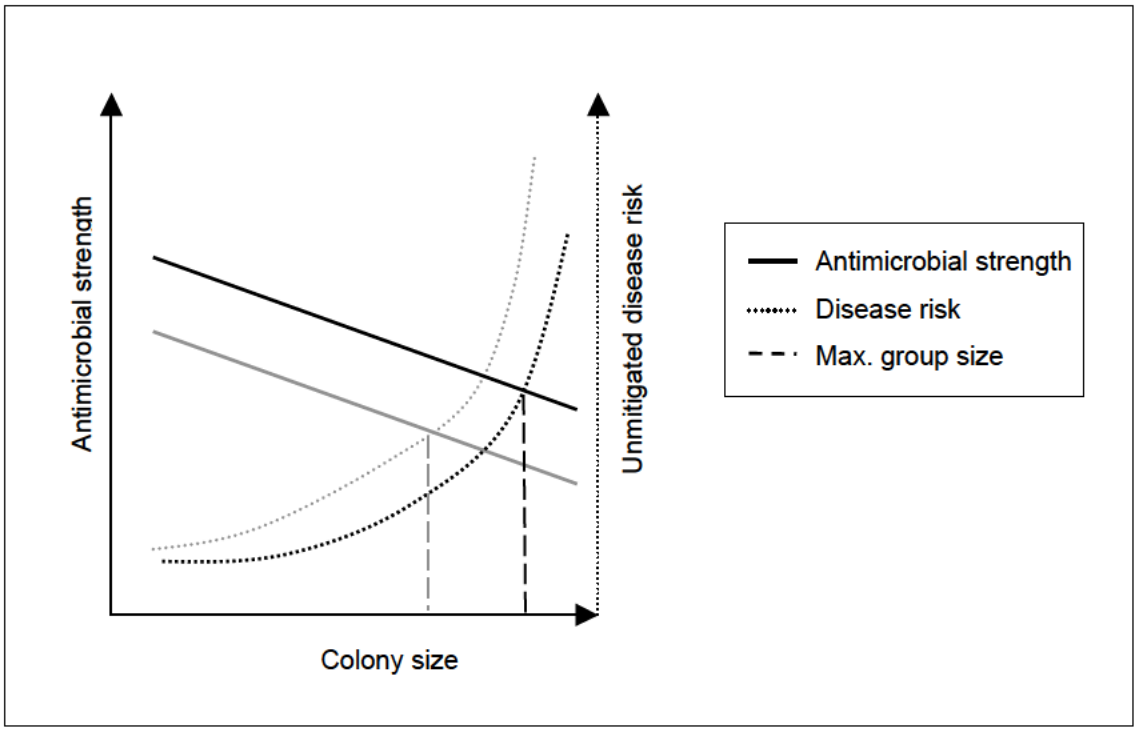


**Figure 4.1: Hypothetical synergistic interactions of disease defence traits**

Black: high genetic diversity scenario; grey: low genetic diversity scenario; max. group size: group size after which disease risk exceeds disease mitigation. It can be seen that genetic diversity both lowers disease risk and improves effectiveness of antimicrobial compounds, raising the threshold for maximum group size.

Overall, our data show that the strength of wasp cuticular antimicrobials is a function of both colony genetic diversity and group size. These findings, along with those of recent related studies (Turnbull *et al.*, 2010; Hoggard *et al.*, 2011) also suggest that disease risk from increasing group size was an important selective pressure driving the evolution of cuticular antimicrobial compounds. The increased protection provided by these compounds then permitted larger maximum attainable group size (increasing colony efficiency and defence: Strassmann *et al.*, 1988; Karsai & Wenzel, 1998) which would otherwise be limited by pathogenic threats. Preliminary evidence in wasps (Hoggard *et al.*, 2011) and thrips (Turnbull *et al.*,

2010) already exists, and may be further investigated by performing similar assays on across social species of the same genus (such as *Polistes*; Reeve, 1991) which show large variation in maximum group.

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# CHAPTER FIVE: MICROBIAL LOAD IN A SOCIAL WASP AND THE IMPLICATIONS FOR THE ROLE OF ANTIMICROBIAL DEFENCES


## Abstract

Cuticular antimicrobial secretions are important defensive compounds which limit microbial infection and transmission among social insects. Recent studies have determined that the effectiveness of these compounds is influenced by both colony size and genetic diversity, however, the real-world effect these compounds have on microbial load has yet to be examined. I explored the hypothesis that variation in the production of antimicrobial microbial compounds will reflect the microbial load, whereby individuals with stronger compounds will have fewer microbial associations. Microbial richness associated with individuals collected from paper wasp colonies (*Polistes humilis*) was assessed using live culture and genetic techniques. These data were then correlated with published data on antimicrobial defences generated from the same colonies. While sample size was low the data were very interesting: There was no evidence of significant bacterial colonisation of the *Polistes* integument, and a very low incidence of fungal colonisation. There was no correlation between microbial load and previously investigated cuticular antimicrobial compounds. This may indicate that selection for stronger antimicrobial compounds provides the broadest possible spectrum of defences, but that maintaining baseline strength is sufficient for the more common pathogenic threats. Behavioural antimicrobial defences such as allogrooming may also act synergistically with compounds to improve overall defences. These findings improve our overall understanding of disease defence in social insects and highlight potentially important avenues for future research.

## Introduction

Cuticular antimicrobial compounds are an important front line defence mechanism in social insects as they restrict microbial growth and thus limit infection (Stow *et al*. 2007). Although our knowledge of the role and evolution of cuticular antimicrobial compounds has steadily increased in recent years (Hoggard *et al*. 2011; Turnbull *et al*. 2010; Stow *et al*. 2007), the real-world effect these compounds have on disease risk in insects has yet to be directly examined.

The relative strength of antimicrobial compounds has been shown to vary greatly among social species, with factors such as maximum group size and nesting habits likely the main influences (Hoggard *et al*. 2011; Turnbull *et al*. 2010). Recently, intra-species variation in antimicrobial strength was observed among different colonies of paper wasps (genus *Polistes*) which was attributed to differences in genetic variation and colony size (Chapter Four). Having identified the source of this variation, the next logical step was to examine what effect, if any, this variation has on the microbial load of social insects. Given that the primary role of these compounds is disease defence, I hypothesised that colonies with less effective antimicrobial compounds will have a heavier microbial load. Unfortunately, data on *Polistes*-specific fungal associations are relatively scarce (Evans 1982; Speare 1920) and more recent studies have reported only larval microbial associations (Fouillaud & Morel 1995; Jeanne & Morgan 1992). There are currently no data available on *Polistes*-specific bacterial interactions. It is not currently known whether this limited knowledge of *Polistes*-associated microbes is indicative of restricted microbial growth in this genus (possibly due to cuticular antimicrobial compounds) or is an artefact of limited scientific inquiry. Further, predicting microbial load for a species is difficult even if data is available for ecologically

similar species; quantitative data available for social termites demonstrates species may range from heavy microbial loads (200 CFUs/cm$^2$; Rosengaus *et al*. 2003) to those which are almost completely clean (Cruse 1998).

As assessing colonies for antimicrobial strength is a resource-intensive process, exploring the viability of related hypotheses using relevant published data sets is preferable as it allows for greater efficiency of investigations. Therefore, as a preliminary investigation of my hypothesis, I quantified bacterial and fungal load (as species richness) of *Polistes humilis* colonies which have been previously assessed for antimicrobial defences and explored these two data sets.

It is known from previous studies that the effectiveness of antimicrobial defences decreases with increasing colony size in *Polistes* (Chapter Four). Further, it is hypothesised that this continued decline creates critical colony size above which antimicrobial defences are insufficient to protect against disease outbreaks, effectively limiting maximum colony sizes. Prior to this point, however, it is unknown how the gradual decline in antimicrobial effectiveness impacts on levels of microbial associations. Assuming no interaction from other disease defence mechanisms, it is likely that microbial load increases as antimicrobial defences decline. External to my hypothesis, these data will improve overall knowledge of *Polistes*; our direct assessment of *Polistes* microbial load, combined with previous incidental commentaries (Evans 1982; Speare 1920), will contribute towards understanding microbial associations with this 'model species' which until now have gone largely unstudied.

## Materials and methods

*Sampling location and methods*

Six *Polistes humilis* colonies were collected from two areas in New South Wales, Australia: Macquarie University (-33.7740, 151.1143) and Hornsby (-33.7013, 151.0969). Wasps were incapacitated using dry ice and collected in sterile containers. Individuals from sampled colonies were also used in simultaneous investigations into the antimicrobial defences in wasps (Chapter Four) and due to the large sample requirements and destructive sampling techniques of these studies, only a single female wasp from each colony was available for these analyses. Because of sexual size dimorphism only females were used for this analysis so that surface areas were more or less standardised (Richards 1978).

Sample size was limited because of factors beyond my control; a major source of frustration for me. The final sampling season, for which the animals for this component of my research were to be collected, was completely disrupted by a four-day heat wave followed by a sudden drop in temperature that resulted in 100% mortality of the colonies marked for collection. This meant that I had to fall back on the residue of specimens remaining from the previous phases of this research.

*Extraction of microbes*

Microbes were washed from the cuticle of wasps using 1.5 mL of 100 mM KCl solution (Stow *et al*. 2010). Washes were then centrifuged and the supernatant was removed. The microbial pellet was resuspended with 100 uL of 100 mM KCl w/v 20% glycerol solution and stored at -20°C.

*DNA extraction*

DNA was extracted from microbial wash solutions using a FastDNA Spin Kit for Soil (MP Biomedicals) following standard protocols with the following exceptions;

780 uL of phosphate buffer was added to the initial sample of 75 uL of microbial wash in Step 2, and Step 7 was repeated to thoroughly remove protein precipitate.

*PCR amplification*

Microbial richness was assessed using two genetic markers; 16S (bacteria; Heuer *et al*. 1997) and ITS2 (fungi; Turenne *et al*. 1999). A 16S PCR screening analysis was performed using universal 16S rRNA primers with a [6-FAM] fluorescent tag attached to the 5' end of the forward primer (Heuer *et al*. 1997). PCRs were performed in 50 μL volumes containing 1x GoTaq Colorless Master Mix (Promega), 0.5 μM forward primer, 0.5 μM reverse primer, 10 μg rNASE and 2.0 mM $MgCl_2$. 5 μL of DNA solution was treated with 5 μL GeneReleaser (BioVentures) following standard protocol prior to the addition of the above reagents in order to maximise template DNA available in the reaction. PCR of ITS2 was performed using universal ITS2 primers with the addition of a [6-FAM] fluorescent tag attached to the 5' end of the forward primer (Turenne *et al*. 1999). PCRs were performed in 10 μL volumes containing 0.5U of GoTaq Flexi DNA polymerase (Promega), 1.0 μM forward primer, 1.0 μM reverse primer, 0.8 μM DNTPs, 1x GoTaq Buffer (Promega) and 2.5 mM $MgCl_2$. PCR amplifications had an initial denaturation at 94°C for 3 min 30 s followed by 36 cycles of 94°C for 30 s, 56°C, 55°C for 30 s and 72°C for 60 s and a final extension step at 72°C for 10 min. PCR product was visualised on a 2% agarose gel prior to being electrophoresed using a 3130xl Genetic Analyzer (Applied Biosystems) and then scored using the software GeneScan (Applied Biosystems) and checked by eye.

*Culturing of cuticular microbes*

Aliquots of 1x, 100x and 1000x dilutions from each sample were plated out onto both LB agar (bacteria) and PDA (fungi) and incubated at 25°C. Plates were regularly checked for growth over a two week period.

*Microbial load and colony traits*

The colonies from which these samples originate were analysed in a related study investigating the effect of colony traits on antimicrobial defences (Chapter Four), therefore data on the corresponding colonies were analysed as part of this study. Published antimicrobial defence data were re-analysed with microbial load data of this study. Due to the sample size, normality of the data could not be accurately assessed and the significance of correlations was assessed using a rank-order correlation test. As unculturable microbes were likely to be present, only data obtained from genetic analysis are used here. Each peak generated by the analyses of the markers was inferred to represent a unique microbial species and separate correlations were performed for fungi (ITS2) and bacterial (16S) loads. Antimicrobial strength data from Chapter Four relevant to the samples analysed in this study are presented in Table 5.1.

**Results**

*Genetic assessment of microbial load*

ITS2 was successfully amplified in three samples, however individually these samples generated very few peaks (Table 5.1). A 405 bp peak was generated using samples from both Colonies 1 and 5. Additionally, Colony 5 produced a peak at 280 bp. Colony 3 produced a single peak at 285 bp. All generated peaks fell within the known size range of ITS2 (Koetschan 2010).

The 16S region was not successfully amplified. Both positive and negative control performed as expected and thus technical issues were eliminated as the cause of this result. However, as no product could be visualised from the primary PCRs we deemed there was insufficient bacterial DNA present in the sample and did not proceed with any further analyses of 16S.

*Microbe cultures*

At the end of the first week an undiluted sample cultured on PDA produced a single fungal colony. This was determined to have originated from the sample for which two peaks were visualised when analysing ITS2 PCR product (see Table 5.1). No growth was recorded on any other PDA plates at any concentration for the other samples. No growth was recorded on any of LB agar plates for any sample at any dilution indicating the absence of viable and culturable bacteria in the samples.

*Microbial load and colony traits*

There was no significant correlation between fungal load and relative antimicrobial strength ($p \gg 0.05$). As 16S could not be amplified, I could not proceed with statistical analyses of bacterial load.

| | **Growth** | | **Peaks** | | **Colony Traits** | |
|---|---|---|---|---|---|---|
| **Colony** | **LB Agar** | **PDA** | **16S** | **ITS2** | ***n*** | **IC50** |
| *1* | - | - | 0 | **1** | 7 | 4.25 |
| *2* | - | - | 0 | 0 | 11 | 7.98 |
| *3* | - | - | 0 | **1** | 17 | 9.00 |
| *4* | - | - | 0 | 0 | 18 | 14.33 |
| *5* | - | + | 0 | **2** | 38 | 4.65 |
| *6* | - | - | 0 | 0 | 179 | 3.67 |

**Table 5.1: Microbial load by colony**

Successful culturing of microbial wash samples on different media (Growth: LB Agar; PDA); numbers of peaks recorded for each genetic marker (Peaks: 16S; ITS2); colony size (*n*) and antimicrobial strength (IC50) of colonies from which samples originate (Chapter Four).

**Discussion**

Overall the results suggest that *Polistes* may have few cuticular microbial associates. Success with amplifying the ITS2 region indicates that the DNA extraction process was successful and suggests that microbes were either absent from the cuticles of these wasps, or that microbial growth was restricted to quantities below which I was able to detect. These data were supported by the live culture component of this study. Although previous studies examining disease resistance in *Polistes* suggest cuticular antimicrobial compounds may prevent microbial colonies from establishing, variance in the strength of these compounds did not appear to affect microbial load across *Polistes* colonies.

*Technical considerations*

At this level of resolution our results for bacterial richness were invariant and I also observed low variation in our assessment of fungal richness. This result could be a sampling error effect but colonies were sampled in two distinct locations and varied considerably with respect to both colony size and relative antimicrobial strength (Table 5.1). Similarly, as fungal and bacterial DNA extractions were performed in the same reaction, and that ITS2 amplification demonstrates that this process was successful, technical error can be similarly excluded as an explanation for these results. As limited sample size is obviously a concern for this study (see Materials and Methods), a larger sample size would be required to fully investigate this finding, however, these results suggests that cuticular microbial growth may be highly restricted in this species, as seen in other social species such as termites (Rosengaus *et al*. 2003; Cruse 1998). If confirmed by future studies, these results have interesting implications for the role of antimicrobial defences of this and other

social insect species. Among the colonies examined in this study, there is up to a four-fold difference in relative antimicrobial strength (Table 5.1) with no observable impact on microbial load. There are two likely explanations for these results. Firstly, wasps may possess more than one disease defence mechanism which may act synergistically to control microbial load. Secondly, strong antimicrobial defences may be selected to provide the broadest possible defence against pathogens (i.e. more virulent pathogens may only be contained by stronger antimicrobials), however maintenance of a baseline strength of antimicrobial compounds is sufficient to control more common pathogens. It is also possible that pathogens were present in quantities too small to detect, but their presence provoked a facultative host response increasing production of antimicrobial compounds to combat the threat of infection.

*Opportunistic pathogens*

It is unlikely that the fungi observed here were mutualistic or symbiotic as to-date there are no known cases of external microbial associations with *Polistes*, and further they were not present on all individuals. Entomopathogenic fungi are known to parasitise *Polistes* species (Evans 1982; Speare 1920), particularly in humid conditions (Gibo 1977), however, studies reporting specific interactions are similarly limited. Conversely, *Polistes* nest material has been shown to host a wide range of fungal species (including entomopathogenic species; Fouillaud & Morel 1995) which suggests that individuals may be constantly exposed to potential pathogens, but actual incidence of infection is low. Therefore, the presence of fungal species with no correlation with antimicrobial defences may be indicative of an ongoing 'arms race' (Roy *et al*. 2006) whereby antimicrobial defences are

generally effective, but are occasionally bypassed by emerging or particularly virulent strains. Overall this suggests that the fungal species observed here are opportunistic (or incidental) and unaffected by the antimicrobial compounds present on the cuticle of these wasps.

*Interactions with other antimicrobial strategies*

Behavioural response to microbes should also be considered when interpreting these results. As with most social insects, *Polistes* engage in allogrooming (O'Donnell 1998) which may work synergistically with antimicrobial compounds; i.e. cuticular antimicrobial compounds restrict the growth of microbes but, if they grow, are then removed by allogrooming. This has also been hypothesised to occur in a eusocial bee species, *Trigona carbonaria* (Stow *et al*. 2010). This provides an alternate explanation as to why there was little genetic evidence of microbes; if microbes were restricted without being removed then we may expect a much higher microbial richness to be recorded. If true, this would alter our conclusions; stronger antimicrobial compounds may be selected to restrict heavier microbial loads until they can be removed by grooming. This may also explain why declining antimicrobial defences did not appear to lead to an increasing in microbial load; reduced chemical antimicrobial defences may be compensated for by an increase in allogrooming behaviour. Testing this hypothesis, however, would require direct manipulation of microbial load and observing subsequent antimicrobial strength and behavioural interactions.

Use of more sensitive DNA extraction techniques may reveal whether cuticular microbial growth in this species is restricted or absent. Combined with a broader

study investigating microbial load and antimicrobial compounds associated with larvae and nesting materials as well as adult individuals, this could greatly improve our knowledge of natural disease risk and mitigation by social species and clarify the role of disease defence strategies in the evolution of sociality. These results provide some new insight into as to how these compounds affect microbial load in the natural environment and thus provide a basis for future research in this area.

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# CHAPTER SIX: CONCLUSIONS

The complexity of social insect societies has intrigued scientists for decades, reaching back to the inception of evolutionary theory itself (Darwin 1859). While there are likely numerous factors which have influenced the evolution of these taxa, those which drove the evolution of social insects' arguably most identifiable characteristic, altruism, have been the focuses of most theoretical social insect research for the last fifty years (e.g. Leadbeater *et al*. 2011: Nowak *et al*. 2010; Boomsma 2009; Hughes *et al*. 2008; Hamilton 1972; Lin & Michener 1972; Hamilton 1964). As altruism in social insects is both ecologically successful and *prima facie* counter-intuitive, it has presented multiple interesting research opportunities over the years, however, this focus on altruism has led to the neglect of traits prior in evolutionary history. Specifically, neglect of those traits which stabilised early aggregations of insects before the evolution of complex social behaviours, such as, but not limited to, altruism.

Group size and within-group relatedness are presumed to increase disease risk in social insects (Stow *et al*. 2007) and both these traits increase with social complexity. Although this is easily apparent in highly social species, their pre-social (i.e. non-solitary) predecessors would have also faced an increased risk of disease as they formed aggregations and group size steadily increased. These early groups may have been quickly selected against unless they possessed some manner of disease resistance to protect them from an increasing likelihood of disease epidemic. In this thesis, I investigated the hypothesis that disease resistance traits, specifically cuticular antimicrobial compounds, evolved either prior to, or co-

evolved with sociality, stabilising increasingly larger and more complex aggregations of insects against disease risk.

**The origin of antimicrobial defences**

Wasps were of great utility for investigating all aspects of my hypothesis. Although bees, like wasps, contain species from solitary to eusocial (Stow *et al*. 2007; O'Donnell 1998), wasps are also ancestral to all social Hymenoptera lineages (Brothers 1999). As such, wasps provided both a wide range of species variables to examine, and allowed for inferences to be made regarding some of the earliest selective pressures which drove the evolution of sociality.

Increasing group size has been a persistent disease risk factor throughout the evolution of all levels of sociality (Chapter Three), however, risks associated with high within-group relatedness are not a concern for solitary or aggregating species as these do not form family groups. For these species I hypothesised that environmental disease risk, particularly choice of nesting substrate, carries a greater risk and therefore acted as a strong, early selective pressure (Chapter Three). Comparison of cuticular antimicrobial strength across a range of species of different levels of social complexity and nesting substrates revealed three interesting patterns. First, social species possessed the strongest antimicrobial compounds, suggesting that group size is a strong selective force. Second, the antimicrobial compounds produced by solitary ground-dwelling species were significantly stronger than their counterparts which create mud-nests on above-ground substrates, indicating that disease risk from nesting substrate is also a significant selective pressure. Finally, phylogenetic reconstruction of the species assayed in this study revealed that the solitary, ground-dwelling species diverged at the most basal nodes of constructed

phylogenies. These data suggest that antimicrobial defences were present prior to the evolution of sociality, and initially arose as a defence against soil-borne microbes in solitary, ground-dwelling wasps. These compounds appear to have increased in strength over time, co-evolving with increasing group sizes in social lineages; where species remained solitary and ceased using terrestrial substrates, these compounds appear to have been lost as I detected no antimicrobial activity in these species. Wasp species representing sister taxa to the bee and ant lineages (Apoidea and Vespiodea, respectively; Brothers 1999) were included in this study and were found to possess antimicrobial compounds. These species were derived from more recent nodes in constructed phylogenies. Therefore it is likely that the origin of antimicrobial defences in wasps is also the origin of antimicrobial defences in bees and ants.

All social species used in this study create above-ground paper nests, thus it remains unknown if nest substrate remains an important factor in disease risk once a species reaches the higher levels of social complexity. Although this information is not necessary for inferences about the origins of antimicrobial defences in social insects, it may be of use in bio-prospecting when attempting to locate the strongest possible natural antimicrobial compounds. All social wasps create their nests from pulp material (Gadagkar 1991; Greene 1991; Matsuura 1991; Reeve 1991; Turillazzi 1991), however, different taxa build nests in different locations; hover wasps (subfamily Stenogastrinae), paper wasps (subfamily Polistinae), some yellowjackets (genus *Dolichovespula*) and true hornets (genus *Vespa*) create aerial nests connected to trees or buildings (Gadagkar 1991; Matsuura 1991; Reeve 1991; Turillazzi 1991), while other yellowjackets (genus *Vespula*) create nests in concealed areas such as within trees or underground (Greene 1991). I hypothesise

that species which create nests underground would have stronger antimicrobial compounds driven by an overall higher microbial load in soil compared to air. Further, as pulp nest material can be easily colonised by microbes (Fouillaud & Morel 1995) it is possible that defensive compounds may be incorporated into nest material. This could be tested by replicating the experiments performed in Chapter Three, comparing antimicrobial strength and nesting substrates of the closely related yellowjacket genera (*Dolichovespula* and *Vespula*). It may also prove interesting to compare the strength of antimicrobial compounds obtained from subterranean *Vespula* colonies, and those constructed in above-ground concealed locations to determine if there is a facultative response to nesting substrates.

**Social disease risk: defence and variability**

Social species exhibit high levels of variation among colonies with respect to group size and genetic diversity, which in turn may lead to variation in disease risk. My original hypotheses predicted that those colonies at greater risk from disease outbreaks (low genetic diversity or large group size) may increase production of antimicrobial compounds in response to the heightened disease risk (Chapter Four). However, correlations of genetic and population traits with the antimicrobial strength of a range of different paper wasp (*Polistes humilis*) colonies revealed the opposite trends; antimicrobial defences increased with increasing genetic diversity, and decreased with increasing colony sizes. Further, microbial load was low and did not reflect variation in antimicrobial strength (Chapter Five).

Genetic diversity and group size were revealed to be significant (but opposing) influences on the strength of antimicrobial compounds, however, genetic diversity

appears to have the stronger effect (Chapter Four). Given this, it is possible that selection may favour recruitment of genetically dissimilar reproductives to colonies so as to increase genetic diversity and thus improve antimicrobial defences. Under inclusive fitness theory, however, this would be selected against as it would also reduce inclusive fitness benefits of altruistic behaviour (Boomsma 2009; Hamilton 1964). In spite of this, unrelated helpers are known in *Polistes* (Queller *et al*. 2000) and it has been suggested that the direct fitness benefits of potentially inheriting a nest are sufficient to outweigh loss of inclusive fitness benefits from lower relatedness (Leadbeater *et al*. 2011). If true, this may also act as a buffer to loss of inclusive fitness benefits as a result of improving antimicrobial defences via genetic diversity: itself another direct fitness benefit. This scenario may also allow for selection of larger group sizes as the higher disease risk they incur may be compensated for by increased antimicrobial and innate (immune) defence arising from greater genetic diversity (Chapter Four; Cremer *et al.* 2007)

This proposed influence of genetic on antimicrobial defences may help to explain observed phenomena in the invasive social wasp populations of North America. *Polistes dominulus* is invasive in North America where it is sympatric with the native *P. fuscatus* (Gamboa *et al*. 2002). Despite having a weaker immune response, lower levels of allogrooming (Wilson-Rich & Starks 2010) and larger colony sizes (Gamboa *et al*. 2002), *P. dominulus* is more ecologically successful (Wilson-Rich & Starks 2010; Gamboa *et al*. 2002). This success is not attributed to direct antagonism between the two species (Gamboa *et al*. 2002), and although enemy-release has been suggested as possible cause (Wilson-Rich & Starks 2010), there have since been documented cases of infection by brood parasites in *P. dominulus* in North America (Madden *et al*. 2010). One hypothesis is that strong

immune responses are unnecessary and thus low immunocompetence has been selected for (Wilson-Rich & Starks 2010). Despite being an invasive species, there is no evidence to suggest that this is the result of a genetic bottleneck; in fact, *P. dominulus* shows unexpectedly high levels of genetic diversity (Johnson & Starks 2004). In light of the findings within my thesis, I hypothesise that the high levels of genetic diversity present in the invasive population of *P. dominulus* have greatly improved the antimicrobial defences in this wasp, reducing the need for higher levels of allogrooming or immunocompetence. Although maintaining all three strategies may maximise protection, a 'trade-off' between disease defence and other metabolically expensive systems may exist, making efficiency of disease defence the more advantageous strategy.

**Research applications**

Overall, the cuticular compounds obtained from social wasps appear to have the strongest antimicrobial qualities (Chapter Three). Their response against an evolutionary novel pathogen in the bioassays is indicative of a broad-spectrum defence (Chapters Three and Four), and generally low microbial load observed on individuals (Chapter Five) provides preliminary support for this. Although larger sample sizes and assays against different pathogens would be required to confirm this, this research indicates that social wasps are a potential source of strong, natural, broad-spectrum antimicrobial compounds. Specifically, for bio-prospecting purposes, social species of wasps which exhibit high levels of genetic diversity (Chapter Four) may be of particular value. Pending further investigation, nesting substrate (particularly subterranean) may also prove to be an ecological variable of interest. Finally, an alterative approach to isolating the compounds observed on *P.*

*humilis* during this research may be available given the observed correlation between genetic diversity at the Pc80 locus and antimicrobial strength (Chapter Four). While I do not suggest that the Pc80 locus itself codes for functional genetic information, this correlation suggests that the genes which code for antimicrobial compounds in this species may be linked to the Pc80 locus. As an alternative to biochemical isolation and identification of these antimicrobial compounds, exploration of the *P. humilis* genome surrounding the Pc80 locus may reveal loci responsible for their production. Similar discoveries have been made linking specific genes (*Relish*) to antimicrobial production the honey bee (*Apis mellifera*; Schlüns & Crozier 2007) and several species of ant (Schlüns & Crozier 2009). This may present an easier method of identification for these compounds, but may also allow for their production in transgenic organisms at a later stage.

**Conclusion**

The findings within this thesis demonstrate both qualitative and quantitatively, the influences of disease risk on the evolution of social insects. Cuticular antimicrobial compounds likely arose in a solitary wasp as a response to soil-borne pathogens, and were conserved throughout the evolution of the social Hymenoptera. The effectiveness of these compounds increased with group size, appearing strongest in social species with large group sizes. In social species, antimicrobial compounds appear to provide broad-spectrum microbial defence which improves with genetic diversity and is weakened by increasing group size. This apparent 'trade-off' between pathogen defence and colony efficiency, arising from larger group size, suggests that disease risk has remained a strong selective pressure throughout the evolution of sociality in these insects.

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# APPENDIX A: ANTIMICROBIAL STRENGTH INCREASES WITH GROUP SIZE: IMPLICATIONS FOR SOCIAL EVOLUTION

The following manuscript was published in *Biology Letters*. It is presented in the format required for publication in said journal, excepting tables and figures which are labelled sequentially throughout this thesis. Full citation for this paper is as follows:

Turnbull, C., **Hoggard, S.**, Gillings, M., Palmer, C., Stow, A., Beattie, D., Briscoe, D., Smith, S., Wilson, P., & Beattie, A. (2010). Antimicrobial strength increases with group size: Implications for social evolution. *Biology Letters* doi:10.1098/rsbl.2010.0719

**Summary**

We hypothesize that aggregations of animals are likely to attract pathogenic microorganisms and that this is especially the case for semi-social and eusocial insects where selection ultimately led to group sizes in the thousands or even millions, attracting the epithet 'superorganism'. Here we analyze antimicrobial strength, per individual, in eight thrips species (Insecta: Thysanoptera) that present increasing innate group sizes and show that species with the largest (100-700) had the strongest antimicrobials, those with smaller groups (10-80) had lower antimicrobial activity, while solitary species showed none. Species with large innate group sizes always showed strong antimicrobial activity while the sub-social species showed no activity until group size increased sufficiently to make activity detectable. The eusocial species behaved in a similar way with detectable activity appearing once group size exceeded 120. These analyses show that antimicrobial strength is determined by innate group size. This suggests that the evolution of sociality which, by definition, increases group size, may have had particular requirements for defences against microbial pathogens. Thus, increase in group size, accompanied by increased antibiotic strength, may have been a critical factor determining the 'point of no return', early in the evolution of social insects, beyond which the evolution of social anatomical and morphological traits was irreversible. Our data suggest that traits that increase group size in general are accompanied by increased antimicrobial strength and that this was critical to transitions from solitary to social and eusocial organisation.

**Keywords:** antimicrobial, evolution, sociality, thrip

**Short Title:** Antibiotic production and sociality

## Introduction

When animals live together in increasing numbers and proximity it is likely that they provide increasingly attractive substrates for pathogenic microorganisms. This would seem to be the case especially for social animals such as social insects. Indeed, since Darwin wrote that the existence of social insects was his "one special difficulty", potentially fatal to his theory, the scientific literature has addressed a variety of explanations for their evolution [1-3]. There were probably many factors that contributed to it [4], however, because eusocial insect colonies are comprised of relatively closely related individuals that co-occur in high densities, they represent ideal environments for pathogenic microorganisms and are highly vulnerable to microbial attack [5,6]. Thus, entomopathogens may be strong selective agents wherever group size increases and in response, effective front line antimicrobials may have been selected for, especially in social insects. Our previous research showed that the strength of antimicrobials in bees increased with the level of sociality [6]. However, the increase was non-linear, with the greatest increment being between the solitary and semi-social species, that is, among the smallest group sizes, strongly suggesting that the very emergence of social traits required defenses against microbial pathogens[6]. Thrips provide an ideal group to investigate this transition further.

Thrips belong to the insect Order Thysanoptera which includes 5500 species globally with many more awaiting description[7]. They include species that are solitary, many of them living in flowers and some well known because they are horticultural pests. Others exhibit a range of group sizes, frequently through the construction of more or less enclosed domiciles made of leaves or other plant structures. A further assemblage of species induces a variety of plant galls,

frequently on *Acacia* phyllodes, often inhabited by several hundred tightly packed individuals. Some of these colonies are not eusocial, consisting of a single female and one generation of her offspring. Other species show eusocial traits, especially the presence of a soldier caste[8,9].

## Material and methods

We examined 8 species and the group sizes shown (*group size* $\bar{x} \pm SE$) were those from freshly collected specimens: The non-eusocial gall makers*, Kladothrips arotrum (385±70, n=8),* and *Kladothrips antennatus (130±9, n=15)* were species where the group consists of a single female with large numbers of her offspring, there being no castes or other eusocial traits. In sociobiological terminology such single females surrounded by hundreds of offspring are regarded technically as 'solitary' but here our interest is in the group size. *Dunatothrips vestitor* forms loose associations of individuals within 'domiciles' formed when the animal pulls leaves (phyllodes) together with silk for shelter *(46±7, n=9)*. These four species, plus the solitary *Haplothrips froggatti (1)*, were all collected from the same habitats near Alice Springs, Northern Territory, Australia. The eusocial gall-maker *Kladothrips intermedius (146±6, n=15)* was collected from near Tomingley, New South Wales, Australia. All these sites, selected to minimize as many variables as possible, were characterized by semi-arid, shrub-dominated vegetation. The activity of both *Teuchothrips ater (23±4, n=20)* and *Klambothrips oleariae (45±8, n=9)* deforms leaves which curl or roll to form a tube that may or may not be closed. These species, which form small aggregations, were collected from the Canberra area. The solitary *Odontothripiella aloba (1)* was collected from coastal sclerophyll near Sydney (Table A.1). In summary, the provenance of the groups was: large, non-eusocial groups living within galls, large, eusocial groups within galls, smaller aggregations within leaf domiciles and, finally, solitary animals. These groups then fell within one of three innate group size classes: hundreds, tens or solitary. These classes were derived from both our observations and the literature (see Table A.1 and references therein).

There were no significant differences in the size of individuals among species.

Cuticular antimicrobials were obtained from living animals and bioassays were carried out according to established methods[5,6,10]. Thrips were washed in 90% ethanol for 5minutes followed by

3 rinses to ensure maximum extraction. The solvent was then removed by rotary evaporator and the residual extract re-suspended in LB broth. Assays against *Staphyloccocus aureus* were performed using opposing gradients of microbial extract concentration and bacterial cell numbers across rows of 12 wells in 96-well microtitre plates. This produced concentration-growth response curves for each species. There were 3 controls: broth alone, *S. aureus* with broth and extract with broth. In all cases but one the greatest concentration of thrip extract used was equivalent to 80 thrips with more than 320 insects being used to produce a ¾ dilution series and 80 thrip equivalents within the first well, 60 in the second and so on. For *Odontothripella aloba* the concentration of thrip extract used was equivalent to 150 thrips using a ½ dilution series. Following incubation at 37°C for 18 hours, growth in the treatments and controls was measured as increase in optical density (OD) and expressed as: increase in OD test well/increase in OD of *S. aureus* control. We performed additional tests for species that showed no activity in the dilution series: In the solitary species, and *D. vestitor*, which showed no activity, additional wells containing artificial aggregations of 150 individuals (n=5) were tested for activity. In the eusocial *K. intermedius,* the additional wells contained 120 individuals (n=5).

| **Species** | **Group Sizes** | | | **GPS** | **References** |
|---|---|---|---|---|---|
| | Mean/SE | Maximum | Innate | | |
| *Kladothrips arotrum* | 385±70 | 770 | 100s | 23º42′26″S 133º50′11″E | 750 adults + 500 juveniles[14]; 433[12] |
| *K. antennatus* | 130±9 | 176 | 100s | Same as *K.arotrum* | 313[12] |
| *K. intermedius* | 146±6 | 190 | 100s | 32º45′06″S 148º22′30″E | 57±1.74 - 101±20[14] |
| *Teuchothrips ater* | 23±4 | 50 | 10s | 33º44′28″S 151º02′11″E | L.A. Mound (pers.comm.) |
| *Klambothrips oleariae* | 45±8 | 80 | 10s | 35º27′39″S 149º00′59″E | L.A. Mound (pers. comm.) |
| *Dunatothrips vestitor* | 46±7 | 98 | 10s | 23º45′59″S 133º52′36″E | 21, 50[15] |
| *Haplothrips froggatti* | n/a | 1 | 1 | 23º42′19″S 133º53′19″E | n/a |
| *Odontothripiella aloba* | n/a | 1 | 1 | 33º39′12″S 151º13′27″E | n/a |

**Table A.1. Group sizes and sampling locations**

**Results**

*Staphylococcus aureus* was most strongly inhibited by *Kladothrips arotrum* and *K. antennatus* (Figure A.1A), the species with the highest innate group sizes. Extracts from 20 individuals inhibited bacterial growth. Gall controls showed no activity. The leaf-rollers *Teuchothrips ater* and *Klambothrips oleariae,* with smaller group sizes, showed only weak antimicrobial activity, and then only at the highest aggregations (Figure A.1B). The leaf-spinner *Dunatothrips vestitor*, and the solitary species *Odontothripiella aloba* and *Haplothrips froggatti* showed no activity at all (Figure A.1C) even when artificial concentrations of 150 individuals (n=5) were assayed separately. The eusocial gall-maker, *Kladothrips intermedius,* also showed no activity (Figure A.1D) but, in additional assay wells containing extract from 120 individuals (n=5), representing the largest galls, there was some activity: a decline of 20% in bacterial growth (not shown in Figure A.1D as individual wells rather than dilution series were used, see methods).

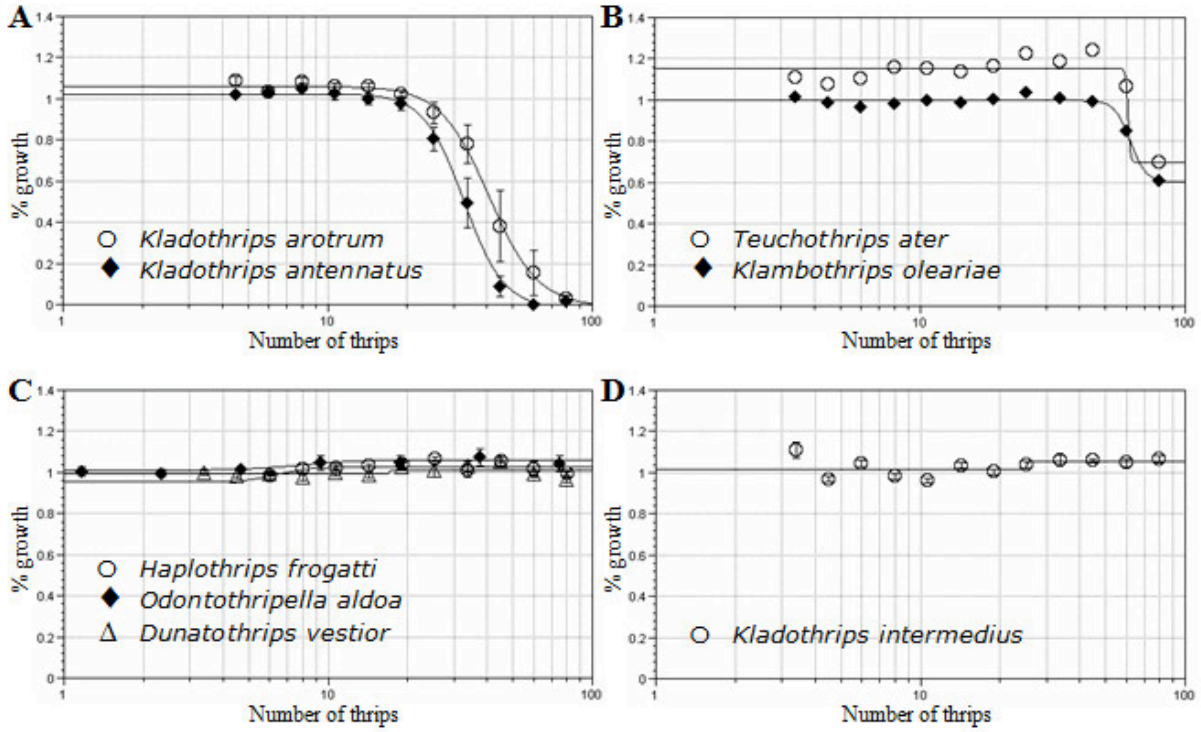


**Figure A.1. Concentration-growth response curves showing activity of extracts from 8 thrips species against *Staphylococcus aureus*.**

x: number of thrips, y: ratio of test well to control well growth. The highest extract concentration was equivalent to 80 individuals with a ¾ dilution series, except for *Odontothripella aloba* where the highest concentration was equivalent to 150 individuals with a ½ dilution series. Following incubation at 37°C for 18 hours, growth measured as increase in optical density (OD) and expressed as: increased OD test well/increased OD *S. aureus* control. The curves were compared by functional analysis of variance [16] and differed significantly between group size classes ($F = 55.108$. $df = 2$. $P < 0.005$).

## Discussion

Our data show that the innate group size of these species is a predictor of its antimicrobial strength. Thus, when it was large, as in *K. arotrum* and *K. antennatus,* antimicrobials were strongest. This may be especially important when within-colony genetic diversity is low, as in these species[11]. For species with smaller innate

group sizes, as in the leaf-rollers, antimicrobial activity was detectable only in the largest groups, suggesting a threshold for activity for any species where group size increases beyond a critical level.

The same applied in the eusocial *K. intermedius* which showed no activity until group size approached 120. This apparent threshold was unexpected as, in a previous study, eusocial bees showed very strong activity[6]. However, while it has a soldier caste, placing it squarely in the eusocial category[8], *K. intermedius* exhibits intermediate group size with small brood numbers, small galls and small numbers of soldiers[9,12,13] and therefore possesses modest antimicrobial strength, appropriate to its group size class. Group size probably set this species apart from other strongly eusocial insect species such as the stingless bees *Trigona* whose colonies contain thousands of individuals and secrete very strong antimicrobials[6].

Our data show that once group size reaches a critical point, there is powerful selection for an increase in the strength of antimicrobials. We see this especially in *K. arotrum* and *K. antennatus* but also in *K. oleariae* and *T. ater.* In this context, it is interesting to consider the case for species with eusocial traits which, by definition, drive increase in group size. The results for the eusocial *K. intermedius* are consistent with the others as antimicrobial activity increases at larger group sizes. Together, the three *Kladothrips* species represent a range of group size within a single genus and demonstrate evolutionary lability for antimicrobial defence, militating against phylogenetic constraints.

Wilson[3] presented evidence that social insects descended from species adapted to well-protected microhabitats that could be defended against enemies, especially

predators, parasites and competitors. We note that this list did not include microbial pathogens, which is ironical in this context, as they may have been the most important and ubiquitous class of enemy and one that could take advantage of the very defences assembled against all the other types of enemy. Our experimental data may shed light on the early stages of such social evolution, suggesting that species evolving social or eusocial traits may have experienced group sizes at which pathogenic microorganisms were the most important selective agents. Further, strong antimicrobials may have been necessary to traverse the 'point of no return' early in social evolution, begging the question as to how far it would have got in their absence.


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## APPENDIX B: MATING SYSTEM AND GENETIC STRUCTURE IN THE PAPER WASP (*Polistes humilis*)

The following manuscript was published in *Australian Journal of Zoology*. It is presented in the format required for publication in said journal, excepting tables and figures which are labelled sequentially throughout this thesis. Full citation for this paper is as follows:

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**Abstract**

We provide the first description of the mating system, genetic structuring and dispersal in the Australian paper wasp, *Polistes humilis.* Individuals were collected from 13 colonies which were within 700 metres of each other at a location near Sydney, Australia. Analysis of genotypic data from four microsatellite loci provided no evidence of males siring offspring in their natal colony and heterozygote excesses within most colonies suggest that this form of outbreeding is typical in *P. humilis*. The same data show that queens are singly mated and that the number of queens and therefore groups of full siblings increase with colony size. Consequently, genetic variation also increases with colony size. The relationship between the number of queens and colony size in *P. humilis* may be indicative of a breakdown in dominance hierarchies in larger colonies or a defence against disease transmission.

**Introduction**

The genus *Polistes* consists of over 200 species and is widely distributed in a variety of environments (Reeve 1991). Studies of the genus have made important contributions to the understanding of the early evolution of sociality as it is considered primitively social since it does not exhibit the complex caste differentiation or strict division of labour found in other eusocial Hymenoptera (Sayama and Takahashi 2005; Tibbetts 2007). Many aspects of *Polistes* biology have been investigated by observational and behavioural studies but it has been difficult to assess mating frequency or parentage from them (Reeve 1991). More recently, genetic studies have overcome such deficiencies, particularly in the study of social insects where microsatellite markers have been deployed to determine

colony relatedness, maternity of broods and levels of genetic variation (Queller and Goodnight 1989; Queller *et al* 1993; Peters *et al* 1995).

Here we examine *Polistes humilis*, a paper wasp endemic to Australia (and invasive in New Zealand; Clapperton *et al*. 1996) which is distributed from southern Queensland, through New South Wales to Victoria and west to South Australia (Richards 1978). In *P. humilis*, as in other *Polistes* species, there is no observable morphological differentiation between reproductive and sterile castes (Reeve 1991). However, little else is known of its reproductive behaviour. Here we examine the mating system and dispersal of *P. humilis* using molecular methods.

**Materials and methods**

*Sampling*

Thirteen colonies of *P. humilis* were collected from Macquarie University campus near Sydney, Australia (S 33'46'26 E 151'06'). Collections were carried out between February and April 2007 at temperatures below 15ºC or during periods of precipitation when individuals do not leave the colony to forage. The location for each colony was recorded by GPS (GARMIN) and the minimum and maximum pairwise distances between colonies was 0.3 meters and 700 meters respectively. The number of individuals within colonies ranged from 4 to 95 with a total of 245 adult and 179 immature wasps collected. Individuals were placed in 90% ethanol soon after removal from their colonies.

*Species identification*

Wasps were initially identified as *P. humilis* by morphological examination. Males were identified by yellow markings on the mesoternal area (Richards 1978). DNA was extracted from internal tissues in the thoracic region of adult wasps and from internal tissue of immature individuals using proteinase-K and salting-out (Sunnucks and Hales 1996). CO1 gene fragments were amplified using standard procedures (Folmer *et al.* 1994) and amplicons purified using ExoSap-IT (USB) according to the manufacturer instructions. Purified products from Polymerase Chain Reaction (PCR) were sequenced using the original PCR primers with dye terminator reactions on a 3130x1 Genetic Analyser (Applied Biosystems). Wasps were confirmed as *P. humilis* by comparison to known *P. humilis* CO1 gene sequences in Genbank. The sex of individuals was confirmed by microsatellite allele counts (see below). Because *Polistes* are haplodiploid, individuals heterozygous for at least one locus were recorded as female (see Table B.1). The chance of error in gender allocation was calculated using allele frequencies, comparing expected and observed numbers of females which were homozygous for all loci.

*Genotyping*

Wasps were genotyped at four microsatellite loci (see Table B.1). PCRs were performed in 10 μL volumes containing 0.5 U of *Taq* DNA polymerase (Promega), 1.25 μM forward primer, 10 μM reverse primer, 0.4 μM -29 M13 fluorescent tag primer, 8μM DNTPs, 1x *Taq* Buffer (Promega) and 2.0 mM $MgCl_2$. PCR amplifications had an initial denaturation at 94 ºC for three minutes followed by six 'touch down' cycles of 94 ºC denaturation for 30 seconds, annealing temperatures

(60 ºC, 58 ºC, 56 ºC, 54 ºC, 52 ºC, 50 ºC) for 30 seconds and an extension step of 72 ºC for 45 seconds. On the completion of the last touchdown cycle, another 35 cycles were carried out at 50 ºC annealing temperature and a final extension of 10 minutes at 72 ºC. PCR products were electrophoresed using a 3130xl Genetic Analyzer (Applied Biosystems) and allele sizes were scored using the software GeneScan (Applied Biosystems) and checked by eye.

| Locus | Repeat | Alleles | $H_O$ | $H_E$ | *P* | Reference |
|---|---|---|---|---|---|---|
| Pdom7 | $(AAG)CAG(AAG)_9$ | 10 | 0.668 | 0.798 | 0.080 | Henshaw 2000 |
| Pc68 | $(CT)_{13}$ | 4 | 0.487 | 0.635 | 0.057 | Tsuchida *et al*. 2003 |
| Pc80 | $(CT)_{32}$ | 11 | 0.587 | 0.651 | 0.001 | Tsuchida *et al*. 2003 |
| Pdom93 | $(AAG)_2ACG(AAG)_2$ $ACG(AAG)_5$ | 3 | 0.463 | 0.504 | 0.000 | Henshaw 2000 |

**Table B.1: Characteristics of the microsatellite markers in *Polistes humilis* including observed heterozygosity ($H_O$), expected heterozygosity ($H_E$) and probability of deviation from Hardy-Weinberg Equilibrium (*P*).**

Allele frequencies used in calculations were generated from all sampled adult females using GENEPOP 1.2 (Raymond and Rousset 1995). The homozygote excess evident for each locus is likely to be the result of a spatial Wahlund effect because samples were obtained from groups of highly related individuals.

*Mating system*

To examine the mating system, all individuals from five colonies were genotyped. These colonies were selected to provide a gradient of colony sizes from 10 to 50 individuals to assess whether the number of queens per colony varied with colony size. To provide additional data on the mating system, we genotyped all adult females in a further eight colonies which we selected because they contained at least four adult females. In each of these 13 colonies the presence of multiple queens was inferred by identifying groups of full siblings (Table B.2). Because the alleles that

were shared among full siblings were used to infer the parent genotypes the probability of a falsely-deduced homozygous parental genotype from four offspring is approximately $0.5^4$ (0.06). *Polistes* queens are singly mated (Strassmann 2001) therefore full siblings will share the paternal allele and inherit either one of the maternal alleles. Our method is conservative as it may underestimate the number of full sibling groups in cases where a male has sired offspring with more than one queen. The likelihood of underestimating queen number is influenced by the genetic distinctiveness of the queen. To examine this, we calculated the probability of two unrelated individuals and of two full siblings sharing the same genotype at each of the four microsatellite loci using the program CERVUS 3.0 (Kalinowski *et al*. 2007). The chance that two unrelated individuals and two full siblings are genetically identical in our data set is low (0.0004 and 0.041 respectively).

*Dispersal*

In each of the five colonies that were exhaustively sampled we examined for the presence of the sire by matching each male genotype with the paternal alleles that were identified in the groups of full siblings. The role of dispersal in the mating system was further assessed by calculating the inbreeding coefficient ($F_{IS}$) for each of the 13 colonies. $F_{IS}$ at the colony level was calculated using allele frequencies generated from all adult females with the software FSTAT 2.9.3.2 (Goudet 1995).

**Results**

*Gender assignment*

Overall 144 adults and 23 immature individuals were scored as female. The individuals scored morphologically as female but homozygous across all four loci

was not significantly different to the number predicted by the allele frequencies ($\chi^2$; Obs. = 2; Exp. = 1.3; $P$ » 0.05). There were no individuals morphologically assigned as male that were heterozygous at any locus, indicating that diploid males were not present. Consequently, the sexes assigned to individuals were considered accurate.

*Summary statistics for microsatellite data*

Microsatellite loci were tested for linkage disequilibrium using the software FSTAT 2.9.3.2 (Goudet 1995) and were found to be segregating independently. We found no evidence for null alleles, PCR amplification was achieved with all DNA samples and there was no particular loci exhibiting an unexpected homozygote excess. Details of the microsatellite repeat type and number of alleles per locus are presented in Table B.1.

*Multiple queens in colonies of P. humilis*

With a single reproductive pair, a maximum of three alleles per locus were expected among female progeny, and two among male progeny. Our data shows that within colonies, up to seven alleles were found at a single locus. The number of full sibling groups detected within a colony ranged between one and six, with most having at least two. A single full sibling group was observed in only one colony (Table B.2).

| Colony | *n* | *Full-sib* | *Pdom7* | *Pc68* | *Pc80* | *Pdom93* | $F_{IS}$ | *p* |
|---|---|---|---|---|---|---|---|---|
| *1* | 8 | 2 | 3 | 2 | 2 | 2 | -0.052 | 0.358 |
| *2* | 7 | 3 | 4 | 2 | 5 | 3 | 0.143 | 0.895 |
| *3* | 17 | 6 | 6 | 2 | 4 | 3 | 0.245 | 0.991 |
| *4* | 35 | 5 | 5 | 1 | 5 | 4 | -0.212 | 0.001 |
| *5* | 8 | 3 | 3 | 3 | 5 | 2 | -0.244 | 0.021 |
| *6* | 6 | 2 | 3 | 2 | 2 | 2 | -0.262 | 0.184 |
| *7* | 11 | 2 | 3 | 2 | 3 | 2 | -0.545 | 0.000 |
| *8* | 9 | 1 | 3 | 2 | 5 | 2 | 0.053 | 0.560 |
| *9* | 5 | 2 | 2 | 3 | 3 | 3 | -0.179 | 0.172 |
| *10* | 5 | 2 | 3 | 2 | 4 | 2 | -0.318 | 0.064 |
| *11* | 77 | 5 | 7 | 3 | 5 | 3 | -0.334 | 0.000 |
| *12* | 4 | 2 | 2 | 2 | 2 | 2 | 0.125 | 0.525 |
| *13* | 19 | 4 | 5 | 3 | 6 | 3 | 0.070 | 0.784 |

**Table B.2: Summary statistics for colonies of *Polistes humilis***

Within each of the 13 colonies analysed we provide the adult colony size (*n*), number of full sibling groups (*Full-sib***)**, number of alleles at each locus, the inbreeding coefficient ($F_{IS}$) and probability of deviation from Hardy-Weinberg (*P*). Allele frequencies used in calculations were generated from all adult female samples using GENEPOP 1.2 (Raymond and Rousset 1995).

*Number of queens and genetic variation increases with respect to colony size*

The minimum number of full sibling groups per colony increased with colony size (Spearman-Rank, $P = 0.0086$; Fig B.1). Correspondingly, the number of alleles present within a colony increased with colony size (Spearman-Rank, $P = 0.0015$). Parental genotypes were unique to each full sibling group which suggests that queens mate once and that males mate with a single queen within a colony. Comparison of queen genotypes shows that reproductive females within colonies are not full siblings because they did not have at least one allele in common at each locus.

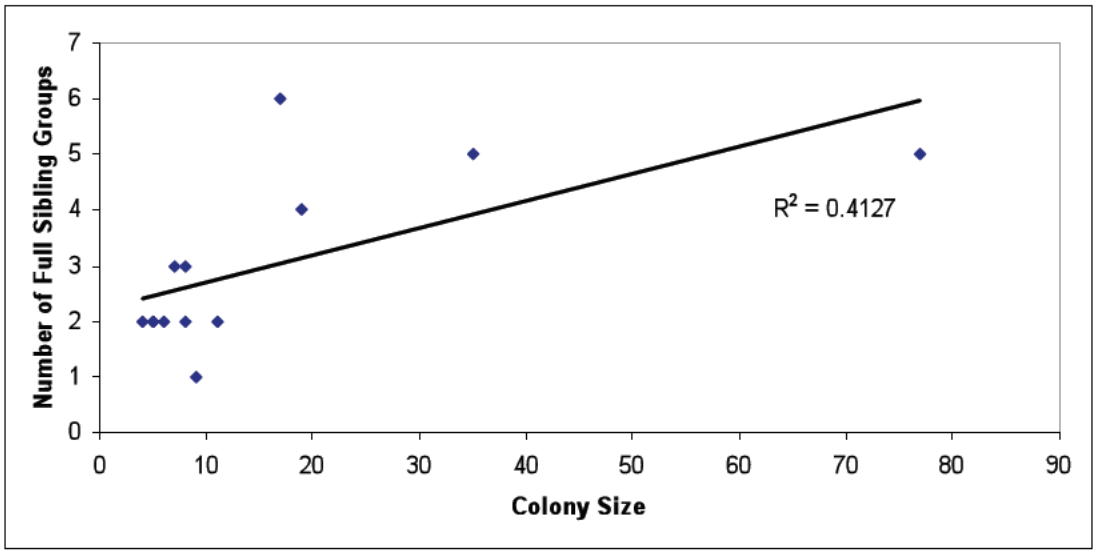


**Figure B.1: Number of full sibling groups within a colony versus the total number of adult individuals within a colony**

*Dispersal*

Negative $F_{IS}$ values were present in eight of thirteen colonies indicating that outbreeding is commonplace at the colony level (see Table B.2). In the five completely genotyped colonies the paternity of immature individuals could not be assigned to any of the adult males present within the colony. Conversely, within these same colonies, the maternity of immature individuals could always be assigned to at least one of the adult females present in the colony. These results suggest that outbreeding may be achieved largely by male dispersal.

## Discussion

Our genetic data show that colonies of *Polistes humilis* are characterized by multiple queens and that queen number increases with colony size. Within a colony each queen is singly mated by a different male and this may explain why reproductive females are not full sisters. Because our estimates of queen number are

inferred from the genotypes of their offspring we cannot be certain whether queens were reproducing concurrently or consecutively. In the case of the former, increasing numbers of queens in larger colonies may be a result of a breakdown in dominance hierarchies (Reeve 1991) or possibly a defence against disease transmission. However, there was no evidence of a larger proportion of non-reproductive adult females in smaller colonies (Spearman-Rank; $P = 0.218$). This result suggests that either the dominant female relinquishes any attempt at controlling subordinates above a certain small group size or that other factors, such as defence against disease are important. Increased genetic variability has been shown to reduce disease risk in several hymenopteran species, such as bumble bees (Baer and Schmid-Hempel 2001) and honey bees (Tarpy 2003) and we show that larger colonies of *P. humilis* have greater genetic variation. In future work we aim to ascertain whether genetic variation is associated with disease resilience in *P. humilis*.

It is also possible that we have identified several queens that have reproduced consecutively. However, analysis of each of the five colonies that were completely genotyped indicates that, at least in some cases, two or more queens were contributing to brood at the same stage of development. We therefore suspect that the simultaneous presence of multiple queens within nests of *P. humilis* may be typical. This has also been concluded by others who have observed multiple reproductive *P. humilis* females laying eggs at the same time within colonies (Itô 1986; Liebert 2008). Further, in another Australian paper wasp, *Ropalidia revolutionalis*, there is genetic evidence of queen replacement by several simultaneously reproducing females (Henshaw *et al*. 2004).

We detected no inbreeding in *P. humilis* and found that dispersal is primarily male biased. In each of the fully genotyped colonies, none of the adult male genotypes were consistent with the genotypes of immature individuals, which suggest that males are not reproducing in their natal colony. This form of outbreeding is supported by the negative $F_{IS}$ values observed within many colonies as well as the lack of diploid males which are symptomatic of inbreeding (Gerloff *et al*. 2003). It is suggested that the mating system of several eusocial species has evolved to minimise inbreeding (e.g. Crozier and Page 1985), especially in response to the problem of diploid male production (Paxton *et al.* 2000). It is possible that *P. humilis* can detect and avoid mating with nestmates as a means of inbreeding avoidance, or perhaps dispersal is carried out prior to mating, as in the parasitoid wasp *Bracon hebetor* (review by Cook and Crozier 1995).

Overall, the genetic data confirm *Polistes* behavioural observations suggesting that there are multiple queens per colony. The same data suggest that the multiple queens are genetically diverse and/or are mating with genetically distinct males. Further investigation into the increase in queen number with colony size and similarly increasing levels of genetic variation may reveal whether this occurs as a method of offsetting disease risk within a colony or is due to a break down of dominance hierarchies. Either way, the maintenance of genetic diversity appears to be important in this primitively eusocial species.

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## APPENDIX C: R SCRIPT FOR GOMPERTZ CURVE CALCULATIONS

```
# Script to perform a NLS fit of a modified Gompertz function to
Stephen
# Hoggard's wasp data. The curves to be fitted are "inhibition" or
"kill"
# curves.
#
# NOTE: IC99 represents the concentration that kills or inhibits to
99% of the
# initial value.
#
# Version 7 :  23 August 2010
# Peter D. Wilson
# Department of Biological Sciences
# Macquarie University, New South Wales Australia 2109

ModGompertz <- function (C,a,k,m)
 {
  return(a*exp(-exp(-k*(C - m))))      #  (log10( )
 }

FourParamLogistic <- function(C,a,b,c,d)
 {
  return(a +(b/(1+exp(c*(C - d)))))
 }

Inv4ParamLogistic <- function(f,a,b,c,d)
 {
  return(d + log((d-f+a)/(f - a))/c)
 }


WorkDir <- "G:/Wasp curves/"    #  Problem files/
#WorkDir <- "/media/BLACKBEAST/Wasp curves/"

BulkData <-
read.table(paste(WorkDir,"/WaspData2.txt",sep=""),header=T,sep="\t"
)
BulkData <- BulkData[nrow(BulkData):1,]
#BulkData

nItems <- ncol(BulkData) - 1
xvals <- log10(BulkData[,1])
ItemNames <- names(BulkData)[-1]

FitResults <- data.frame(FileName=I(rep("",nItems)),
                         a.fitted = rep(0,nItems),a.SE =
rep(0,nItems),
                         k.fitted = rep(0,nItems),k.SE =
rep(0,nItems),
                         m.fitted = rep(0,nItems),m.SE =
rep(0,nItems),
                         Logistic.a = rep(0,nItems),Logistic.a.SE =
rep(0,nItems),
                         Logistic.b = rep(0,nItems),Logistic.b.SE =
rep(0,nItems),
```

```
                          Logistic.c = rep(0,nItems),Logistic.c.SE =
rep(0,nItems),
                          Logistic.d = rep(0,nItems),Logistic.d.SE =
rep(0,nItems),
                          residual.SE = rep(0,nItems),numiter =
rep(0,nItems),
                          df = rep(0,nItems),IC01=rep(0,nItems),

IC50=rep(0,nItems),IC99=rep(0,nItems),FitType=I(rep(" ",nItems)))

FitResults$FileName <- ItemNames
xplot <- seq(-3.5,1,0.01)

######par(mfrow=c(5,7))
for (i in 1:nItems)
 {
  pdf(paste(WorkDir,ItemNames[i],".pdf",sep=""))
  theData <- data.frame("x"=BulkData[,1],"y"=BulkData[,i+1])
  #names(theData) <- c("x","y")

plot(log10(theData$x),theData$y,main=ItemNames[i],xlab="log10(Conc)
",ylab="Rel. Growth",ylim=c(0,1.2),xlim=c(-3,1))
  print("  ")
  print("==============================")
  print(ItemNames[i])
  #print(theData)

  big_y <- which(theData$y > 0.7)
  m_big <- mean(theData[big_y,"y"])
  z1 <- which(theData$y <= 0.7)
  z2 <- which(theData$y >= 0.3)
  mid_y <- z2[match(z1,z2)]
  small_y <- which(theData$y < 0.3)

  #print("big_y:")
  #print(big_y)
  #print("mid_y:")
  #print(mid_y)
  #print("small_y:")
  #print(small_y)

  # Estimate the upper asymptote, a, as the mean of "big" values
  a_init <- mean(theData[big_y,])[2]

  # Produce reasonable initial values for parameters k and m:
  # Find the log10(Conc) values for the last "big" and first
"small" values in
  # the observed data. These points span the region into which the
point of
  # inflection falls:
  m1 <- log10(theData[big_y[length(big_y)],1])
  m2 <- log10(theData[small_y[1],1])
  #print(paste("m1 = ",m1,"    m2 = ",m2,sep=""))

  # Take the mean of the "small" values:
  m_small <- mean(theData[small_y,])[2]
  sd_small <- sd(theData[small_y,])[2]
  zzz <- m_small + 1.96*sd_small
  #print(paste("m_small =",m_small,", sd_small =",sd_small,", Upper
CL = ",zzz))
```

```
  if (!is.nan(m_small)) # Deal with totally degenerate data
   {
    # Estimate the inital k-value as the linear slope spanning the
interval
    # identified by m1 and m2,and a_init and aa:
    k_init <- (m_small - a_init)/(m2 - m1)
    #if (k_init < -3.5) { k_init <- -3.5 }
    #print(paste("k_init =",k_init))
    #lines(c(m1,m2),c(a_init,m_small),lty=3)

    # Estimate initial m-value as the mid-point of the interval:
    m_init <- (m1+m2)/2
    #print(paste("m_init ",m_init))
    #abline(v=m_init,lty=2)

    #print("Trying Gompertz fit...")

    # Fit the modified Gompertz function. Preliminary trials
indicate that
    # convergence is slow, therefore boost maxiter, and
occassionally there is
    # a need to use a very small minimum factor value:
    try(Gompertz.fit <- nls(y ~ a*exp(-exp(-k*(log10(x)-m))), data
= theData,
                        start = list(a = a_init, k = k_init, m =
m_init),

control=nls.control(maxiter=1000,tol=0.00001,minFactor=0.00000009,
                        warnOnly=T),trace=F),silent=T)

    if (exists("Gompertz.fit") &&
(Gompertz.fit$convInfo$stopMessage == "converged"))
     {
      FitResults[i,"FitType"] <- "Gompertz"
      z1 <- summary(Gompertz.fit)
      #print("Gompertz fit succeeded:")
      #print(z1)

      FitResults[i,"a.fitted"] <-
round(z1$coefficients["a","Estimate"],4)
      FitResults[i,"a.SE"] <- round(z1$coefficients["a","Std.
Error"],4)

      FitResults[i,"k.fitted"] <-
round(z1$coefficients["k","Estimate"],4)
      FitResults[i,"k.SE"] <- round(z1$coefficients["k","Std.
Error"],4)

      FitResults[i,"m.fitted"] <-
round(z1$coefficients["m","Estimate"],4)
      FitResults[i,"m.SE"] <- round(z1$coefficients["m","Std.
Error"],4)

      FitResults[i,"residual.SE"] <- round(z1$sigma,4)
      FitResults[i,"df"] <- z1$df[2]
      FitResults[i,"numiter"] <- z1$convInfo$finIter

      yplot <-
ModGompertz(xplot,FitResults[i,"a.fitted"],FitResults[i,"k.fitted"]
,FitResults[i,"m.fitted"])
      lines(xplot,yplot)
```

```
      # If the fitted k-value is not equal to zero, compute
estimates of IC99,
      # IC50 and IC01
      if (FitResults[i,"k.fitted"] != 0)
        {
         FitResults[i,"IC01"] <- round(10^(FitResults[i,"m.fitted"]
- log(log(1/0.99))/FitResults[i,"k.fitted"]),4)

points(log10(FitResults[i,"IC01"]),0.99*FitResults[i,"a.fitted"],pc
h=16)
         FitResults[i,"IC50"] <- round(10^(FitResults[i,"m.fitted"]
- log(log(2))/FitResults[i,"k.fitted"]),4)

points(log10(FitResults[i,"IC50"]),0.5*FitResults[i,"a.fitted"],pch
=16)
         FitResults[i,"IC99"] <- round(10^(FitResults[i,"m.fitted"]
- log(log(1/0.01))/FitResults[i,"k.fitted"]),4)

points(log10(FitResults[i,"IC99"]),0.01*FitResults[i,"a.fitted"],pc
h=16)
        }
       }
      else
       {
        # Gompertz fit has failed because k.fitted -> vertical.
Estimate IC50 by similar triangles
        #print("Gompertz fit failed...")
        #xx <- log10(theData$x)
        #text(min(xx) + (max(xx) -
min(xx))/2,0.5,"Vertical\nslope1") # min(theData$y) +
(max(theData$y) - min(theData$y))/2
        FitResults[i,"a.fitted"] <- a_init
        FitResults[i,"k.fitted"] <- k_init
        FitResults[i,"IC50"] <- round(10^((m1+m2)/2),6)

points(log10(FitResults[i,"IC50"]),0.5*(a_init+m_small),pch=16)
        #print("Trying Four Paramater Logistic fit...")

        try(Logistic.fit <- nls(y ~ a +(b/(1+exp(c*(log10(x) -
d)))), data = theData,
                                start = list(a = m_small, b = m_big -
m_small, c = k_init, d = 10^((m1+m2)/2)),

control=nls.control(maxiter=1000,tol=0.00001,minFactor=0.00000009,
                                warnOnly=T),trace=F),silent=T)

        ##############################################
        if (exists("Logistic.fit") &&
(Logistic.fit$convInfo$stopMessage == "converged"))
         {
          #print("Logistic fit succeded:")
          #summary(Logistic.fit)
          ### Do stuff here
          FitResults[i,"FitType"] <- "Four-param Logistic"
          z1 <- summary(Logistic.fit)
          FitResults[i,"Logistic.a"] <-
round(z1$coefficients["a","Estimate"],4)
          FitResults[i,"Logistic.a.SE"] <-
round(z1$coefficients["a","Std. Error"],4)
```

```
          FitResults[i,"Logistic.b"] <-
round(z1$coefficients["b","Estimate"],4)
          FitResults[i,"Logistic.b.SE"] <-
round(z1$coefficients["b","Std. Error"],4)
          FitResults[i,"Logistic.c"] <-
round(z1$coefficients["c","Estimate"],4)
          FitResults[i,"Logistic.c.SE"] <-
round(z1$coefficients["c","Std. Error"],4)
          FitResults[i,"Logistic.d"] <-
round(z1$coefficients["d","Estimate"],4)
          FitResults[i,"Logistic.d.SE"] <-
round(z1$coefficients["d","Std. Error"],4)

          yplot <-
ModGompertz(xplot,FitResults[i,"a.fitted"],FitResults[i,"k.fitted"]
,FitResults[i,"m.fitted"])
          lines(xplot,yplot)

          FitResults[i,"IC01"] <-
round(10^(Inv4ParamLogistic(0.99*(FitResults[i,"Logistic.a"]+FitRes
ults[i,"Logistic.b"]),

FitResults[i,"Logistic.a"],FitResults[i,"Logistic.b"],

FitResults[i,"Logistic.c"],FitResults[i,"Logistic.d"])),4)

points(log10(FitResults[i,"IC01"]),0.99*FitResults[i,"a.fitted"],pc
h=16)
          FitResults[i,"IC50"] <-
round(10^(FitResults[i,"Logistic.d"]),4)

points(log10(FitResults[i,"IC50"]),0.5*FitResults[i,"a.fitted"],pch
=16)
          FitResults[i,"IC99"] <-
round(10^(Inv4ParamLogistic(0.01*(FitResults[i,"Logistic.a"]+FitRes
ults[i,"Logistic.b"]),

FitResults[i,"Logistic.a"],FitResults[i,"Logistic.b"],

FitResults[i,"Logistic.c"],FitResults[i,"Logistic.d"])),4)

points(log10(FitResults[i,"IC99"]),0.01*FitResults[i,"a.fitted"],pc
h=16)
         }
        else
         {
          #print("Logistic fit failed...resort to piece-wise linear
fit v1...")

          FitResults[i,"FitType"] <- "Piece-wise Linear"

lines(c(log10(theData[1,1]),m1),c(m_big,m_big),lty=1,col="black")
          lines(c(m1,m2),c(m_big,m_small),lty=1,col="black")

lines(c(m2,log10(theData[nrow(theData),1])),c(m_small,m_small),lty=
1,col="black")

          x_IC99 <- m2 - (1-0.99)*(m2 - m1)
          FitResults[i,"IC99"] <- round(10^x_IC99,6)
          y_IC99 <- m_big - 0.99*(m_big- m_small)
          points(x_IC99,y_IC99,pch=16)
```

```
          x_IC01 <- m2 - (0.99)*(m2 - m1)
          FitResults[i,"IC01"] <- round(10^x_IC01,6)
          y_IC01 <- m_big - 0.01*(m_big- m_small)
          points(x_IC01,y_IC01,pch=16)

         }
       }
    }
  else
   {

    if (length(mid_y) != 0)
     {
      # Fudge zero level
      #if (length(theData$y) ==1)
      # {
        theData$y[(mid_y[1]+1):nrow(theData)] <- 0
      # }
      #else
      # {
      #  theData$y[mid_y[length(mid_y)+1]:nrow(theData)] <- 0
      # }
      #print("Mid-level values(s) exist: Trying Gompertz fit with
estimated zero-level values")
      try(Gompertz.fit <- nls(y ~ a*exp(-exp(-k*(log10(x)-m))),
data = theData,
                        start = list(a = a_init, k = k_init, m =
m_init),

control=nls.control(maxiter=1000,tol=0.00001,minFactor=0.000000005,
                        warnOnly=T),trace=F),silent=T)
      if (exists("Gompertz.fit") &&
(Gompertz.fit$convInfo$stopMessage == "converged"))
       {
        #print("Fudged Gompertz fit worked...")

        z1 <- summary(Gompertz.fit)
        #print(z1)
        FitResults[i,"FitType"] <- "Fudged Gompertz"
        FitResults[i,"a.fitted"] <-
round(z1$coefficients["a","Estimate"],4)
        FitResults[i,"a.SE"] <- round(z1$coefficients["a","Std.
Error"],4)

        FitResults[i,"k.fitted"] <-
round(z1$coefficients["k","Estimate"],4)
        FitResults[i,"k.SE"] <- round(z1$coefficients["k","Std.
Error"],4)

        FitResults[i,"m.fitted"] <-
round(z1$coefficients["m","Estimate"],4)
        FitResults[i,"m.SE"] <- round(z1$coefficients["m","Std.
Error"],4)

        FitResults[i,"residual.SE"] <- round(z1$sigma,4)
        FitResults[i,"df"] <- z1$df[2]
        FitResults[i,"numiter"] <- z1$convInfo$finIter
```

```
        yplot <-
ModGompertz(xplot,FitResults[i,"a.fitted"],FitResults[i,"k.fitted"]
,FitResults[i,"m.fitted"])
        lines(xplot,yplot)

        # If the fitted k-value is not equal to zero, compute
estimates of IC99,
        # IC50 and IC01
        if (FitResults[i,"k.fitted"] != 0)
          {
           FitResults[i,"IC01"] <-
round(10^(FitResults[i,"m.fitted"] -
log(log(1/0.99))/FitResults[i,"k.fitted"]),4)

points(log10(FitResults[i,"IC01"]),0.99*FitResults[i,"a.fitted"],pc
h=16)
           FitResults[i,"IC50"] <-
round(10^(FitResults[i,"m.fitted"] -
log(log(2))/FitResults[i,"k.fitted"]),4)

points(log10(FitResults[i,"IC50"]),0.5*FitResults[i,"a.fitted"],pch
=16)
           FitResults[i,"IC99"] <-
round(10^(FitResults[i,"m.fitted"] -
log(log(1/0.01))/FitResults[i,"k.fitted"]),4)

points(log10(FitResults[i,"IC99"]),0.01*FitResults[i,"a.fitted"],pc
h=16)
          }
       }
      else
       {  ##############################
        #print("Fudged Gompertz fit failed...")
        # Gompertz fit has failed because k.fitted -> vertical.
Estimate IC50 by similar triangles
        #xx <- log10(theData$x)
        #text(min(xx) + (max(xx) -
min(xx))/2,0.5,"Vertical\nslope2") # min(theData$y) +
(max(theData$y) - min(theData$y))/2
        FitResults[i,"a.fitted"] <- a_init
        FitResults[i,"k.fitted"] <- k_init
        FitResults[i,"IC50"] <- round(10^((m1+m2)/2),6)

points(log10(FitResults[i,"IC50"]),0.5*(a_init+m_small),pch=16)
        #print("Trying Four-paramater Logistic fit...")

        try(Logistic.fit <- nls(y ~ a +(b/(1+exp(c*(log10(x) -
d)))), data = theData,
                            start = list(a = m_small, b = m_big -
m_small, c = k_init, d = 10^((m1+m2)/2)),

control=nls.control(maxiter=1000,tol=0.00001,minFactor=0.00000009,
                            warnOnly=T),trace=F),silent=T)

        if (exists("Logistic.fit") &&
(Logistic.fit$convInfo$stopMessage == "converged"))
         {
          #print("Logistic fit succeded:")
          #summary(Logistic.fit)
          ## Do stuff here
          FitResults[i,"FitType"] <- "Four-param Logistic"
```

```
          FitResults[i,"FitType"] <- "Four-param Logistic"
          z1 <- summary(Logistic.fit)
          FitResults[i,"Logistic.a"] <-
round(z1$coefficients["a","Estimate"],4)
          FitResults[i,"Logistic.a.SE"] <-
round(z1$coefficients["a","Std. Error"],4)
          FitResults[i,"Logistic.b"] <-
round(z1$coefficients["b","Estimate"],4)
          FitResults[i,"Logistic.b.SE"] <-
round(z1$coefficients["b","Std. Error"],4)
          FitResults[i,"Logistic.c"] <-
round(z1$coefficients["c","Estimate"],4)
          FitResults[i,"Logistic.c.SE"] <-
round(z1$coefficients["c","Std. Error"],4)
          FitResults[i,"Logistic.d"] <-
round(z1$coefficients["d","Estimate"],4)
          FitResults[i,"Logistic.d.SE"] <-
round(z1$coefficients["d","Std. Error"],4)

          yplot <-
ModGompertz(xplot,FitResults[i,"a.fitted"],FitResults[i,"k.fitted"]
,FitResults[i,"m.fitted"])
          lines(xplot,yplot)

          FitResults[i,"IC01"] <-
round(10^(Inv4ParamLogistic(0.99*(FitResults[i,"Logistic.a"]+FitRes
ults[i,"Logistic.b"]),

FitResults[i,"Logistic.a"],FitResults[i,"Logistic.b"],

FitResults[i,"Logistic.c"],FitResults[i,"Logistic.d"])),4)

points(log10(FitResults[i,"IC01"]),0.99*FitResults[i,"a.fitted"],pc
h=16)
          FitResults[i,"IC50"] <-
round(10^(FitResults[i,"Logistic.d"]),4)

points(log10(FitResults[i,"IC50"]),0.5*FitResults[i,"a.fitted"],pch
=16)
          FitResults[i,"IC99"] <-
round(10^(Inv4ParamLogistic(0.01*(FitResults[i,"Logistic.a"]+FitRes
ults[i,"Logistic.b"]),

FitResults[i,"Logistic.a"],FitResults[i,"Logistic.b"],

FitResults[i,"Logistic.c"],FitResults[i,"Logistic.d"])),4)

points(log10(FitResults[i,"IC99"]),0.01*FitResults[i,"a.fitted"],pc
h=16)

         }
        else
         {
          #print("Logistic fit failed...resort to piece-wise linear
fit v2...")

          FitResults[i,"FitType"] <- "Piece-wise Linear"

          x_IC99 <- m2 - (1-0.99)*(m2 - m1)
          FitResults[i,"IC99"] <- round(10^x_IC99,6)
          y_IC99 <- m_big - 0.99*(m_big- m_small)
```

```
          points(x_IC99,y_IC99,pch=16)

          x_IC01 <- m2 - (0.99)*(m2 - m1)
          FitResults[i,"IC01"] <- round(10^x_IC01,6)
          y_IC01 <- m_big - 0.01*(m_big- m_small)
          points(x_IC01,y_IC01,pch=16)

lines(c(log10(theData[1,1]),m1),c(m_big,m_big),lty=1,col="black")
          lines(c(m1,m2),c(m_big,m_small),lty=1,col="black")

lines(c(m2,log10(theData[nrow(theData),1])),c(m_small,m_small),lty=
1,col="black")
         }
       }
     }
    else
     {
      #print("No zero- and mid-level values...abandon all hope")
      xx <- log10(theData$x)
      text(min(xx) + (max(xx) - min(xx))/2,0.5,"No fit\npossible")
# min(theData$y) + (max(theData$y) - min(theData$y))/2
      FitResults[i,"FitType"] <- "No fit possible"
     }
   }

  dev.off()
 }

# Save the FitResults table to file. NOTE: Change the file name for
each data set
# to avoid overwriting the last set of results.
write.table(FitResults,paste(WorkDir,"FitResults3.txt",sep=""),quot
e=F,row.names=F,sep="\t")
```

## APPENDIX D: BIOSAFETY APPROVAL

*Biosafety application ref 5201001071 - formal letter of final approval*

Dear Dr Stow

Re: The evolution of sociality and antimicrobial defences (REF: 5201001071 LAB)

Thank you for your correspondence dated September 8 2010 responding to the Committee's request for further information concerning the above application. Your responses were reviewed and Final Approval of the above application was granted, effective 8 September 2010.

You must inform the Committee of your willingness to accept and comply with any conditions by signing the Agreement Statement attached to this email, and returning one copy of this Agreement to the Committee Secretary, Ms Nicola Myton, in the Research Office at Macquarie University.

The following personnel are authorised to conduct this research:

Dr Adam Stow – Chief Investigator/Supervisor
Mr Stephen Hoggard – Co-Investigator

Please note the following standard requirements of approval:

1. Approval will be for a period of twelve (12) months. At the end of this period, if the project is continuing then a progress report must be submitted. If, at the end of this period the project has been completed, abandoned, discontinued or not commenced for any reason, you are required to submit a Final Report. If you complete the work earlier than you had planned you must submit a Final Report as soon as the work is completed. These reports are located at the following address:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/forms

A Progress/Final Report for this study will be due on: 1 September 2011.

2. Please remember the Committee must be notified of any proposed alteration to the project. This can be done by downloading a 'Request for Amendment' form from the following address:

http://www.research.mq.edu.au/for/researchers/how_to_obtain_ethics_approval/biosafety_research_ethics/forms

3. If you will be applying for or have applied for internal or external funding for the above project it is your responsibility to provide the Macquarie University's Research Grants Management

Assistant with a copy of this email as soon as possible. Internal and External funding agencies will not be informed that you have final approval for your project and funds will not be released until the Research Grants Management Assistant has received a copy of this email.

If you need to provide a hard copy letter of Final Approval to an external organisation as evidence that you have Final Approval, please do not hesitate to contact the Committee Secretary at the address below.

Please retain a copy of this email as this is your formal notification of final Biosafety approval.

Yours Sincerely

Dr Sinan Ali
Chair, Macquarie University Biosafety Committee

--
Office of the Deputy Vice Chancellor (Research)

Macquarie University Biosafety Secretariat

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