The Antifungal Defences of Australian *Acacia* thrips

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Declaration

I hereby declare that, to my knowledge, no content of this thesis has been submitted for the award of any other degree or diploma at any other institution. The work contained within this thesis is my own except for contributions as stated in the acknowledgements of each chapter and as stated here. Chris Hammill provided contributions towards the statistical analysis for the work presented in Chapter 2 and 3. Assistance in fieldwork for Chapter 3 was provided by Assistant Professor Thomas Chapman and Holly Caravan. Associate Professor Adam Stow collected field samples for Chapter 4. Experimental design for all chapters were aided by Emeritus Professor Andrew Beattie, Christine Turnbull, Associate Professor Adam Stow, and Assistant Professor Thomas Chapman. Contributions towards the writing and review of this thesis were made by Associate Professor Adam Stow, Assistant Professor Thomas Chapman, and Emeritus Professor Andrew Beattie and Christine Turnbull.

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Thesis Abstract

Social living exposes organisms to an increased risk of pathogen infection, a risk that is amplified in social insects due to several life history traits, including high population densities within colonies and high relatedness between individuals. To offset disease risk, social insects have developed specialized antimicrobial defences. The Australian *Acacia* thrips, a model lineage for the study of eusocial evolution, vary greatly in their life history traits, and thus provide an excellent model system to investigate how disease mitigation measures might vary in accordance with species-level characteristics, and across developmental stages for particular species. Previous studies have already described links between antimicrobial production and increasing social complexity in the thrips, but much of the basic biology surrounding this antimicrobial defence remains unknown. This thesis aims to increase our knowledge of antimicrobial production in the *Acacia* thrips by describing the source and structure of their antifungals, testing whether group-size and colony maturity affect antifungal strength, and by characterising antifungal production for different castes. The research presented here provides important basic information on how *Acacia* thrips defend against entomopathogenic fungi, and may be useful in future comparative analysis focusing on how these antifungal defences are tied to the evolution of sociality.

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Chapter 1

General introduction

Social Insect pathogen defence and the development of eusociality

This thesis attempts to help clarify the role antimicrobial production plays in the development of sociality in the insects. Living within a social group inherently increases the risk of pathogen transmission and infection to an organism (Schmid-Hempel 1998). The insect societies are the oldest on earth and to combat this increased threat, have developed sophisticated antimicrobial defences. This chapter provides the necessary background in social insect biology, a basic introduction to the pathogens which target these societies and examples of the complex behavioural and chemical defences which have been selected for. It then presents an introduction to the Australian *Acacia* thrips, their importance in studying the evolution of eusociality, and how their antimicrobial defences are now being explored to this end. Understanding how antimicrobial defences aid in the development of social living is important not only for the study of social insects but also may lead to novel methods which human societies can use to defend themselves against the increasing threat of pathogen epidemics.

Social living in insects

Over evolutionary time, arguably the most successful and complex societies have developed within the insects. These societies make up the majority of insect biomass on earth (Wilson 1990) and influence nearly every ecosystem through the pollination of plants (Tepedino 1979; Potts et al. 2010), soil turnover (De Bruyn and Conacher 1990), and degradation of dead plant and animal matter (Bignell and Eggleton 2000; De Toro et al. 2015; Warren and Bradford 2012). The social insects include some species of bees (Cameron and Mardulyn 2001), wasps (Carpenter 1991), aphids (Aphidoidea; Abbot 2009), thrips (Thysanoptera; Crespi 1992), beetles (Coleoptera; Kent and Simpson 1992), and all species of ants and termites (Wilson 1971). Living within a social group provides many benefits including increased foraging efficiency (Traniello and Beshers 1991), cooperative raising of brood (Brian 1953; Cassill et al. 2002), and group level

defence against predators (Vulinec 1990). It is partially because of these benefits which have allowed the social insect societies to become so successful.

Despite these benefits, social living also has one major disadvantage: the increased risk of pathogen infection (Schmid-Hempel 1998). Social insects produce colonies in a long-lived, densely populated, communal nest where relatedness between individuals is usually very high. These life history traits produce the ideal environment for pathogen spread, representing a major threat to the insect societies (Stow and Beattie 2008).

Population density and a common nest

The number of individuals which make up a mature social insect colony varies widely. Allodapine bees and relatives (Xylocopinae), hover wasps (Stenogastrinae), and sphecid wasps (Pemphredoninae) will form groups of ~10 individuals. However, honeybees (Apini), ants (Formicidae) and termites (Isoptera) can form colonies which are 4 – 6 orders of magnitude larger (Bourke 1999). These large populations of individuals interact within a common nest and come in frequent contact with one another (Fewell 2003). The combination of high host population density and contact between susceptible individuals, increases the likelihood of pathogen transmission from an infected individual to a healthy individual (Anderson and May 1978; May and Anderson 1978; McCallum et al. 2001). This threat can be reduced by low worker movement and nest architecture (Pie et al. 2004) but remains a problem for most insect societies.

Genetic diversity

Most individuals within a social insect colony are kin and therefore are highly related (Hamilton 1964). Relatedness among individuals is even higher in the social Hymenoptera and Thysanoptera where haplo-diploid sex determination increases relatedness between sisters to 0.75 (Berkelhamer 1983; Crozier 1970; Crespi 1991; Hamilton 1964) or higher, depending on the

inbreeding coefficient (Sundström et al. 2003; Chapman et al. 2000). The importance of this is that reduced genetic diversity is linked to a decrease in disease resistance (Spielman et al. 2004; Whiteman et al. 2006). Social insects, especially the social hymenoptera, have developed behaviours which increase genetic variation within a colony, possibly in response to pathogenic pressures (Stow and Beattie 2008). Polygyny (multiple queens) is present in many ant species (Hölldobler and Wilson 1977; Ross and Carpenter 1991; Keller 1995) while polyandry (multiple mating of queens) is more common in bees (Page 1980; Boomsma and Ratnieks 1996; Strassmann 2001). The effect of increased genetic variation on pathogen resistance is clear; colonies with higher genetic diversity have reduced parasite loads (Liersch and Schmid-Hempel 1998; Calleri et al. 2006) and are quicker to respond to pathogen threats (Ugelvig et al. 2010).

Entomopathogenic microbes

Entomopathogens are microorganisms which infect and carry out a portion of their lifecycle within insects and include species of bacteria, fungi, and viruses (Schmid-Hempel 1998). The most widely studied aspect of entomopathogen biology is the culture of strains which target either insect pests of major crops (Jackson et al. 2010; Ruiu et al 2013; Gul et al. 2014; Angus 1965; Roberts 1989) or species that cause considerable harm to humans (Kanzok and Jacobs-Lorena 2006; Scholte et al. 2004; Shah and Pell 2004; Butt and Copping 2000) or their infrastructure (Williams et al. 2003; Chouvenc et al. 2011). However, entomopathogens represent a major threat to insect societies and attention towards the evolutionary implications of insect pathogens has therefore increased, particularly with reference to group living and the evolution of eusociality in the insects (Stow et al. 2007; Stow and Beattie 2008; Turnbull et al. 2011).

Species of fungi, bacteria, viruses, nematodes, and protozoa survive as entomopathogens on social insect colonies. Each of these groups present unique dangers to social insects and subsequently have selected for specialized defensive adaptations (Schmid-Hempel 1998). Of

these groups however, fungi and bacteria are arguably the most prevalent and important entomopathogens of social insects, with the latter developing a wide range of complementary external antimicrobial defences (Cremer et al. 2007). For this reason this chapter will focus primarily on these two groups.

Entomopathogenic fungi

Pathogenic fungi are regarded as an increasing threat to a range of animal and plant species (Fisher et al. 2012), including human populations (Brown et al. 2012). Approximately 90 genera and at least 700 species of fungi specialize in infecting insect species (Gul et al. 2014). Unlike bacterial and viral entomopathogens, fungi do not need to be ingested to infect a host. Instead, they penetrate the host cuticle using enzymes and a specialized germ-tube to push through the cuticle into the body cavity before proliferating a hyphal network (Leger et al. 1986; Hajek and Leger 1994; reviewed in Pedrini et al. 2007 and Ortiz-Urquiza and Keyhani 2013). Once inside, fungal hyphae spread throughout the body cavity producing toxins which eventually kill the infected host (Charnley 2003). In many species the fungal fruiting body then emerges from the cadaver and releases spores (Roy et al. 2006; Shahid et al. 2012). Pathogenic fungi are highly virulent and once established within a host, infections are near impossible to completely remove (Hajek and Leger 1994).

Entomopathogenic bacteria

Both gram positive and gram negative bacterial groups have developed entomopathogenic strains (Hurst 2016; Aronson et al. 1986). Bacteria usually infect a host through the gastro-intestinal tract after being ingested but also may enter with pathogenic nematodes or through a wound (Vallet-Gely et al. 2008). Once inside the hemolymph, some bacterial species produce toxic compounds which eventually lead to host death (Hurst 2016; De Maagd et al. 2003). The

spore-forming, *Bacillus thuringiensis* (*Bt*) and its subspecies are one such group, and are arguably the most widely studied bacterial entomopathogens (Ruiu 2015, Aronson et al. 1986). The toxic 'cry' proteins that *Bt* strains produce are effective against a range of pest insect species (Höfte and Whitely 1989; Schnepf et al. 1998). Due to difficulties in culturing many native bacterial species (Stewart 2012) it is likely that only a small percentage of biologically relevant entomopathogenic strains have been discovered.

Social insect pathogens

Several pathogens have been found to target social insect colonies, however, the majority of research has focused on pathogens of the economically important honey bee. Some of the more well studied pathogens of bees include Chalkbrood disease caused by the *Ascosphaera apis* (Aronstein and Murray 2010), Stonebrood caused by multiple species of *Aspergillus* (Shoreit and Bagy 1995), and American and European foulbrood brought on by *Paenibacillus larvae* and *Melissococcus plutonius* respectively (Genersch 2010; Forsgren 2010). Ant and termite colonies are likely targeted by a more diverse assemblage of pathogens due to many species nesting in soil or decaying plant material (Rosengaus et al. 2003, 2011; Schmid-Hempel 1998; Roose-Amsaleg et al. 2004), however, relatively less is known about their specific pathogens. Entomopathogenic species of Clavicipitaceae including *Metarhizium anisopliae* (Milner et al. 1998; Hughes et al. 2004), *Cordyceps* or *Beauveria* (Evans 1982; Evans and Samson 1982, 1984), and *Cordycepioideus* species (Blackwell and Gilbertson 1984; Ochiel et al. 1997), have been found to infect ant and termite species. More than 1.31 pathogen species exist for every social insect host species, thus these pathogenic pressures select for increasingly complex antimicrobial defences drive evolutionary changes in these lineages (Schmid-Hempel 1998).

Antimicrobial adaptations

A fundamental aspect of social insect colonies is the ability to defend the nest and its contents from predators and parasites (Lin and Michener 1972). The evolutionary pressure produced by pathogens has selected for defensive adaptations in all arthropods (Roy et al. 2006), with arguably the most complex and variable adaptations occurring in the social insect lineages (Cremer et al. 2007; Cremer and Sixt 2009). The variety and complexity of the defences developed to combat entomopathogens suggests that microbial invaders may in fact be a more serious threat to social living than that of large predators (Turubull et al. 2012a; Schmid-Hempel 1998). Understanding these defences is therefore vital for revealing how the insect societies first developed and how they continue to survive today.

Microbial defence and social insects

Insect immunity was initially thought to be composed of a simple, innate immune response (Hoffmann 1995; Gillespie et al. 1997). However, with the discovery of trans-generational immune priming (Sadd et al. 2005; Moret 2006), immunological memory (Sun et al. 2014; Sadd and Schmid-Hempel 2006), and the push to include external defences, like the secretion of antimicrobial compounds (Otti et al. 2014), the insect immune system is much more complex than previously stated (Schmid-Hempel 2005). In the social insects, specialized prophylactic secretions, active behavioural responses and group level defences have also evolved to limit pathogen transmission. The investment in these defences by all individuals within a colony produces a ‘social immune system’, where at the cost of personal immunity (Cotter et al. 2013) individuals collectively defend against harmful microbes (Cremer et al. 2007). Although the internal immune response is a key aspect of insect pathogen defence, for the purposes here, this chapter will focus on external, front line defences, both prophylactic and active, due in part to their increased importance in social insect colonies (Stow and Beattie 2008).

Social insect colonies provide the ideal environment for pathogen transmission. In particular, the high relatedness and thus shared resistance genes among individuals, produce a colony-wide susceptibility to novel infections (Stow and Beattie 2008; Schmid-Hempel 1994, 1998). Therefore, external defences which inhibit or remove pathogens before they infect individuals, limiting the spread of a pathogen through a colony are highly important (Cremer et al. 2007). For example, entomopathogenic fungal spores can be removed from the cuticle through allogrooming (Rosengaus et al. 1998) or inhibited by secreted antimicrobial compounds (Rosengaus et al. 2000), however, once within the hemolymph it is difficult for the insect immune system to combat fungal hyphae (Hajek and Leger 1994). Thus, external defences are crucial for the colony survival and represent an important area of study in the social insects.

Social insect external defences include both prophylactic and active defences that include specialized behaviours and external antimicrobial compounds, which can be produced by the individual (Bot et al. 2002), harvested from the environment (Chapuisat et al. 2007) or produced by symbiotic bacterial species (Hughes et al. 2008). Passive defences produce an environment which is unsuitable for the establishment of a pathogen, while active defences are used in response to the presence of infective material and require the ability to detect pathogens (Cremer et al. 2007). Pathogen detection is present in all major social insect groups (Yanagawa et al. 2009, 2010, 2011; Swanson et al. 2009; Ugelvig et al. 2010; Wilson-Rich et al. 2009). In addition, honey bees and termites have also developed the ability to communicate that they are infected through a specialized dance (Land and Seeley 2004) and vibratory display (Rosengaus et al. 1999), respectively. It is clear that the threat of pathogen infection has selected for many specialized behaviours and compounds which limit microbial growth. Below, a variety of antimicrobial defences present in the social insects are reviewed.

Antimicrobial behaviours

The most widely studied antimicrobial behaviour in the social insects is allogrooming, where pathogens and parasites are physically removed from the cuticle of conspecifics (Hart 1990). Allogrooming has been observed in many species of ant (Tranter and Hughes 2015; Reber et al. 2011), bee (Evans and Spivak 2010; Boecking and Spivak 1999), and termite (Rosengaus et al. 1998; Hamilton et al. 2011) and is highly effective in removing pathogens (Reber et al. 2011; Rosengaus et al. 2010) and lowering the risk of infection to others in the colony (Traniello et al. 2002; Cremer et al. 2007; Evans and Spivak 2010). In the ants and termites, the removal of pathogens is made more effective through the use of secreted antimicrobial compounds which are spread on the cuticle during allogrooming, removing any pathogenic material and also protecting that individual from future infection (Rosengaus et al. 2000; Fernández-Marín et al. 2006; Tragust et al. 2013).

Other common antimicrobial behaviours include nest cleaning and social isolation which help to limit pathogen spread within a colony (Gilliam et al. 1983; Oi and Pereira 1993; Fefferman et al. 2007). Nest cleaning is present in all major social insect groups and includes the removal of corpses and other potentially harmful debris from the nest structure (Sun and Zhou 2013). Corpse removal behaviours ensure that possible pathogens or parasites inside deceased individuals are removed before they are able to spread and have been documented in all major social insect lineages (Diez et al. 2012; Wilson et al. 1958; Gilliam et al. 1983, 1988; Visscher 1983; Sun and Zhou 2013). Social isolation is particularly common in the ants and occurs when an infected individual is excluded from the colony, either by choice or forcibly by nest-mates (Hart and Ratnieks 2001; Heinze and Walter 2010; Bos et al. 2012; Chapuisat 2010).

Antimicrobial secretions

Antimicrobial secretions make up a large proportion of the social immunity of a social insect colony. Antimicrobial secretions originate from the venom gland in the social Hymenoptera (Baracchi et al. 2011, 2012; Graystock and Hughes 2011), however, most ant species also possess a metapleural gland, which specializes in producing numerous antimicrobial compounds (Veal et al. 1992; Beattie et al. 1986; Ortius-Lechner et al. 2000; Bot et al. 2002). Venom is used to coat eggs and larvae (Obin and Vander Meer 1985; Vander Meer and Morel 1995), sprayed on the nest structure (Tranter et al. 2014; Baracchi et al. 2011; Baracchi and Turillazzi 2010), groomed onto the cuticle (Baracchi et al. 2011), and is taken up into the infrabuccal pocket to aid in allogrooming (Tragust et al. 2013). Termites produce antimicrobials through several different means including from the sternal (Rosengaus et al. 2004; 2011), frontal (Rosengaus et al. 2000; Zhou et al. 2004), and mandibular glands (Hamilton et al. 2011), through fecal pellets (Rosengaus et al. 1998), and by impregnating their nest materials with naphthalene, a hydrocarbon with antifungal activity (Chen et al. 1998).

The active antimicrobial compounds present in social insect secretions differ widely between lineages (Table 1). In general, bees and wasps rely on antimicrobial peptides which they produce in the venom gland (Baracchi et al. 2011, 2012). Organic acids make up a large proportion of metapleural gland secretions in ants but peptides, ketones, and alkaloids, produced in other glands also have antimicrobial activity (Bot et al. 2002; Zelezetsky et al. 2005; Mendonça et al. 2009; Storey et al. 1991). Termite secretions contain mixtures of enzymes, terpines, and other compounds which inhibit pathogens (Table 1). This variation in antimicrobial compounds is likely due, in part, to differing pathogen pressures experienced by each lineage. For example ground nesting species likely encounter a wider variety of harmful fungi and bacteria than species which nest above ground or in trees (Schmid-Hempel 1998).

Environmentally derived antimicrobials

Social Hymenoptera species in addition to producing their own antimicrobial compounds also rely on compounds which are derived from plant resins. When founding a new hive, usually within a hollow of a tree, honey bees will coat the inside of the cavity with a propolis made of plant resins (Seeley and Morse 1976) with antimicrobial properties (Simone-Finstrom and Spivak 2010; Evans and Spivak 2010). The presence of resins lowers the expression of honey bee immune genes (Simone et al. 2009) and, in response to pathogen attack, individuals will increase resin collection likely as a form of self-medication (Simone-Finstrom and Spivak 2012). Antimicrobial plant resins are also collected by wood ants (*Formica paralugubris*) and placed in the nest as a prophylactic defence against entomopathogens (Chapuisat et al. 2007; Castella et al. 2008a, b). The presence of resin also reduces some aspects of the immune system, similar to that observed in honey bees (Castella et al. 2008b). The use of environmentally derived antimicrobial compounds suggests a significant cost is associated with normal prophylactic and immune defences, and highlights the importance of external microbial defences in the social insects.

Symbiotic bacteria derived antimicrobials

Similar to research uncovering the role symbiotic microbial communities play in human well-being (Foster and Neufeld 2013; Guarner and Malagelada 2003), recent studies have outlined the importance of symbiotic relationships fostered between social insects and antimicrobial producing bacteria. The first description of a mutualistic association between antimicrobial producing bacteria and social insects was between *Streptomyces* and fungus-growing ants (Attini: Formicidae) which were cultured in special pits on the cuticle (Currie et al. 1999). Since then, mutualistic relationships have been described in multiple species of ants (Currie et al. 2006), termites (Visser et al. 2012; Chouvenc et al. 2013; Peterson and Scharf 2016), honeybees (Evans and Armstrong 2006; Promnuan et al. 2009), wasps (Madden et al. 2013), ambrosia beetles (Grubbs et al. 2011), and Australian *Acacia* thrips (Alteen 2012).

Antimicrobial producing bacterial symbionts are cultured by the insects in specialized glands on the cuticle (Mattoso et al. 2012; Currie et al. 2006), in the gut (Vásquez et al. 2012; Evans and Armstrong 2006; Koch and Schmid-Hempel 2011) or in the nest material itself (Chouvenc et al. 2013; Madden et al. 2013). In addition to protecting adult individuals against pathogens (Mattoso et al. 2012; Visser et al. 2012), bacterial antibiotics are used to protect eggs and larvae in wasps (Madden et al. 2013), and fungal food sources in ants (Currie et al. 1999, 2003, 2006; Currie 2001), termites (Visser et al. 2012) and probably beetles (Grubbs et al. 2011). With the exception of gut symbionts, many of the mutualistic relationships are formed with Actinomycetes, principally Streptomyces. Actinomycetes are filamentous bacteria commonly found in soil which produce a wide array of antibiotic compounds (Kaltenpoth 2009; Baltz 2008; Watve et al. 2001) including many which are used in pharmaceuticals (Raja and Prabakarana 2011; Lam 2007). The prevalence of these symbiotic relationships in all social insects suggests that they are necessary for living in large colonies, an adaptation which could aid in reducing pathogen threats to human societies.

Antimicrobial defences and the development of eusociality

Eusociality first developed in the ants and termites over 100 million years ago (Engel et al. 2016; LaPolla et al. 2013; Wilson 1971), a transition that was likely met with increases in pathogen threat and subsequently an increased need for antimicrobial defences (Stow et al. 2007). The complex physiological and behavioural adaptations developed in response to pathogen stresses in social insects have been covered earlier in this chapter, however, the initial defences which allowed these lineages to overcome the increased threat present in the transition from solitary to social living are thus far unknown. Although considerable research has focused on eusociality in ant and termite lineages, the traits important for the development of eusociality are obscured due to the absence of closely related non-social species and an ancient origin of

eusociality (Wilson 1971). This necessitates the study of model lineages like the *Acacia* thrips, social aphids, wasps, and some bee lineages for discovering traits important for the evolution of eusociality. Principally, the *Acacia* thrips provide a useful study system for comparative studies regarding the potential effects of antimicrobial production on the development of group-living and sociality.

The *Acacia* thrips

The Thysanoptera are an insect Order commonly referred to as thrips. All thrips species are relatively small in size (majority of species are roughly 1mm), darkly pigmented, possess fringed wings, and most feed on fungi or plant cells (Gullan and Cranston 2010). The most highly studied thrips species are pests which target a variety of economically important crops (Reitz 2009; Kirk and Terry 2003). However, thrips are found world-wide and form a diverse order which includes many species with differing life histories (Lewis 1973). The Australian *Acacia* thrips, for example, are a clade which survives the harsh climate of arid and semi-arid Australia by living on and within *Acacia* phyllodes. Comprised of at least 30 genera and 235 species, the *Acacia* thrips have developed several unique life history suites making them highly suitable for evolutionary studies (Crespi et al. 2004).

Acacia thrips species can be roughly split into four different groups based on their life history and ecology: 1) Gall-inducing thrips, 2) Phyllode-gluers, 3) Opportunists, and 4) Exploiters (Kleptoparasites and inquilines) (Crespi et al. 2004). Gall-inducers and phyllode gluers build domiciles in which they survive while the opportunists and exploiter species attempt to either parasitize domiciles or move into domiciles which have already been abandoned. These vastly differing life histories present in the *Acacia* thrips has made them an excellent model lineage for the comparative study of parasite – host dynamics (Chapman et al. 2006) ,

diversification (McLeish et al. 2007a,b), and particularly the development of group living and eusociality present in the gall-inducing *Kladothrips* and *Oncothrips* (McLeish and Chapman 2007).

Why study Kladothrips?

Kladothrips contain at least 24 described species, which form galls on growing *Acacia* phyllodes (a modified leaf) (Crespi et al. 2004; Wills et al. 2004; McLeish et al. 2006). The gall is formed by a previously mated foundress whereby once she is fully enclosed, deposits her eggs. After hatching, the brood feed on the gall interior by consuming the contents of singular plant cells. The brood either mature fully within the gall and then disperse or leave the gall as instars to pupate in the soil, depending on species. A gall can be long lived (1 – 2 years) and densely populated, with some species producing colonies of over 750 adults living together within a ~1cm^3 gall (Turnbull et al. 2011; Mound 1971). Relatedness within a colony is high (between 0.65 and 0.92) due to a combination of haplo-diploid sex determination and a high instance of inbreeding (McLeish et al 2006; Chapman et al. 2000).

The *Kladothrips* are useful for studying the evolution of eusociality for several reasons. Eusociality likely developed only once in the *Kladothrips* lineage roughly 6 million years ago (McLeish and Chapman 2007). To date, eusociality has been described in six species (Chapman et al. 2008; Crespi et al. 2004; Crespi 1992), each producing a soldier caste which protects the reproductive caste (Perry et al. 2004) and have reduced but varying levels of reproductive skew (Chapman et al. 2002). The remaining *Kladothrips* species form semi-social groups or are solitary (Crespi et al. 2004). The recent development of eusociality and presence of closely related sub-social species produces the ideal conditions for the comparative analysis of traits which are likely important for the development of complex sociality.

Similar to the allodapine and halictid bees, galling aphids, and wasps, *Acacia* thrips are now used as a model clade to study the life-history traits and environmental factors which may influence the development of eusociality (Chapman et al. 2008). So far, high relatedness and inbreeding (Chapman et al. 2000; McLeish et al. 2006), high kleptoparasitic pressure (Chapman et al. 2006), small brood (Kranz et al. 2002) and gall size (Kranz 2005; Wills et al. 2001) and a long duration within the gall (Chapman et al. 2008) were likely present and important for the origin of soldier production and thus eusociality in the *Kladothrips*. Recently, the role of pathogen defence in the development of eusociality has also been explored in this lineage, detailing how thrips must first overcome the increased microbial threat associated with social living before eusociality can develop.

Acacia thrips microbial defences

Antimicrobial defence has recently been described in several species of *Acacia* thrips and other thrips species (Turnbull et al. 2011, 2012a). The *Acacia* thrips protect themselves from microbial threats through the production of exterior antimicrobial compounds (Turnbull et al. 2011) with a portion of this antimicrobial activity likely being produced by symbiotic, antimicrobial producing *Streptomyces* species (Alteen 2012). The strength of these antimicrobials is positively correlated with increasing group size and social complexity within the thrips (Turnbull et al. 2011, 2012a), a trend also present in bees (Stow et al. 2007, 2010), and wasps (Hoggard et al. 2011), suggesting that more effective microbial defences are necessary for the development of social living. In the social *Kladothrips intermedius* (Froggatt), antimicrobial production may be a caste specific trait where washes of soldier caste individuals contain higher antifungal activity compared to dispersers (Turnbull et al. 2012b). This suggests that the production of these compounds may have developed as an altruistic trait in this species, similar to

the development of specialized forelimbs for the defence against *Koptothrips* invasions (Crespi 1992).

Although the ecological and evolutionary importance of *Acacia* thrips antimicrobial defences are beginning to be explored, more basic research is needed to fully understand how antimicrobial defence could affect the development of eusociality in this clade. Antimicrobial assessments have been carried out on relatively few species, and despite thrips secretions from distantly related thrips receiving in depth study (Suzuki et al. 2004), the antimicrobials and their source(s) in *Acacia* thrips remain unknown. This information would be useful for corroborating the findings of previous studies, comparing *Acacia* thrips antimicrobial strength and compounds with that produced by other social insect groups, and providing a source and structure to the antimicrobials allowing for comparison between solitary and social species. Uncovering how this lineage defends against pathogen attack will help clarify our understanding of how antimicrobial defence maybe linked to the development of eusociality in the *Acacia* thrips.

My research aims to determine if antifungal production is more widespread in the *Acacia* thrips by extracting and quantifying the antifungal activity of three gall-inducing species (*Kladothrips arotrum* and *K. tepperi*) and one Kleptoparasitic *Koptothrips* species (*Ko. dyskritus*) against *Cordyceps bassiana* spores, a common entomopathogen. Additionally, the effect colony maturity and size had on antifungal activity was tested to determine if a colonies antifungal defence changes as a colony matures. Chapter three focuses on the antifungal production of *K. sterni* morphs and attempts to determine if caste-specific antifungal production, similar to that found in *K. intermedius* soldiers, is also present in the non-dispersing gall-morph. The study presented in chapter four aims to determine the source and structure of the antifungal compounds in the *Acacia* thrips. This is done by extracting the defensive secretion produced by most *Kladothrips* species and testing it for antifungal activity against *C. bassiana* spores.

Subsequently, the main chemical compounds present in the secretion were then tested in the same manner for antifungal activity at biologically relevant concentrations. These studies attempt to increase our understanding of how the gall-inducing thrips protect themselves from fungal pathogens. The source, compounds, and factors which influence the strength of this defence are important for further studies involving the link between *Acacia* thrips antifungals and the development of sociality. Due to chapter two being published prior to the submission of this thesis, (Coates et al. 2017) this chapter is presented in manuscript format. The two subsequent data chapters are presented in thesis chapter format. Following the data chapters is a brief general discussion on the importance of this work and supplementary material.

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Table legend

Table 1.1: Examples of externally secreted compounds produced by social insect groups which have been found to have antimicrobial effects.

Table list

Table 1.1

Group	Source	Compound	Effective against	Reference
Ants	Metaplural gland	Phenylacetic acid Indolacetic acid Hexanoic acid Octanoic acid	*Candida albicans* *Staphylococcus aureus* *Escherichia coli*	Bot et al. 2002 Mendonça et al. 2009
			*Bacillus subtilis** *Beauveria bassiana* *Leucoagaricus gongylophorus* *M. anisopliae* *Aspergillus niger** *Pseudomonas stutzeri* *Escovopsis spores* *B. bassiana spores* *M. anisopliae spores* *Aspergillus niger spores* *Gliocladium virens spores*+* *Trichoderma sp. spores*+* *Gliocladium virens**	Bot et al. 2002
		Phenylactic acid 3-hydroxydecanoic acid Indoleacetic acid	*Bacillus sphaericus* *Pseudomonas putida* *Bacillus cereus* *P. fluorescens* *E. coli* *S. aureus*	Nascimento et al. 1996
	Mandibular gland	Citral 4 – Methyl – 3 heptanol 2 – heptanone 3 – Octanone 4-Methyl-2-heptanone β-Citronellol Geraniol	*Candida albicans* *Staphylococcus aureus* *Escherichia coli*	Cole et al. 1975 Mendonça et al. 2009
	Venom gland	Alkaloids	*B. bassiana* *M. anisopliae* *Paecilomyces fumosoroseus*	Storey et al. 1991
		Pilosulin (peptide)	*E. coli* *S. aureus* *C. albicans*	Zelezetsky et al. 2005

Termites	Queen pheromone	*n*-butyl-*n*-butyrate 2-methyl-1-butanol	*Fibularhizoctonia sp.*	Matsuura and Matsunaga 2015
	Frontal gland	α-pinene β- pinene limonene	*M. anisopliae*	Rosengaus et al. 2000
		trinervitane diterpenoids	*Bacillus subtilis*	Zhao et al. 2004
	Salivary gland	β-1,3-glucanase	*M. anisopliae*	Hamilton et al. 2011
		β-1,3-glucanase Termicin	*M. anisopliae*	Hamilton and Bulmer 2012
	Sternal gland	*n*-hexanoic acid$^{\theta}$	*M. anisopliae*	Rosengaus et al. 2004
Bees	Venom gland	Mellitine venom	*E.coli* *S. aureus* *Salmonella typhyimurium*	Zolfagharian et al. 2016
		Lasioglossins (peptides)	*P. aeruginosa* *S. aureus*	Čeřovský et al. 2009
		Halictines (peptides)	*P. aeruginosa* *S. aureus*	Monincová et al. 2010
Wasps	Venom gland	Peptides (Hover wasp)	*Bacillus subtilis* *Escherichia coli* *Saccharomyces cerevisiae*	Baracchi et al. 2012
		Dominulin A$^{\Psi}$ Dominulin B$^{\Psi}$	*B. subtilis* *E. coli*	Turillazzi et al. 2006
		Peptides (Mastoparans)	*B. subtilis* *Staphylococcus aureus* *E. coli* *Pseudomonas aeruginosa*	Čeřovský et al. 2008
	Salivary gland	Unknown	*B. subtilis* *E.coli*	Turillazzi et al. 2004

*= no effect by Indoleacetic acid, + = No effect by Acetic acid, Ψ = also present in Dufour's gland, θ = First described as a secretion from the sternal gland however this was redacted in Rosengaus et al. 2011 instead stating *n*-hexanoic acid was present on the cuticle.

Chapter 2

High density brood of Australian gall-inducing *Acacia* thrips aid in fungal control

Running title: Antifungal Production in *Acacia* thrips

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Abstract

Kladothrips (Froggatt) is a genus of gall-inducing thrips that develop broods, and in some species, long-lived colonies within galls they form on phyllodes of *Acacia* in arid and semi-arid Australia. The gall interior is a stable environment for thrips in an otherwise inhospitable environment, but these conditions may also be favorable for fungal parasites. This fungal threat is corroborated by the observation that *Kladothrips* produce highly effective antifungal compounds. Here we investigated antifungal production in three *Acacia* thrips species, two gall-inducers: *Kladothrips arotrum* and *Kladothrips tepperi*, and one kleptoparasitic thrips, *Koptothrips dyskritus*. Using a spectrophotometer, the germination of a suspension of *Cordyceps bassiana* spores (an entomopathogenic fungus) can be detected by observing an abrupt increase in light absorption by the suspension. The addition of thrips exterior washes to these fungal spore suspensions resulted in significant delays in fungal germination for all three species. Foundresses in both *Kladothrips* species strongly delay fungal germination before their brood has matured. Young and maturing colonies with less than 50 adult individuals (the remainder of the brood are in juvenile stages of development) produced some antifungal effects but within the range produced by the foundress alone. Mature colonies (>100 adults) delayed germination for the duration of our observational window (48 hours), suggesting a possible group size threshold for antifungal effectiveness. *Koptothrips dyskritus* antifungals were observed to be the strongest of the three species when <50 individuals were present. The strong antifungal abilities of the invading *Ko. dyskritus* would allow this species to invade older or damaged galls, which has been observed in the field. This pattern of antifungal activity in *Acacia* thrips suggests that effective defence against fungal pathogens is strongly associated with group size and colony maturity.

Introduction

Among the two dozen described species, the gall-inducing *Kladothrips* show significant variation in life history ranging from solitary to eusocial (Crespi et al. 2004). Gaining information on the natural history of taxonomic groups that show variation in sociality is valuable to identifying characteristics associated with the transitions from solitary to social living. A model clades approach (Chapman et al. 2008) has revealed social transitions, and species-level characteristics that are potentially associated with the evolution of sociality including, origins and losses of sociality (Crespi 1996), relatedness (Chapman et al. 2000), gall volume (Wills et al. 2001), soldier reproduction (Chapman et al. 2002), inbreeding (McLeish et al. 2006), and origins of soldiering (McLeish and Chapman 2007). In this current study we focus on social immunity; that is, communal nest site protection from microbes (Cremer et al. 2007). Social immunity could play a role in social evolution by extending the lifespan of colonies and, thus, setting the stage for cooperation to evolve among conspecifics. Two solitary and one eusocial species of gall-inducer have already been shown to have the ability to retard fungal growth (Turnbull et al. 2012a, b). In this present study we extend this comparative data-base by describing antifungal production in an additional solitary gall-inducer, and a kleptoparasitic *Acacia* thrips species that specializes in gall invasions.

Kladothrips (Froggatt) is a genus of small-bodied (<2mm) thrips that survive the harsh environment of arid and semi-arid Australia by living within galls they induce on *Acacia* phyllodes (Mound et al. 1996; Morris et al. 1999; McLeish et al. 2007). As the colony grows, individuals live in the gall at high densities, come in frequent physical contact with one another, and are highly related (Crespi et al. 1997, 2004), producing ideal conditions for the spread of pathogens, similar to that experienced by the major social insect groups (Schmid-hempel 1998; Zasloff 2002; Stow and Beattie 2008). To offset this increased microbial threat, the social insects

invest in social immunity (Cremer et al. 2007), where individuals cooperatively defend against harmful microbes through behaviours such as allogrooming (Walker and Hughes 2009, 2011; Reber et al. 2011; Evans and Spivak 2010; Rosengaus et al. 1998), and/or the production of externally secreted antimicrobials (Baracchi et al. 2011; Rosengaus et al. 2000, 2004; Rosengaus and Savoie 2002; Hamilton et al. 2011; Beattie et al. 1986; Veal et al. 1992; Stow and Beattie 2008). Recently, the Australian *Acacia* thrips have also been found to invest in social immunity through the production of antimicrobial compounds, providing a model lineage to explore how this defence could possibly be linked to the development of sociality (Turnbull et al. 2011, 2012a).

Antimicrobial (antibacterial and/or antifungal) production has been described in nine thrips species, including three gall-inducing thrips: *Kladothrips arotrum* (Mound 1971), *K. antennatus* (Moulton), and *K. intermedius* (Bagnall) and one domicile-builder: *Dunatothrips vestitor* (Mound and Morris) (Turnbull et al. 2011, 2012a). In the *Acacia* thrips, antimicrobial strength increases with increasing group size and social complexity (Turnbull et al. 2011, 2012a) suggesting that microbial defence may be important for social evolution, a trend also described in Allodapine bees (Stow et al. 2007, 2010) and wasps (Hoggard et al. 2011). Antifungal production might also be associated with altruistic behaviour in the eusocial *Kladothrips intermedius*, where antifungal production of the altruistic soldier caste is significantly higher than that of the reproductive dispersing caste (Turnbull et al. 2012b). These studies suggest that antimicrobial production increases, and is specialized in more socially complex species however, a description of the microbial defence in additional species will provide a more comprehensive knowledge of the extent that social living in the thrips is associated with antimicrobial production.

In addition to the gall-inducers, kleptoparasitic thrips species also rely on the gall structure for survival (Crespi et al 2004), and therefore are likely to experience similar selective

pressures from microbial pathogens. Foundresses of *Koptothrips* (a non-sister genus to the *Kladothrips*) specialize in usurping the gall from the original gall-inducer and raising their brood within the structure (McLeish et al. 2007; Morris et al. 2002; Crespi and Abbot 1999). All of the gall-inducing species which have been tested for antifungal production (Turnbull et al. 2012b) are also invaded by *Koptothrips* species (Crespi and Abbot 1999), raising the question of whether the invader makes use of the host compounds or produces its own. There is some evidence that *Koptothrips* invasions occur either when a gall is very young (Chapman et al. 2006), or old (Crespi and Abbott 1999) depending on species therefore, the timing of antifungal production in the host thrips may affect the invaders investment in their own antifungal defence. If *Koptothrips* produce their own antifungals, it suggests that this defence may be widespread in the *Acacia* thrips and important for gall living in both parasite and host.

Although the evolutionary aspects of antimicrobial production in the *Acacia* thrips are becoming clearer, the ecological patterns of production, like how microbial defence varies during a colonies lifecycle, remain unknown. Young *Kladothrips* colonies have a high density of larvae which, like most immature insects, likely do not possess developed immune systems (Gillespie et al. 1997), and are unable to self-groom or produce antimicrobial compounds (Tranter et al 2014). For these reasons *Kladothrips* colonies may be especially vulnerable to microbial attack early in its lifecycle. In other social insects, larvae rely on the social immunity provided by adult individuals including allogrooming and the application of antimicrobial compounds (Tragust et al. 2013; Obin and Vander Meer 1985; Baracchi et al. 2011). *Kladothrips intermedius* may similarly protect the immature kin through increasing antifungal production in the first eclosing adults, in this case the soldier caste (Turnbull et al. 2012b). Increased antifungal production of the first eclosing adults may be widespread in *Acacia* thrips, however the pattern of antifungal production as colonies mature has not been tested in other species.

In the present study, we describe the pattern of antifungal production in *Kladothrips arotrum* and *Kladothrips tepperi* (Uzel 1905) colonies at differing maturities, from single foundresses to fully mature colonies with over 800 adults. We also gathered data on antifungal production of the invading parasite *Koptothrips dyskritus* (Mound 1971), especially for comparison with the antifungal production of the host species. This study provides the first report of antifungal production in *K. tepperi* and *Ko. dyskritus* and further describes the relationship between colony maturity and antifungal production in the *Kladothrips*, highlighting the potential importance of social immunity for these group living insects. Admittedly, we are unable to make any conclusions on the role social immunity plays in the evolution of sociality in this clade, but our focus here on the demographics of antifungal production within a gall, will help refine the evolutionary questions that could be answered using this system in future studies.

Methods

Sample collection

Spherical galls of *Kladothrips arotrum* (Mound) and *Kladothrips tepperi* (Uzel) were collected from 50 *Acacia aneura* (F. Muell.) trees (all plant specimens were over 2 metres in height) within 100 km of Quilpie, Queensland, Australia (26.6167° S, 144.2667° E). Sampled trees were in close proximity so that the galls were expected to have experienced similar climatic conditions and risk of invasion for both macro and microbial enemies. Galls were placed in site-labeled individual plastic bags, kept cool and then transported to Macquarie University, Sydney, NSW where they were maintained at 4°C until extraction took place (within 3 weeks). Previous work has shown that thrips galls can be kept for several weeks with no harm to the occupants. Thrips were identified both by gall morphology and species descriptions and photographs (Crespi et al. 2004). *Koptothrips dyskritus* (Mound) samples were collected from *Kladothrips* galls or

taken from the plastic bags in which galls were temporarily stored. In galls which contained *Koptothrips* the original gall-inducer species is unknown because it is expelled or killed in the invasion. In these cases, we attempted to use gall morphometrics to identify the original gall-inducer. The external length, width and height from identified *Kladothrips* species were compared to that of invaded galls through the use of a kernalized support vector machine (SVM) with a radial basis function kernal using the *kernlab* package (Karatzoglou et al. 2004) in R (R Core Team 2014) to predict which gall-inducer was most likely to have originally occupied the gall. Cleared slide mounted vouchers were produced for all thrips species collected and are now stored at Memorial University in St. John's, Newfoundland and Labrador.

Fungal culture

Cordyceps bassiana (bals.) was cultured from natural populations of *Exoneura nigrescens* (an allodapine bee) found in Victoria, Australia (Stow et al. 2010). *Cordyceps bassiana* has been found associated with other thrips species and is used as an effective biocontrol agent against thrips species (Shaw and Pell 2003). Previous studies involving thrips antifungals have also used this strain as a proxy for natural fungal pathogens (Turnbull et al. 2012a, b). For this study, the fungus was cultured on Luria Broth (LB) agar plates at 25°C in an incubation chamber for 2 – 4 weeks to allow the formation of spores. The culture was then left at room temperature until needed.

Antifungal extraction and quantification

Galls were randomly selected from the original samples. Sterile techniques were used to remove thrips from the galls and established laboratory protocols were utilized to extract possible antifungal compounds from the mature adult individuals (see Turnbull et al. 2011). Each extract contained the antifungal compounds from mature adults of one gall only; no pooling of extracts

was carried out. The adult and larval bodies were stored in 90% ethanol until species and brood size could be determined.

The antifungal assays we used are described in Smith et al. (2008). Antifungal suspensions were kept on ice until dried into a Quickfit ® tube by adding 150µL at a time and then subjecting this portion of the suspension to 25 millibar pressure at 24°C using the Rotavapor R-210 (BÜCHI, Switzerland) until all the liquid evaporated. Each sample was dried in this way until no suspension was left in the original microcentrifuge tube. That tube was washed three times with 150µL of 90% ethanol and dried in the same way to minimize the amount of suspension lost via transfer. After the suspension and subsequent washes were dried, the Quickfit ® tube was sealed with parafilm®, labeled, and kept on ice.

In a UV light sterilized laminar flow hood 130µL of LB was added to each Quickfit ® tube and then sealed with parafilm before being vortexed for 2 – 3 minutes to suspend any antifungal elements. The LB suspension was then pipetted from the Quickfit ® tube into a 1.5mL microcentrifuge tube and then centrifuged at 14,000 rpm for 2 minutes to pellet any debris. 100µL of the LB suspension was carefully pipetted away from the debris and placed in a well of a 96 well culture plate.

The fungal spore solution was prepared on the day that samples were run by first combining 10mL of LB and 10µL of 0.1% Tween 80 solution into a sterile McCartney vial. The LB and Tween solution then received 1mg of *Cordyceps bassiana* spores and vortexed. 100µL of this fungal spore solution was added to each sample and positive control wells. Positive control wells received an additional 100µL of pure LB, matching the volume in treatment wells. The wells surrounding treatment and positive control wells were filled with 200µL LB as negative

controls. The plate lid was sealed with parafilm® to prevent evaporation during the spectrophotometry analysis.

The loaded plate was placed in a VERSA max tunable microplate reader (Molecular Devices) and the change of optical density (OD) over time was measured using the SoftMax Pro program. The spectrophotometer was set to analyze at a 405 nm wavelength for 48 hours while incubating at 25°C for the duration.

Fungal growth in wells containing only fungal spores and growth media (positive controls), began between 16 to 24 hours after plating with optical densities (OD) close to zero in the 0 to ~16 hour lag phase. Once there was germination and hyphal growth a linear growth phase was observed. Some treatments, containing thrips washes, showed some minor stochasticity in optical density readings before germination took place, presumably caused by fine particulate matter that was not removed completely by the centrifugation step of the thrips washing procedure. Time of germination was determined as the point in time when a sample surpassed 0.05 OD and showed a strong linear increase in OD thereafter (junction between the lag and linear growth phase). The delay of germination caused by experimental samples was calculated by subtracting the average positive control germination time from the germination time of each experimental well. At least 20 negative controls (LB with no fungal spores added) were included for all microplate readings.

Statistical analysis

Eight microplates contained the entirety of our treatments. Each plate was prepared and run on the spectrophotometer on separate days. In each case, ten positive controls (LB broth and fungal spores) were added to each plate. One plate contained six positive control wells only, and for the purposes of the subsequent analysis was made equivalent to ten by generating four

additional bootstrap samples. These controls had the same fungal spore concentration as each other and the treatments on the same plate because they were prepared from the same fungal spore / LB broth preparation on that day. A repeated measures analysis of the positive control germination times was used to test the within plate repeatability of these samples (outlined in Arnqvist and Mårtensson 1998).

Initially the dataset was analysed as a whole and the delay of germination was plotted against the number of adults present within a gall (Fig1a). At the 100 individual mark the samples effectively delayed germination past our 48 hour sampling window for both *Kladothrips* species. (No *Koptothrips* samples exceeded this number of individuals). Samples which delayed germination past our 48 hour sampling window provided evidence for the presence of antifungal compounds, however, they cannot provide any information on antifungal effects with respect to group size and were therefore removed from further analysis. In order to visualize the effectiveness of antifungals in young broods and the effects of group size across all three species the data were truncated to include only samples with up to 50 adults present within a gall (Fig1b). Using R (R core team 2014) a linear mixed effects model was fit to this truncated data set to assess the effect of species on germination time. The model included species as a fixed effect and number of adults and the plate (batch) as random intercept random effects. The average delay of germination for each species is presented with standard deviation as the measure of variability.

Results

One hundred and ninety-nine *Kladothrips tepperi* or *K. arotrum* galls were randomly opened and inspected. Galls that did not contain the gall-maker were either empty (9/199, mostly containing frass and/or fungus), contained unidentified immature stages of flies (Diptera) and moths (Lepidoptera) (66/199) or contained the gall-invader *Koptothrips dyskritus* (11/199).

Based on gall morphometrics, the SVM classified the species of gall-inducer with an overall accuracy of 78%, species-specific accuracies were 90% (*K. tepperi*) and 67% (*K. arotrum*) as determined by leave-one-out cross-validation. Measurements for the galls in which *Ko. dyskritus* colonies were found, were subsequently analysed through the SVM to predict the likely identity of the original gall-inducer. Of the 11 galls which contained invaders, the model predicted 9 were induced by *K. tepperi* and 2 by *K. arotrum*. The combination of a low number of *K. arotrum* galls invaded with the lower accuracy for SVM determination for this species provides some support for this population of *Ko. dyskritus* to be a specialist invader of *K. tepperi* galls. However, a previous study found that a population of *Ko. dyskritus* was able to invade the galls of multiple gall-inducer species on a different *Acacia* host (Gonsalves 2010); therefore, we assert here that, regardless of whether this population of invader is specializing on *K. tepperi* or not, our collection of *Ko. dyskritus* represents a single population.

The germination time of fungal controls within plates was highly repeatable (r =0.983), showing that within plate error was minimal. No growth or change in OD was observed in any of the 208 negative control samples tested.

Thrips-washes showed an inhibitory effect on *Cordyceps bassiana* spore germination in all but three samples delaying germination compared to the positive controls. Many of the *Kladothrips* samples delayed germination longer than our 48 hour sampling window (*K. arotrum*: 14/30; *K. tepperi*: 13/ 29 (Fig.2.1)). The occurrence of samples containing thrips extract germinating before the fungal spore positive controls was rare, however, three samples germinated before the positive controls in their respective plates: one in *K. tepperi* (3.6 hours) and two in *K. arotrum* (0.85 and 6.73 hours)(Fig 2.1). For the two *Kladothrips* species, small group sizes (<100 individuals) had varying effectiveness against fungal germination, but when a

group contained more than 100 individuals, nearly all samples inhibited germination past the 48 hour sampling window (Fig 2.1a).

With a focus on the *Kladothrips* (host) galls which contained fewer total individuals, a comparison with the invading parasite *Ko. dyskritus* whose numbers were always low could be made. Thus, for those host galls with less than 50 individuals (Fig 2.2), we fitted a linear mixed effect model to determine if species had an effect on fungal spore germination, while controlling for brood size and random variation between plates. The model explained 26.49% of the variation in the data (conditional R^2; Nakagawa and Schielzeth 2013) with the fixed effect of species accounting for 26.19% of the total variation (marginal R^2; Nakagawa and Schielzeth 2013). This result suggests that random effects play a very small role relative to the effect of species. Other variables which could explain the variation in the data may include micro-environmental differences between galls, the colonies health at the time of sampling, previous pathogen attack causing changes to antifungal production, and differences in inherited antifungal ability, among others. In most cases these variables are difficult or even impossible to measure during sampling, for this reason we chose to focus on variables established in previous studies (Turnbull et al. 2011, 2012a, b; Stow et al. 2007, 2010). The effect of species on the delay of germination was significant, χ^2 (df = 2, N = 82) = 15, p< 0.001, *Kladothrips arotrum* caused 4.74 ± 3.94 hours of delay, *K. tepperi* delayed germination by 1.70 hours longer (6.44 ± 5.04 hours) and *Ko. dyskritus* even longer, taking an extra 6.64 hours to germinate compared to *K. arotrum* (11.4 ± 5.13 hours)(Fig 2.2).

Discussion

The washes of *K. arotrum*, *K. tepperi* and *Ko. dyskritus* effectively slow or inhibit the germination of *Cordyceps bassiana* spores within our observation window (Fig 2.1, Fig 2.2). This study adds *K. tepperi* and *Ko. dyskritus* (*K. arotrum* having been previously tested) to the

growing list of *Acacia* thrips which produce antifungals (Turnbull et al. 2012b). Similar to Turnbull and colleagues (2012b) previous study, antifungals extracted from larger groups of *K. arotrum* (>100 individuals) were highly effective at slowing fungal germination and growth however we found that small groups also produced significant delays in fungal growth (Fig2.1b, Fig 2.2). The observation of all three gall-living species producing effective antifungals is consistent with an increased threat of microbial attack when living in high density groups within a common 'nest' structure (Schmid-Hempel 1998). A more in-depth analysis of antimicrobial production in additional *Acacia* thrips species, both gall-living and not, would be required to reinforce this supposition, and to provide evidence for the importance of social immunity to the development of group-living and sociality in the thrips.

Antifungal production was observed in all group sizes including samples which contained only the foundress and *Kladothrips* colonies with 50 individuals or less (Fig 2.1b). These small colonies delayed *C. bassiana* spore germination within the range of that produced by singular foundress samples (Fig 2.1b). In the *Kladothrips* species studied here, no discernable increase in antifungal production was present in the first eclosing individuals. However, the pattern we did observe suggests that antifungal production is required from the very start of the colony, previously not thought to occur until group size was 50 individuals or more (Turnbull et al. 2012b). Immature *K. arotrum* and *K. tepperi* individuals are initially protected by the foundress who effectively produces a base line of antifungal protection until the brood begin to eclose. Antimicrobial maternal care has been documented in several insect groups including the European earwig (*Forficula auricularia*) (Boos et al. 2014), the fire ant (*Solenopsis invicta*) (Vander Meer and Morel 1995) and the subterranean termite (*Reticulitermes speratus*) (Matsuura and Matsunaga 2015) which protect their eggs using antimicrobial secretions. Parental care has

been previously described in other thrips species (Tallamy 1984), but this is the first report of *Acacia* thrips foundresses providing antifungal protection to their young.

As a colony matures and the number of adults approaches 100 individuals, antifungal effectiveness in *K. arotrum* and *K. tepperi* colonies sharply increases (Fig 2.1a). The majority of *Kladothrips* colonies with over 100 adult individuals inhibited the germination of *C. bassiana* spores past our 48 hour observation window. At this size, a colony may produce enough antifungals to completely inhibit fungal germination, effectively protecting it from natural fungal invasions (Fig 2.1a). A similar threshold has been proposed in earlier studies where the effectiveness of antibacterials (Turnbull et al. 2011) and antifungals (Turnbull et al. 2012b) sharply increased when 50 – 100 individuals are present. If there is a group-size threshold for effective social immunity in the *Kladothrips*, this may have selected for increasing colony size, and a longer time spent within the gall, thus producing ideal conditions for the development of cooperation among kin.

When compared to their gall-inducing hosts, *Ko. dyskritus* antifungals were significantly more effective in delaying the germination of *C. bassiana* spores (Fig 2.2). The increased delay in fungal germination, produced by *Ko. dyskritus* washes, suggests that this species may produce its own antifungal compounds. *Ko. dyskritus* are known to invade old, abandoned *Kladothrips* galls (Crespi and Abbott 1999) where harmful fungi may already be present, necessitating the production of their own antifungals. Alternatively, this species may preferentially invade mature *Kladothrips* colonies effectively usurping the gall structure and any residual antifungal compounds. Gall tissue has previously been found to have no antimicrobial activity (Turnbull et al. 2011) however, no studies have directly focused on how long residual antimicrobials remain active within a gall once the host *Kladothrips* have left. The *Koptothrips* and *Kladothrips* are non-sister taxa (McLeish et al. 2007; Morris et al. 2002; Crespi and Abbot 1999) and the

potential discovery of effective antifungals in the *Koptothrips* reinforces the supposition that fungal defence is widespread in group-living *Acacia* thrips and may be important for living within the gall.

An ancestral ability to produce antifungal compounds likely aided the development of group living and larger colony sizes in the *Acacia* thrips. The protothysanopteran has been proposed to be a fungal feeder that inhabited leaf litter (Mound et al. 1980; Nel et al. 2013) where defence against fungal pathogens would be necessary. This defence likely remains important in many extant thrips species (including some *Kladothrips*) which pupate in the soil (Crespi et al 2004; Kranz et al. 2001; Crespi and Abbot 1999), where pathogenic fungus would be a threat. The production of compounds thought to act as antifungals has been observed in many different species of thysanoptera (Blum 1991; Blum et al 1992; Suzuki et al 2004; De Facci et al 2014) suggesting that microbial defence is widespread and important to this lineage. Studying this co-evolutionary relationship appears to be critical for understanding the development of group- and gall-living in the *Acacia* thrips, however the source of microbial defence in this lineage has yet to be fully described.

Although the effectiveness of *Acacia* thrips antimicrobials is being revealed, the source and structure of the compounds remains relatively unknown. A possible source of antimicrobials is an anal secretion, which in other thrips genera has been found to contain compounds likely used for communication (Teerling et al 1993, Suzuki et al 1988), defence against predators (Suzuki et al. 2004; Blum et al. 1992; Blum 1991; Howard et al. 1983), and fumigation against harmful microbes (Suzuki et al. 2004; De Facci et al. 2014). Several strains of *Streptomyces* with extensive antibiotic activity against bacteria and fungi have also been cultured from whole *K. intermedius* galls (Alteen 2012), a bacterial genus also used by the European beewolf (*Philanthus triangulum*) for antifungal defence of their larvae (Kaltenpoth et al. 2005). How closely these

microbes are associated with the thrips is not known, but their presence suggests a possible source of the antimicrobial substances involved in this gall-inducer story.

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Figure Legends

Figure 2.1: A scatterplot for the delay of germination in hours (h) (y-axis) as a function of the number of adults sampled (x-axis) in each of the three thrips species (see figure key). (a) Shows all samples in our study ranging from a single foundress to broods up to a maximum of 800 individuals. (b) Shows a subset of our complete data set of germination delay (h) relative to number of adults. In this panel, the maximum number of individuals was restricted to 50 or less. Lines of best fit included to clarify the trend observed from each species.

Figure 2.2: The delay of germination in hours (h) caused by the experimental washes of each thrips species when group size was below 50 individuals (truncated data).

Figures

(a)
Delay (Hours)
Number of Adults
Species
K. arotrum
K. tepperi
Ko. dyskritus
(b)
Delay (Hours)
Number of Adults

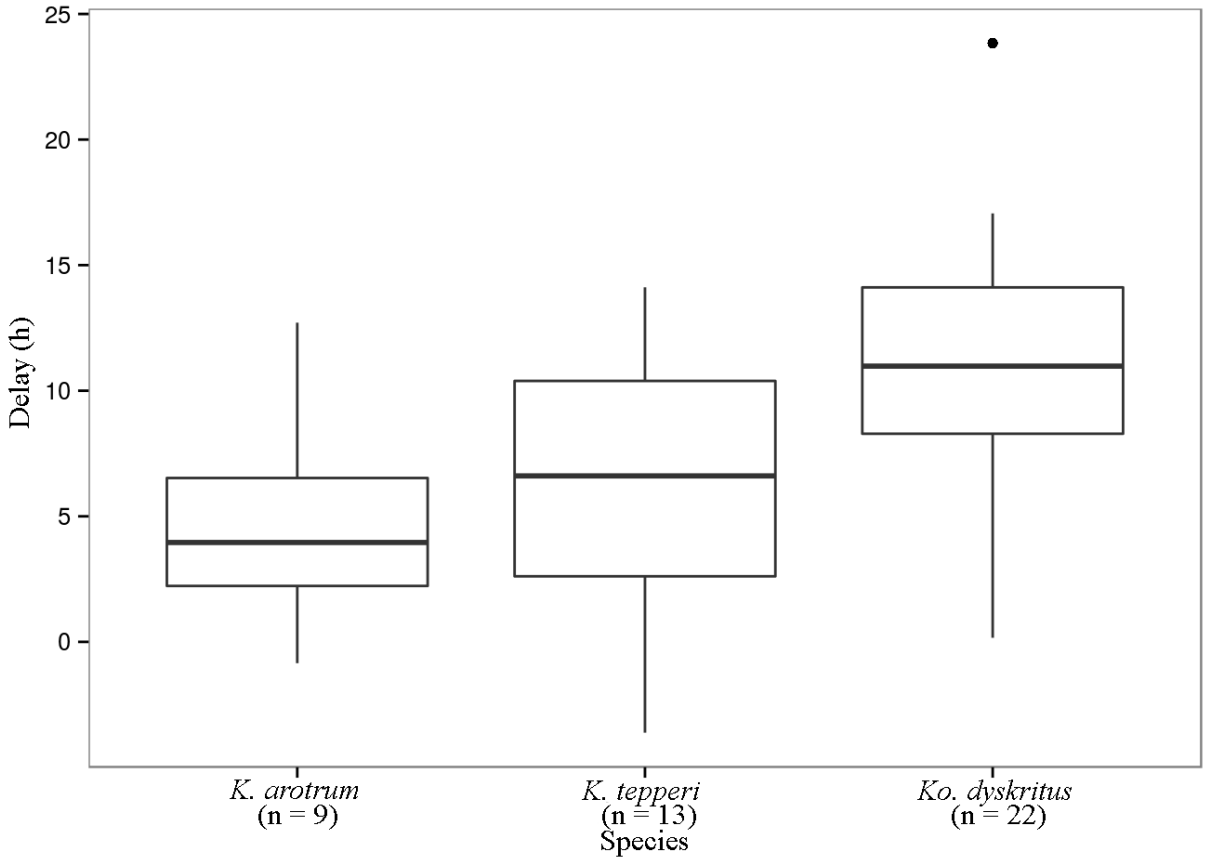
25
20
15
10
5
0
Delay (h)
K. arotrum
(n = 9)
K. tepperi
(n = 13)
Ko. dyskritus
(n = 22)
Species

Figure 2.2

Chapter 3

Patterns of antifungal activity in *Kladothrips sterni* foundresses, gall-morphs, and larvae

Abstract

In addition to defending the colony from invading *Koptothrips*, soldier caste individuals in *Kladothrips intermedius* produce more antifungals than that of the reproductive caste, raising the question of which defence, morphological or antifungal developed first in this caste. This study focuses on *Kladothrips sterni*, a species that produces a gall-morph caste which is morphologically similar to the soldiers of eusocial species but lacks the enlarged forelimbs and behaviour for macro defence. If *K. sterni* gall-morphs produce higher antifungal activity than others in the gall, it suggests that microbial defence develops before macro defences and may be important for the initial development of group-living and subsequently sociality. Here, we test extractions taken from *K. sterni* foundresses, gall-morphs and larvae for antifungal activity against *Cordyceps bassiana* spores using spectrophotometry. Extractions from all morphs produced antifungal activity. Foundresses produced significantly more antifungal activity compared to gall-morphs. These data therefore provide no evidence that gall-morphs have comparatively strong antimicrobials, such as is present in soldiers of other species, however, evidence for maternal antimicrobial care and larval investment in microbial defence was present.

Introduction

Social insect colonies are long-lived, and contain highly related individuals occurring in high population densities (Wilson 1971). Due to these traits, social insects experience an increased risk of pathogen infection (Schmid-Hempel 1998). In addition to developing antimicrobial behaviours and secretions (Cremer et al. 2007), some species have offset this threat by producing castes that specialize in defending the colony from harmful microbes. Caste-specific antimicrobial defences are common within the fungus-farming ants, where the minor workers principally weed the fungus garden of microbial pest species (Currie and Stuart 2001; Gerstner et al. 2011) and possess larger antimicrobial-producing metapleural glands compared to larger workers respective to body size (Hughes et al. 2010). Caste-specific immune responses are also present in ant (Koch et al. 2013) and termite species (Rosengaus et al. 2007), suggesting that these individuals are more likely to undertake pathogenically risky behaviours. Caste-specific antifungal production has also been discovered in the eusocial *Kladothrips* where the altruistic soldier caste in *K. intermedius* defends the colony against both large and microbial invaders.

Eusocial *Kladothrips* species produce two distinct castes within the colony, dispersers and soldiers (Crespi 1992). Dispersers make up the reproductive caste which will eventually become either foundresses or mates of foundresses. Soldiers eclose before the dispersing caste and defend the gall from *Koptothrips* invasion using enlarged forelimbs (Crespi 1992; Perry et al. 2004). Morphologically, soldiers differ from dispersers in having reduced or missing wings and flight musculature (Chaulk et al. 2013), reduced melanisation of the cuticle, and a reduced reproductive potential (Chapman et al. 2002). In the eusocial *Kladothrips intermedius*, the soldier caste has been found to also produce significantly more antifungals than that of the dispersing caste (Turnbull et al. 2012b), thus likely representing a caste-specific antimicrobial defence. The dual role of the soldier caste raises the question of which defence developed first. To potentially

answer this question we focus on *Kladothrips sterni*, a species that produces a gall-morph caste which is morphologically similar to soldiers but does not defend the gall from large invaders.

Kladothrips sterni (Mound, Crespi, and Kranz) induces galls on *Acacia aneura* in Western Australia and Queensland, Australia (Morris et al. 2001, McLeish et al. 2007) and can produce long-lived (~2 years) colonies of over 1400 individuals (Mound et al. 1996). The gall contains multiple partitions inside, forming locules which connect close to the ostiole, unique among *Kladothrips* (Mound et al. 1996; Crespi et al. 2004). Colonies are composed of two castes: dispersers, and a non-dispersing caste known as gall-morphs which are morphologically similar to soldier caste individuals in eusocial species like *Kladothrips intermedius* (Mound et al. 1996). Gall-morphs eclose before the rest of the brood and have reduced cuticular melanisation, do not develop wings, and have reduced eye development, however, they do not possess the enlarged forelimbs of soldiers, used to defend the gall from kleptoparasites (Crespi and Worobey 1998; Crespi et al. 2004; Mound et al. 1996).

Gall-morphs may therefore represent potential proto-soldiers which have developed the non-dispersing morphology but not the enlarged forelimbs for colony defence (Morris et al. 2001; Mound et al. 1996). These individuals may have instead developed as a ‘medic’ caste and have lost their ability to disperse by investing heavily in antimicrobial defence. The development of caste-specific antimicrobials may therefore be the first step in the development of a non-dispersing caste and subsequently soldiers and eusociality in the gall-inducing thrips. To date however, no antifungal studies have been carried out on *K. sterni* to test this hypothesis.

This study aims to determine if *K. sterni* foundresses, gall-morphs and larvae produce effective antifungal compounds. We collected antifungal extractions from all three groups and measured their effect on *Cordyceps bassiana* (bals.) spore germination using spectrophotometry.

If gall-morphs produce more antifungal activity than foundresses, it suggests that increased antifungal production is potentially linked to the non-dispersing morphology and early eclosion in the *Kladothrips*. If however, higher antifungal activity is observed in foundresses, this supports the hypothesis that these individuals invest heavily in sterilizing the gall interior before her brood reach adulthood, as described in *K. arotrum* and *K. tepperi* foundresses (Coates et al. 2017). Finally, we test for antifungal activity in *K. sterni* larvae to determine if immature individuals contribute to pathogen defence in species that produce long-lived colonies. This study is the first description of antifungal production in *K. sterni*. Describing the antifungal patterns of this species is important for understanding the role gall-morphs assume, as well as how caste-specific antifungal production may have developed in this lineage.

Methods

Sample collection

Kladothrips sterni (Mound, Crespi & Kranz) galls were collected from *Acacia aneura* roughly 300 km south of Newman, WA along highway 95 (S25°45’53.3 E119°08’37). On this occasion the galls were extremely scarce with only a single tree found to have living colonies, the majority of which were at an early stage of maturity. Galls were collected from this tree and were transported to Macquarie University in a cooler, making sure that the galls were never in direct contact with ice. Galls were then stored at 4°C until extraction took place.

The distribution and annual life-cycle of many *Kladothrips* species is unpredictable and sporadic. This unpredictable nature, combined with the costs associated with sampling in remote areas of arid Australia produces difficulties in effectively sampling gall-inducing species. Thus, the sample size present within this study is small (n = 11 for foundress, n = 4 for gall-morph, and n = 3 for larvae dilution series) and galls with adult gall-morphs and dispersers were not

collected. Due to this lack of disperser caste individuals within the same gall, we were unable to determine if the gall-morph possesses increased antifungal capabilities similar to that found in *K. intermedius* soldiers. This study therefore serves as a preliminary report documenting the presence of antifungal activity in the extractions of *K. sterni* individuals.

Fungal culture and antifungal extraction

Before opening each gall, the height, length and width (mm) of each gall were measured using calipers. After the gall was opened, individuals were sorted depending on life stage and caste by placing foundresses, gall-morphs and larvae in separate microcentrifuge tubes. Standard sterile techniques were used to remove thrips from the galls and antifungal compounds were extracted using previously established laboratory techniques (Turnbull et al. 2011; Coates et al. 2017). The antifungal suspensions were stored at -20°C until required and the thrips were preserved in 90% ethanol. Identification of *Kladothrips sterni* was carried out using species and gall descriptions (Crespi et al. 2004; Mound et al. 1996).

Fresh *Cordyceps bassiana* cultures were produced from storage stocks as per pre-established laboratory techniques (Turnbull et al. 2012a).

Antifungals were extracted from the suspension and pitted against *Cordyceps bassiana* spores using established protocols (Coates et al. 2017) with some alterations. Most galls only contained a single foundress and her larvae, but four galls also contained gall-morphs. Due to the small brood sizes of the collection, extractions of 10 individuals were pooled for foundress and gall-morph samples and then a 1:2 serial dilution was used to test for antifungal activity. The dilution series tested the effectiveness of 5, 2.5, 1.25, 0.625, 0.312, and 0.156 thrips equivalents against *C. bassiana* spore germination. In this way we were able to maximise the data we were able to extract from relatively small numbers of brood.

Despite low numbers of adult individuals, some galls contained large amounts of larvae. Extractions were carried out on these larvae identically to that explained above. Larval extractions contained 210, 120, and 244 individuals respectively in three separate extractions. These extractions were then tested using a 1:2 dilution series using the same protocol as adult samples but were not compared to foundress or gall-morph samples due to the different concentration gradient.

In total, 12 foundress, 4 gall-morph, and 3 larval dilution series were tested for antifungal activity. Each spectrophotometry plate contained 1 - 4 antifungal dilution series, at least 20 negative controls containing only sterile LB broth to ensure aseptic technique, and 10 - 20 fungal positive controls containing LB broth and fungal spores only. Eight plates contained all of our sampling, one plate contained only larvae experimental samples and was therefore not analyzed with the foundress and gall-morph data.

Data analysis

All data analysis was carried out using R (R core team 2016). A preliminary, visual analysis was carried out by plotting the delay of germination against the concentration gradient for foundress and gall-morph samples (Fig 3.1). Delay of germination was calculated by subtracting the mean fungal spore positive control germination time for each plate from the germination times of experimental wells within the same plate.

A linear mixed-effects model was fitted to our data which allowed the comparison of the germination times between foundress, gall-morph, and fungal spore positive controls. This analysis controls for the fixed effects of morph and concentration and the random effects between the 7 replicate plates. A *post-hoc Tukey test* for multiple comparisons of means was then run on

our model to determine if any significant difference in germination time exists across the concentration gradient between experimental and fungal spore positive control samples.

A separate linear mixed effects model was fitted to the larvae germination time data which controlled for the fixed-effects of sample (either: fungal spore positive control or larvae samples) and the number of individuals which contributed in the extraction and the random effects between plates.

Results

Gall Collection

The galls collected had an average height of 3.7mm, average length of 7.7mm, and average width of 1.4mm) and resembled slightly flattened kidney-shaped pouches (Mound et al. 1996). The partitions and locules in many cases were not fully formed when the galls were opened. Our samples had an average of 4 chambers and 3 partitions, and a maximum of 10 chambers (9 partitions). In all cases the chambers were connected to each other at the ostiole allowing individuals to pass between chambers. Of the 274 galls that were opened, 181 (66%) had living individuals inside. A total of 41 galls had a fungal-like growth within them, with 31 (76%) of these infected galls containing no living brood inside. The invasion rate was low, with only 24 (9%) galls showing signs of invasion. The majority of these (15) were invaded by an unknown organism which had already left the gall, while the remainder were invaded by lepidopterans (9) and Diptera (2).

All 150 fungal spore positive controls germinated within the usual timeframe. No growth curves consistent with contamination were present in any of the negative control samples. One of the gall-morph 5 individual samples was lost during plating (n = 3 at this concentration).

The linear mixed-effects model was fitted to determine if concentration and morph had a significant effect on the germination time of *Cordyceps bassiana* spores while controlling for random variation between plates. Our model explained 79.6% of the variance in the data (Conditional R^2; Nakagawa and Schielzeth 2013) of this 79.6%, the fixed effects of concentration and sample accounted for 19.6% of this variance (marginal R^2; Nakagawa and Schielzeth 2013). An ANOVA test determined that the variance between plates was significant and thus was included in our model (χ^2 (df = 1, N =7) = 208, $p < 0.001$). The effect of sample concentration (χ^2

(df = 1, N =225) = 86, $p < 0.001$) and morph (χ^2 (df = 2, N = 225) = 22.1, $p < 0.001$) were significant. Foundress samples germinated at 31.8 hours ± 1.93 SE, significantly later than both gall-morph samples (29.1 hours ±0.806, $p = 0.00228$) (See Fig 3.1), and fungal spore positive controls (29.5 hours ± 0.558, $p <0.001$). Gall-morph and fungal positive control germination time did not significantly differ (0.4 hours difference ± 0.22, $p = 0.895$). The results of the *post-hoc Tukey test* for multiple comparisons of germination time between morphs and fungal spore positive controls is presented in Table 3.1. Foundress extractions began to have a significant effect on germination time at 0.3135 thrips equivalents but the maximum of 5 thrips equivalents were necessary to significantly slow germination time for the gall-morph samples.

Larval samples had a significantly longer time to germination compared to the fungal spore positive controls. The effect of the number of individuals on germination time was significant χ^2 (df = 1, N =18) = 16.5, $p < 0.001$. The effect of sample (larvae or positive controls) was also significant at the $\alpha = 0.01$ level χ^2 (df = 1, N = 58) = 8.56, $p = 0.003$. Fungal spore positive controls germinated at 34.1 hours ± 4.1 and larvae samples germinated at 38 hours ± 1.36 taking 3.97 hours longer to germinate. Our model explained 77.2% of the variance in the data (Conditional R^2; Nakagawa and Schielzeth 2013). Of this 77.2%, the fixed effects of concentration and sample accounted for 24.8% of this variance (marginal R^2; Nakagawa and Schielzeth 2013). The relatively low amount of variance explained by our fixed effects suggests that random effects play a significant role in germination time relative to the effect of concentration and species.

Discussion

This study provides the first evidence for antifungal production in foundresses, gall-morphs and larvae in *Kladothrips sterni* (Fig 3.1, Table 3.1). Foundress extractions were highly effective at slowing the germination of fungal spores even when fractions of an extraction were present (Table 3.1). At the 1.25 thrips equivalent concentration, *K. sterni* foundresses significantly delayed germination of *C. bassiana* spores by approximately 5.6 hours. This delay is comparable to that found in young *K. arotrum* and *K. tepperi* colonies including singular foundress extractions (Coates et al. 2017). The increased antifungal output of these individuals compared to their brood suggests that the foundress first sterilizes the gall interior, removing any pathogens which may have been present on the phyllode surface, effectively protecting her brood. The higher antifungal production by *K. sterni* foundresses supports the hypothesis that maternal antifungal care is present in the gall-inducing thrips lineage.

Increased antimicrobial potential is also present in leaf-cutter ant gynes (*Acromyrmex spp.*) which possess significantly larger metapleural glands compared to workers (Hughes et al. 2010), fire ant (*Solenopsis invicta*) queens which coat eggs in a special antimicrobial agent (Vander Meer and Morel 1995), and termite (*Reticulitermes speratus*) queens secreting a pheromone which also inhibits egg-mimicking fungi (Matsuura and Matsunaga 2015). Specialized maternal antimicrobial care is an important trait in social insects, the presence of this defence in group-living thrips foundresses suggests that these defences may have been present before the development of sociality in this lineage.

The sample size of this study was small, however, data presented suggest that gall-morph individuals invest significantly less into the microbial defence of the gall compared to foundresses (Fig 3.1, Table 3.1). In the closely related *K. arotrum* and *K. tepperi*, young colonies

with less than 50 individuals did not produce a marked increase in antifungal activity (4.74 and 6.44 hours of delay respectively) compared to single foundresses (Coates et al. 2017). In this study, gall-morph extractions at the highest concentration did produce a significant delay in fungal spore germination (7.29 hours ± 1.83). These delays however, are difficult to compare due to sizeable difference in the maximum group size sampled (50 vs. 5 individuals). Up to 110 gall-morphs have been found in mature colonies (Mound et al. 1996) and if significant antifungal defences are present at five individuals, it is likely that the combined antifungal production of the gall-morphs in a mature colony would produce an effective defence.

This possible difference in antifungal investment between foundresses and gall-morphs may represent a trade-off between reproductive and defensive capabilities. Gall-morphs share a similar non-dispersing morphology to soldiers, however, they have retained the ability to reproduce (Mound et al. 1996; Crespi and Mound 1997). Before founding a gall, foundresses must defend against any pathogens present in the environment and sterilize the interior of their gall before depositing brood. Gall-bound individuals however, hatch into a sterile environment and will not encounter external pathogens associated with dispersing or founding a gall. These individuals may instead invest in increased reproduction potentially leading to the exceptionally large (>1400 individuals) colony sizes present in *K. sterni* (Mound et al. 1996).

The extractions from large numbers of larvae in *K. sterni* colonies produced antifungal activity. This activity may be produced by the larvae themselves or from remnant antifungals secreted by the foundress. Previous studies found no antimicrobial activity in larvae of other *Acacia* thrips species (Christine Turnbull Pers. Comm.). Social insect larvae are the target of many entomopathogens (Schmid-Hempel 1998), however, the antimicrobial defence of the brood is usually undertaken by adults through social immune responses (Cremer et al. 2007). Relatively few studies have found external antimicrobial defences produced by larvae of social (Turillazzi et

al. 2004) or group-living species (Arce et al. 2013). However, *K. intermedius* second instar larvae have been found to produce several compounds in an anal secretion, which have been suggested to act as antifungals (De Facci et al. 2014). Although no *K. sterni* larvae were observed to produce this secretion, the presence of antifungal activity in their washes suggests that they may also share this behaviour (See Chapter 4). A more in-depth analysis on the larval secretion and the potential antifungal effects are therefore necessary. The presence of antifungals in *Kladothrips* larvae suggests that group-living and social thrips species may require all individuals to invest in pathogen defence while the colony is young.

The galls collected in this study were at a young developmental stage, most containing only a single foundress and eggs. Due to the low number of individuals the pooling of antifungal extracts was necessary, leading to a relatively small sample size. This study suggests two directions for further research. The first would be to repeat the procedures described here but with adequate sample sizes for all castes and juveniles. This would further investigate the hypothesis that defences against microbial enemies preceded those against macro-enemies, such as kleptoparasitic thrips. The data gathered here suggest the presence of microbial enemies from the very start of the colony, as foundresses show high antifungal activity. This may have been the case in the early evolution at the very start of sociality and eusociality.

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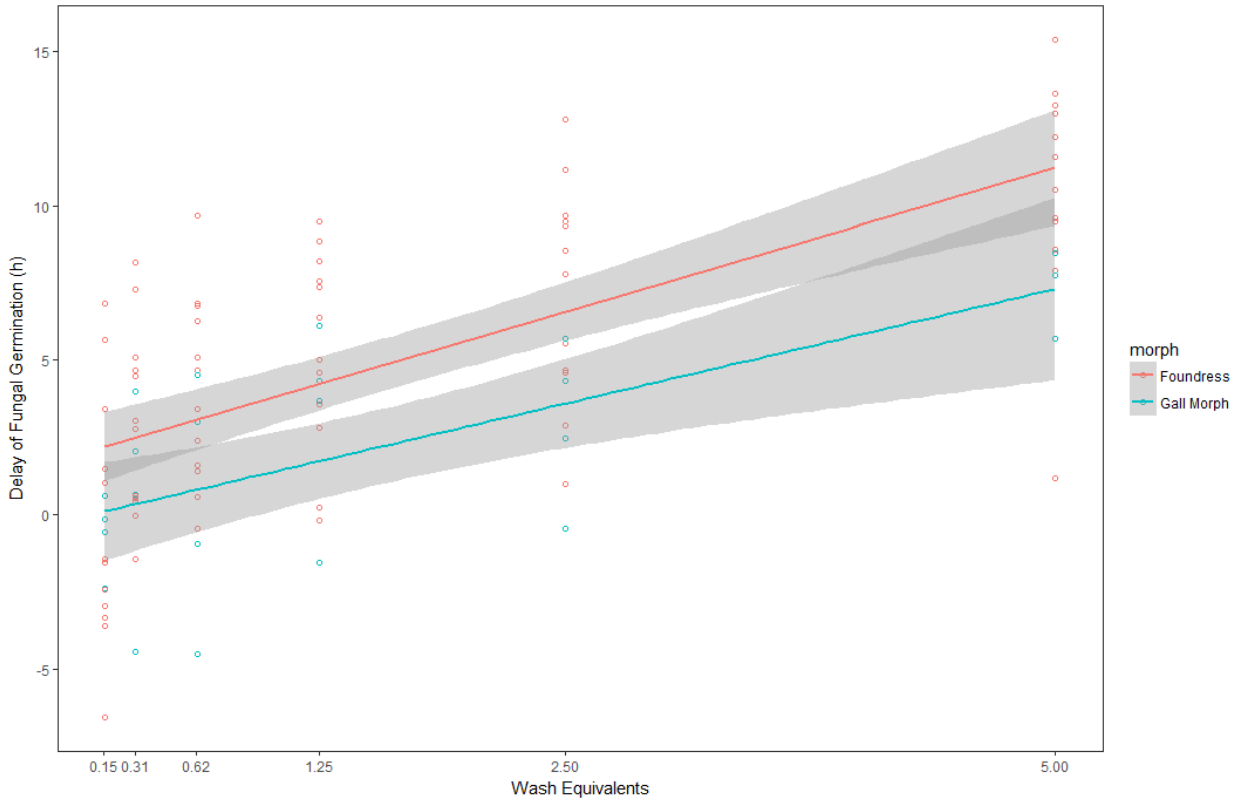
Figure legend

Figure 1: Scatterplot of the delay of *Cordyceps bassiana* spore germination in hours (h) (y-axis) produced by the antifungal washes of *Kladothrips sterni* foundresses and gall-morph castes. Washes were pooled and then tested against the spores in a 1:2 dilution series presented here as 'Wash Equivalents' (x-axis). The trend line presented is a linear regression of the data for each caste, shaded areas denote the 95% confidence intervals.



Figures

Figure 3.1

Table legend

Table 3.1: Results from the *post-hoc Tukey test* which compared the mean germination times of both foundress and gall-morph samples at each concentration to the mean germination time of fungal spore positive controls while controlling for random effects between plates. The null hypothesis being: H_0 = mean experimental sample germination time – mean *Cordyceps bassiana* germination time == 0. The estimated time difference between the two means in hours 't' ± standard error, z-value 'z', and p-value 'p' are provided. * denotes significance at $p < 0.05$, ** denotes significance at $p < 0.01$, *** denotes significance at $p < 0.001$**.**

Tables

Concentration (TE) Morph	0.15625	0.3125	0.625	1.25
Foundress	t = -0.022±0.932 z = -0.023 p = 1.0	t = 3.24±0.932 z = 3.47 p = 0.003**	t = 4.28±0.932 z = 4.59 p = 2.64e-5***	t = 5.59±0.932 z = 5.99 p < 1e-5***
Gall-morph	t = -0.42±1.6 z = -0.263 p = 1.0	t = 0.77±1.6 z = 0.482 p = 1.0	t = 0.734±1.6 z = 0.460 p = 1.0	t = 3.34±1.6 z = 2.09 p = 0.198

Table 3.1

Chapter 4

Source and structure of antifungal compounds in *Kladothrips arotrum* point to possible common antimicrobial defence in group-living insects

Abstract

Kladothrips (Froggatt) is a genus of Thysanoptera that survive in areas of arid and semi-arid Australia by living within galls they induce on *Acacia* phyllodes. Within a gall, brood are highly related and densely populated producing ideal conditions for the spread of pathogens. For group-living insects, increased antimicrobial production has been linked to increasing group size and social complexity leading to the hypothesis that antimicrobials may have facilitated the evolution of sociality. *Kladothrips* species produce antimicrobials, but their structure and source is largely unknown. Some studies have suggested that volatile carboxylic acids within the *Kladothrips* defensive secretion may act as effective fungicides although this has yet to be directly tested. Here, we investigate the *Kladothrips* defensive secretion and its main carboxylic acid components for antifungal activity against a common entomopathogenic fungus using a miniaturized antimicrobial prospecting assay. Removal of the defensive secretion from individuals reduced the antifungal activity of full body extractions, suggesting the secretion possesses antifungal activity. When extracted and tested alone, *Kladothrips arotrum* defensive secretions significantly delayed fungal growth. The principle carboxylic acids present in the closely related, *Kladothrips intermedius* secretions were also found to be effective antifungals at concentrations found within the secretion. Our findings suggest that the hind gut is a likely source of antifungals in *Kladothrips* and that the mixture of carboxylic acids present in the defensive secretion contribute significantly to this effect. Acidic antimicrobial secretions are present throughout the social insect societies and the discovery of a similar mechanism in the *Kladothrips* suggests that this chemical antimicrobial defence may be an important trait for sociality.

Introduction

There are numerous advantages to living within a group, such as more effective foraging (Traniello and Beshers 1991), collective brood care (Wilson 1971), and coordinated defences against predators (Vulinec 1990). However, group-living also presents unique challenges, including an increased threat of pathogen infection and transmission (Schmid-Hempel 1998). This threat is intensified in social insect colonies, where individuals live in shared nests at high densities, come in frequent physical contact, and are highly related, providing ideal conditions for the spread of pathogens (Schmid-Hempel 1998; Zasloff 2002; Stow and Beattie 2008). To offset this increased microbial threat, individuals within a colony will invest in social immunity (Cremer et al. 2007) where specialized behaviours and the production of antimicrobial compounds are used to cooperatively defend against pathogen transmission and infection.

The production of externally secreted antimicrobials is present in all social insect groups and is important for the social immune system (Cremer et al. 2007; Cremer and Sixt 2009). The social Hymenoptera produce venom that contains antimicrobial peptides and acidic compounds (Obin and Vander-Meer 1985; Vander-Meer and Morel 1995; Baracchi et al. 2011, 2012; Tragust et al. 2013). Most ant species, in addition to the venom gland secretions, produce a variety of acidic compounds with antimicrobial properties from a metapleural gland (Beattie et al. 1985; Veal et al. 1992; Ortis-Lechner et al. 2000; Bot et al. 2002). Termites may possess the most sophisticated antimicrobial defences, producing different antimicrobial secretions from sternal, frontal, and salivary glands (Chen et al. 1998; Rosengaus et al. 2000, 2004, 2010; Hamilton et al. 2011), as well as producing antimicrobial fecal pellets (Rosengaus et al. 1998). These secretions are incorporated into nest materials, allogroomed onto conspecifics, or are sprayed on immature individuals to remove or inhibit harmful pathogens present in the environment, and to limit transmission between individuals, effectively protecting the group as a whole (Ortis-Lechner et

al. 2000; Baracchi et al. 2011, 2012; Tragust et al. 2013). Recently, antimicrobial activity has also been detected in the Australian *Acacia* thrips, where the evolutionary and ecological importance of this defence is shedding light on how social immunity may be linked to the development and maintenance of sociality (Turnbull et al. 2011, 2012a, 2012b; Coates et al. 2017).

The Australian gall-inducing *Kladothrips* (Froggatt) are a genus of Thysanoptera that live within galls on *Acacia* phyllodes in parts of arid and semi-arid Australia (Crespi et al 2004). Like the social Hymenoptera, gall-inducing thrips are haplo-diploid and therefore brood relatedness is high, colonies can be long-lived, and galls can be very densely populated (Chapman et al. 2000; Crespi et al. 1997, 2004). For these reasons *Kladothrips* colonies likely experience similar threats from pathogenic microbes as other social insect groups, and might offset these threats through antimicrobial defences. Thrips antimicrobial strength increases with increasing innate group size and social complexity, suggesting that microbial defence may be important for social evolution, a trend also present in the Allodapine bees (Turnbull et al. 2011, 2012a; Stow et al. 2007, 2010) and perhaps the wasps (Hoggard et al. 2011, 2013). Antifungal production may also vary between castes in the eusocial *Kladothrips intermedius* (Bagnall), where antifungal production of soldier caste individuals is significantly higher than that of the reproductive dispersing caste (Turnbull et al. 2012b). Although we are starting to uncover the evolutionary importance of antimicrobial production in the *Kladothrips*, little is known about the possible source and structure of the compounds involved.

To date, over 30 papers have been written on the chemical compounds present in thrips secretions. Depending on species, thrips secretions have been found to contain numerous compounds including hydrocarbons, terpenes, acetates, carboxylic acids, quinones, and a pyranone compound (Suzuki et al. 1986, 1988, 1989, 1990, 1992, 1995, 2000, 2004; Haga et al.

1989a, b; Tschuch et al. 2005, 2008; Teerling et al. 1993; De Facci et al. 2013; 2014). In several species, the secretion is produced in response to a threat and effectively repels ants, functioning as a predator deterrent in these cases (Blum 1991; Blum et al. 1992). However, in some thrips genera, including *Kladothrips,* this secretion may function in communication (Suzuki et al. 1986; De Facci et al. 2013) or possibly as a fumigant, although this specific function has not been directly tested (Suzuki et al. 2004; De Facci et al. 2014). Recently, the volatile components of *Kladothrips intermedius* secretions have been shown to be primarily medium-chain carboxylic acids including caproleic, decanoic, and octanoic acid (De Facci et al. 2014). Decanoic and octanoic acid have been found to have antifungal activity in ant secretions (Bot et al. 2002), but it is unknown if they provide the same defence at the concentrations produced by *Kladothrips*.

This study aims to determine if the *Kladothrips* defensive secretion functions as an effective fungicide. We collected the defensive secretion from *Kladothrips arotrum* (Mound) individuals and used spectrophotometry to quantify the activity against the germination of *Cordyceps bassiana* (bals.) spores. Using the concentrations found in the *K. intermedius* secretions (De Facci et al. 2014), we used the same method to analyze the effect of caproleic, decanoic, and octanoic acid on *C. bassiana* spore germination. A better understanding of the source and structure of *Kladothrips* antimicrobials will allow a more in depth analysis of how this defence is tied to the evolution of sociality in this group and possibly provide insight into common microbial defences shared among social insect lineage

Methods

Gall Collection and Fungal Culture

Kladothrips arotrum galls were collected near Alice Springs in the Northern Territory, Australia (22° 44' 34.897" S; 131° 12' 23.076" E). These galls were then transported back to Macquarie University and stored at 4°C until extraction could take place. Previous studies have found that *Kladothrips* galls can be kept in these conditions for several weeks with no harm to the occupants.

We used a native strain of *Cordyceps bassiana* cultured from populations of *Exoneura nigrescens* (an allodapine bee) found in Victoria, Australia (Stow et al. 2010). *Cordyceps bassiana* is commercially used as a biological control agent against pest thrips species (Shah and Pell 2003) and this *C. bassiana* strain was previously used in studies of *Kladothrips* antifungals (Coates et al. 2017; Turnbull et al. 2012a, b). Storage stocks of this strain were used to produce fresh fungal plates. A small piece of inoculated agar was removed from the storage stock and placed on a fresh Luria Broth (LB) agar plate. This plate was then incubated at 25°C for 2 – 3 weeks to allow the formation of spores. The culture was then left at room temperature until needed. Fungal spore suspensions were made on the day they were needed by combining ~1mg of fungal spores with 10mL of LB and 10µL of Tween 80.

Defensive Secretion Antifungal Analysis

Using standard aseptic techniques, *K. arotrum* galls were bisected through the ostiole and the thrips inside were brushed into a Petri dish using a paintbrush. To induce the production of a defensive secretion, each thrips was lightly pressed on the head and upper thorax with the bristles of a paintbrush. When presented with this stressor, individuals raise their abdomen in a scorpion-like fashion and secret a droplet of clear to yellowish liquid, held at the apical tip of the abdomen by several setae surrounding the anus. This secretion was then quickly absorbed using a sterile,

1cm diameter circle of filter paper (Advantec) held in forceps. Care was taken to not bring the filter paper in contact with the body to avoid collecting any antifungals possibly present on the cuticle. A single individual can be provoked to produce several secretions, therefore we continued collecting from an individual until no liquid was present in the setae after provocation. Each filter paper was used to collect secretions from up to 50 individuals.

The antifungal compounds were extracted from each filter paper, re-suspended in culture media (LB broth), and added to a well in a 96 well flat bottomed cell culture plate using previously established lab protocols (Smith et al. 2008; Coates et al. 2017), with the following modifications: Extractions were also carried out on sterile filter papers to control for any antifungal properties of the filter paper used to collect the defensive secretion. Thus, each plate contained defensive secretion samples, filter paper controls, fungal spore positive controls (fungal spore suspensions with no added antifungals), and negative controls (LB broth alone to verify sterile technique). The loaded plate was then placed in a VERSA max tunable microplate reader (Molecular Devices) and the change in the optical density (OD) of each well was recorded over the course of 48 hours using SoftMax Pro version 6.5.1software. The microplate reader was set to analyze each well at a wavelength of 405nm and to incubate the plate at 25°C. In total 27 defensive secretion extractions, 27 filter paper control extractions, 60 fungal spore controls, and 180 negative controls were analyzed.

Fungal growth in the positive control wells began between 16 and 24 hours. Between 0 and 16 hours after plating the OD of positive controls is roughly zero although minor fluctuations around this point occurred. Due to these minor fluctuations samples were deemed to have germinated when a clear linear growth curve was present and the OD rose by 0.03. The effect of the defensive secretion on *C. bassiana* spore germination was then quantified by comparing the germination times of secretion extract samples, filter paper controls, and fungal control samples.

We first compared the proportion of samples where germination took place within the 48 hour sampling window, between treatments. The samples which did not germinate within the observation window, we scored as germinating at the 48 hour mark. This allowed comparison between treatments. Germination time of the three treatments was first analyzed by a *Kruskal-Wallis Rank Sum* T*est* and subsequently by a *Conover's-test* to compare germination time between treatments. Although we attempted to collect secretions from 50 individuals per filter paper the actual number of secretions collected varied slightly, consequently a *Pearson's Product – Moment Correlation* was used to detect any correlation between the number of individuals and antifungal effectiveness. All analyses used the statistical package R (R Core Team 2014).

The Antifungal Contribution of the Secretion

Previous studies of thrips antifungals involved extracting from whole individuals (Coates et al. 2017; Turnbull et al. 2012a, b). To determine the contribution to the antifungal effect provided by the secretion in full-body washes, we compared the antifungal activity of full-body washes to that of full-body washes of thrips from which the secretion was previously removed. Antifungal extractions were carried out on groups of adult *K. arotrum* which were either undisturbed or had previously had their secretions removed, each group was composed of up to 50 *K. arotrum* adults. We produced 11 and 12 replicates of these 'secretionless' and 'fullbody' extractions respectively. The effect of the extracted antifungals was then quantified using the protocols outlined above. When placed in the spectrophotometer fungal spore positive controls and negative controls were present on each plate with experimental extractions. Again, the proportion of samples which allowed germination to take place within the 48 hour sampling window was calculated and samples which inhibited germination for the full time were conservatively scored to have germinated at the 48 hour mark. A *Kruskal-Wallis rank sum test* and subsequently a *Conover's-test* were used to compare germination time between treatments.

Carboxylic Acid Dilution Series

We used the published solid phase microextraction estimates of the caproleic (25.34ng/secretion), decanoic (11.02ng/secretion), and octanoic acid (8.04ng/secretion) content of *K. intermedius* disperser secretions as our basis for the production of biologically relevant carboxylic acid suspensions. Together these three carboxylic acids account for 99.1% of the secretions (De Facci et al. 2014). Pure stocks of each acid were purchased from Sigma-Aldrich and diluted to 1gram/liter in 90% ethanol. Ethanol was used as the dilution solvent to be consistent with the extraction protocol used to collect the defensive secretions.

For the starting suspension of 250 *Kladothrips intermedius* secretion equivalents the volume of each carboxylic acid stock was calculated to be: 63.35µL of caproleic acid, 27.55 µL of decanoic acid, and 20.1µL of octanoic acid. These three volumes were also combined to produce a "synthetic" thrips secretion to test for synergistic effects. All suspensions were pipetted into respective 1.5mL microcentrifuge tubes and stored at -20°C until needed. The ethanol solvent was dried and the resulting carboxylic acid residues were analyzed using the same protocol described in the defensive secretion analysis with the following modifications: the carboxylic acids were re-suspended in 200µL of LB culture media and a 1:2 dilution series for each sample was loaded into the 96 well cell culture plate so that 125, 62.5, 31.25, 15.63, 7.81, and 3.9 secretion equivalents of each fatty acid were tested against the same concentration of *C. bassiana* fungal spores. Each plate contained two dilution series for experimental carboxylic acid samples, 10 fungal spore positive controls, and 36 negative controls, eight replicates of this configuration were produced on eight separate plates (n = 16 for each acid-concentration combination except for caproleic acid at 62.5 and 125 SE where n = 15). The germination time of fungal spores was determined when a clear linear growth curve was present and OD rose by 0.03.

We then compared the germination time of fungal spores in experimental and fungal positive control samples.

A linear mixed-effects model was fitted to our data which allowed the comparison of germination times between carboxylic acid experimental and fungal spore positive control samples while controlling for the fixed effects of concentration and carboxylic acid and random effects between the 8 replicate plates. A *post-hoc Tukey test* for multiple comparisons of means was then run on our model to determine the difference in mean germination time between fungal positive controls and carboxylic acid samples across the concentration gradient.

Results

Description of Secretion Production Behaviour in Kladothrips arotrum

The initial response of almost all individuals was to move away from the paintbrush (stressor) when it was placed in their path. A defensive secretion was not produced until the paintbrush came in contact with the head and/or thorax. The defensive secretion usually occurred in conjunction with the individual rearing into a "scorpion-like" pose by holding the abdomen over its thorax in an arch. Upon contact with the stressor the individual vigorously rubbed the tip of its abdomen over the stressors surface, presumably to increase the area affected.

Fifty *K. arotrum* adults were taken from one gall and stimulated using a paintbrush and touching them on the head and/or pronotum. Forty eight specimens produced one secretion which was dabbed onto the paintbrush, and 45 of the 48 produced at least a second secretion immediately. The maximum number of secretions produced was 5, observed in 27 cases. Larvae were not observed to produce defensive secretions, instead opting to move away from this stressor.

Antifungal effect of Kladothrips arotrum secretions

The extracted secretions had a significant effect on *Cordyceps bassiana* spore germination. 21/27 (78%) wells containing secretion extractions supressed fungal spore

germination beyond the 48 hour sampling window. In comparison, all fungal spore positive controls and filter paper controls (n = 60 and n = 27 respectively) germinated within the sampling window (Fig 4.1). An *Asymptotic Kruskal-Wallis Test* indicated that germination time between treatments was significantly different (χ^2 = 92.85, df = 2, p < 2.2e-16) which allowed the use of a post-hoc *Conover's-test* which indicated that the germination time of fungal spores treated with secretion extract (mean = 48 hours) was significantly different than both filter paper controls (mean = 30 hours, p = 8.4 e-11) and *C. bassiana* positive controls (mean = 22.5 hours, p < 2e-16). Filter paper and *C. bassiana* positive controls also had significantly different germination times (p < 2e-16). No association was present between number of individuals sampled and antifungal effect (p = 0.886, df = 25, t = -0.1446).

The Antifungal Contribution of the Secretion

Full body washes inhibited germination for the full 48 hour sampling period in 9/12 samples (66.7%) compared to 1/11(9.1%) in secretion removed samples. All *C. bassiana* positive controls (n = 30) germinated within the sampling period. An *Asymptotic Kruskal-Wallis Test* indicated that there was a significant difference in germination time between treatments (χ^2 = 41.0042, df = 2, p-value = 1.248 e-09) the post-hoc *Conover's-test* subsequently established that the germination times of full body wash samples were significantly different to that of both Secretion Removed samples (p = 0.0015) and *Cordyceps* controls (p < 2e-16). Secretion Removed and *Cordyceps* controls also had significantly different germination times (p = 1e-10) suggesting that some antifungal compounds were present elsewhere on *Kladothrips arotrum*. No correlation was present between number of individuals sampled and antifungal effect (p = 0.678, df = 21, t-value = 0.4205).

Carboxylic acid effects on fungal germination

In general all three carboxylic acids produced significant delays in the germination time of *C. bassiana* spores (Fig 4.3, Table 4.1). The random effects of Plate were found to be not significant (χ^2 (df =9, N =7) = 9.25, p = 0.4). The effect of carboxylic acid concentration (χ^2 (df = 5, N = 382) = 813, $p < 0.001$) and carboxylic acid species (χ^2 (df =3, N = 382) = 34.5, $p < 0.001$) were significant. The interaction between concentration and species was also significant (χ^2 (df = 15, N = 382) = 627, $p < 0.001$). The combination of all three acids produced the highest delays at all SE concentrations, including the majority of samples at both 125 SE (14/16) and 62.5 SE (9/16) delaying germination past the sampling window. Individual fatty acids had varying effectiveness roughly correlating with the relative concentration within the secretion. Decanoic, and caproleic acid had increasing effectiveness in higher SE however octanoic acid did not produce more delay at higher SE (Fig4.3)

Discussion

In the present study, we discovered that *K. arotrum* secretions significantly delayed the germination of *Cordyceps bassiana* spores compared to both the fungal positive controls and the filter paper controls (Fig 4.1). Although compounds within the thrips secretion have previously been suggested to act as fumigants (De Facci et al 2014; Suzuki et al. 2004), this study is the first direct evidence of the antifungal properties of the Thysanoptera defensive secretion. As filter paper controls also had a small effect on fungal germination (Fig 4.1) we could not quantify the total delay of germination caused by the secretion. However, we provide evidence that antifungal compounds are likely produced in the hindgut or associated glands (Sharga 1933; Howard et al.1983). Although the accepted function of the defensive secretion in other genera is defence against large predators (Blum 1991; Blum et al. 1992), the gregarious lifestyle of *Kladothrips* may have selected for the production of antifungal compounds. Uncovering the source of

Kladothrips antifungals allows for a more detailed analysis of microbial defences in this lineage and, more specifically, how antimicrobial production differs between solitary and social species.

Kladothrips likely protect their colonies from entomopathogenic microbes through the secretion of carboxylic acids similar to some ant (Nascimento et al. 1996; Schildknecht and Koob1971; Bot et al. 2002) and termite species (Rosengaus et al. 2004) (See Viegas et al. 1989 for a thorough description of how carboxylic acids inhibit microbial growth). The combination of carboxylic acids present in *Kladothrips intermedius* secretions is highly effective against *C. bassiana* spore germination and growth (Fig 4.3, Table 4.1). Independently, all three acids significantly delay fungal growth (Table 4.1). However, caproleic acid produced the longest delays and is likely the primary antifungal compound of the secretion (Fig 4.3). Short, medium and long-chain carboxylic acids have variable effects against bacteria and fungi depending on carbon chain length (Kabara et al 1972; Bot et al. 2002; Huang et al. 2011), therefore a secretion containing a mixture of carboxylic acids would more effectively defend a colony from microbial invasion. One example of such a mixture can be found in the metapleural gland secretion of the leaf-cutter ant *Acromyrmex octospinosus* which contains at least 14 carboxylic acids, including octanoic and decanoic acid (Ortius-Lechner et al. 2000), with variable effects against fungi and bacteria (Bot et al. 2002).

Kladothrips arotrum colonies appear to possess multiple chemical defences against fungal pathogens. The removal of the defensive secretion from individuals reduced the antifungal effects of full body washes, but extractions of individuals which had their secretion removed still significantly delayed germination of fungal spores (Fig 4.2). This result could be explained by incomplete removal or quick synthesis of the secretion, or antifungal compounds being present on the cuticle (Turnbull et al 2011). However, another likely source of microbial defence in the *Kladothrips* could be through a symbiotic relationship with antimicrobial producing bacteria,

several *Streptomyces* strains have recently been found associated with *Kladothrips intermedius* galls which produce wide-spectrum antibiotics effective against gram-negative and gram-positive bacteria, and fungal species (Alteen 2014). Relationships with beneficial, antimicrobial producing bacteria are found in many insect species including ants (Oh et al. 2009; Currie et al. 2006; Little et al. 2006), termites (Visser et al. 2012), wasps (Madden et al. 2013; Goettler et al. 2007; Kaltenpoth et al. 2005) and beetles (Scott et al 2008) and is important for social immunity in group living species (Cremer and Sixt 2009; Cotter and Kilner 2010).

Social immunity innately depends on the group as a whole contributing to the microbial defence of the colony (Cremer et al. 2007); for this reason group size is an important factor in a colonies ability to defend against infection. We observed that the delay of *C. bassiana* spore germination increased with increasing carboxylic acid concentration with full inhibition beginning to take place once more than 62.5 secretion equivalents were present in samples which contained the carboxylic acid combination (Table 4.1, Fig 4.3). Full body and secretion extractions with approximately 50 individuals also significantly slowed the germination of spores (Fig 4.1, Fig 4.2). Our study therefore supports the possibility of a group-size threshold in the *Kladothrips*, described in previous studies, after which a colony would be effectively safe from fungal invasions (Turnbull et al 2011, 2012a; Coates et al. 2017). Such thresholds may drive the development of larger group sizes and allow individuals to co-habit a gall for longer periods of time producing favorable conditions for the development of cooperation and sociality.

The *Kladothrips* thrips represent an ideal lineage for the study of social evolution (Crespi 1992; Chapman et al. 2000, 2008) and this study opens up the possibility for a comparative analysis of the defensive secretion in solitary, group living and social *Acacia* thrips to determine if selection for the production of carboxylic acids is directly linked to increasing social complexity in this lineage. The production of acidic secretions and formation of relationships

with antimicrobial producing bacteria in several social insect lineages suggests that these defences may be a common response to the selection pressures of social living. Uncovering compounds which are selected for through social living can provide information on the intricate changes necessary for the development of complex sociality, not only in the thrips, but in all social living organisms.

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Figure Legends

Figure 4.1: The average germination time of *Cordyceps bassiana* spores in LB liquid culture media with no extractions added (Fungal Positive Control), when extractions from sterile filter papers were added (Filterpaper) and when *Kladothrips arotrum* defensive secretions are present (Secretion). The sampling window lasted for 48 hours and the OD of each sample was measured every 15 minutes. Samples which did not allow germination to take place within the sampling window were conservatively scored as germinating at the 48 hour mark for the purpose of this graph.

Figure 4.2: The average germination time of *Cordyceps bassiana* spores in LB liquid culture media with no antifungal extractions present (Fungal Positive Control), with extractions from whole thrips bodies (Full Body Wash), and with extractions from whole thrips bodies which previously had the defensive secretion removed (Secretion Removed). The sampling window lasted for 48 hours and the OD of each sample was measured every 15 minutes. Samples which did not allow germination to take place within the sampling window were conservatively scored as germinating at the 48 hour mark for the purpose of this graph.

Figure 4.3: The average germination time of *Cordyceps bassiana* spores when biologically relevant concentrations (See methods) of carboxylic acids present in *Kladothrips intermedius* defensive secretions (De Facci et al 2014) were added. The x-axis presents the secretion equivalents tested in the dilution series which in delay of germination caused by each carboxylic acid at each secretion equivalent concentration. The grey line with zero slope denotes the average germination time of *Cordyceps bassiana* fungal control samples present on each plate (n = 80, 20.5 hours). Each acid at each concentration has n = 16 with the exception of caproleic acid at 62.5 and 125 SE where n = 15.

Table Legends

Table 4.1: Using a multiple comparison of means the average time of germination for each carboxylic acid at each concentration was compared to the average germination time of *Cordyceps bassiana* positive control samples. With the H_0 = Carboxylic acid germination time – *Cordyceps bassiana* germination time == 0,'t' denotes the estimated time difference between the two means in hours, 'z' denotes the z-value, 'p' denotes p value, * denotes significance at $p < 0.05$, ** denotes significance at $p < 0.01$, *** denotes significance at $p < 0.001$**.**

Figures

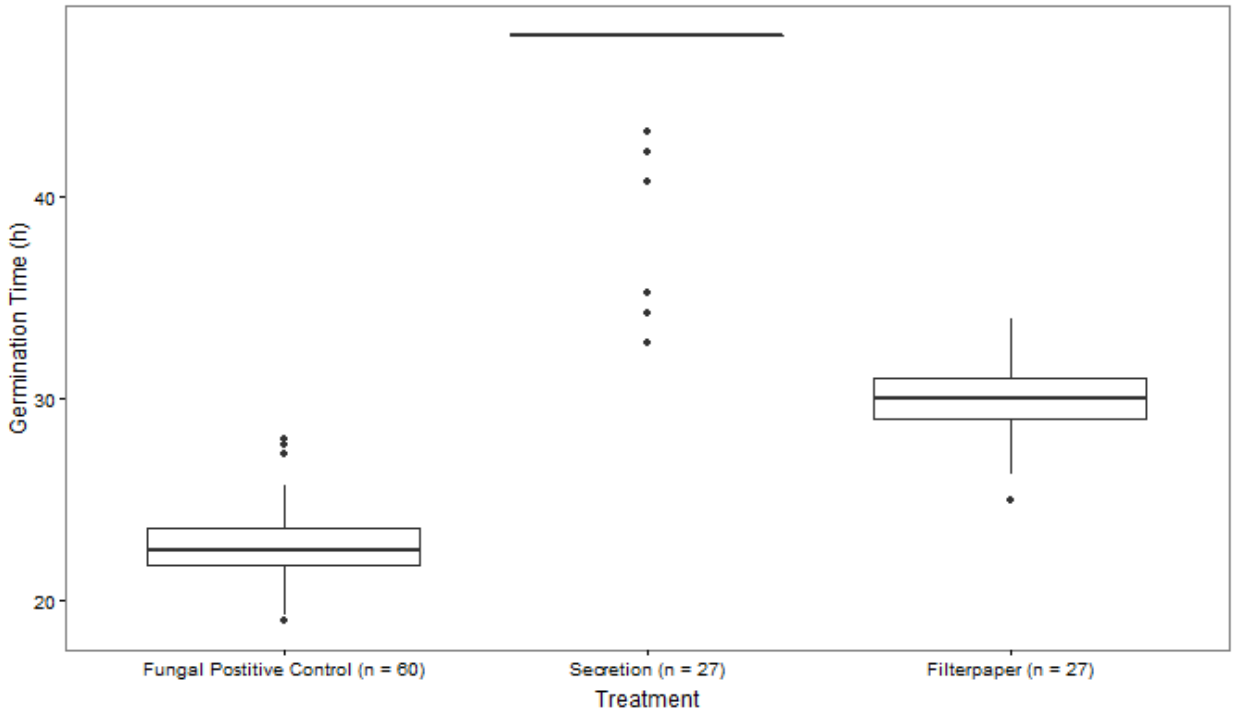

Figure 4.1

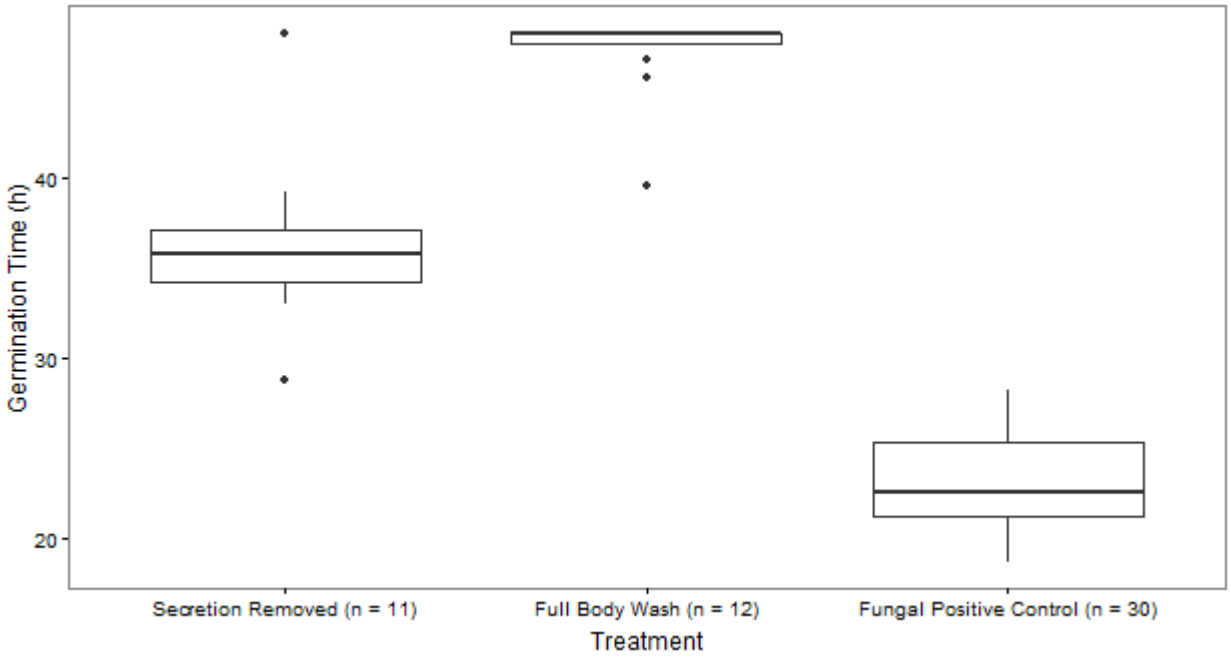
Germination Time (h)
40
30
20
Secretion Removed (n = 11)
Full Body Wash (n = 12)
Fungal Positive Control (n = 30)
Treatment

Figure 4.2

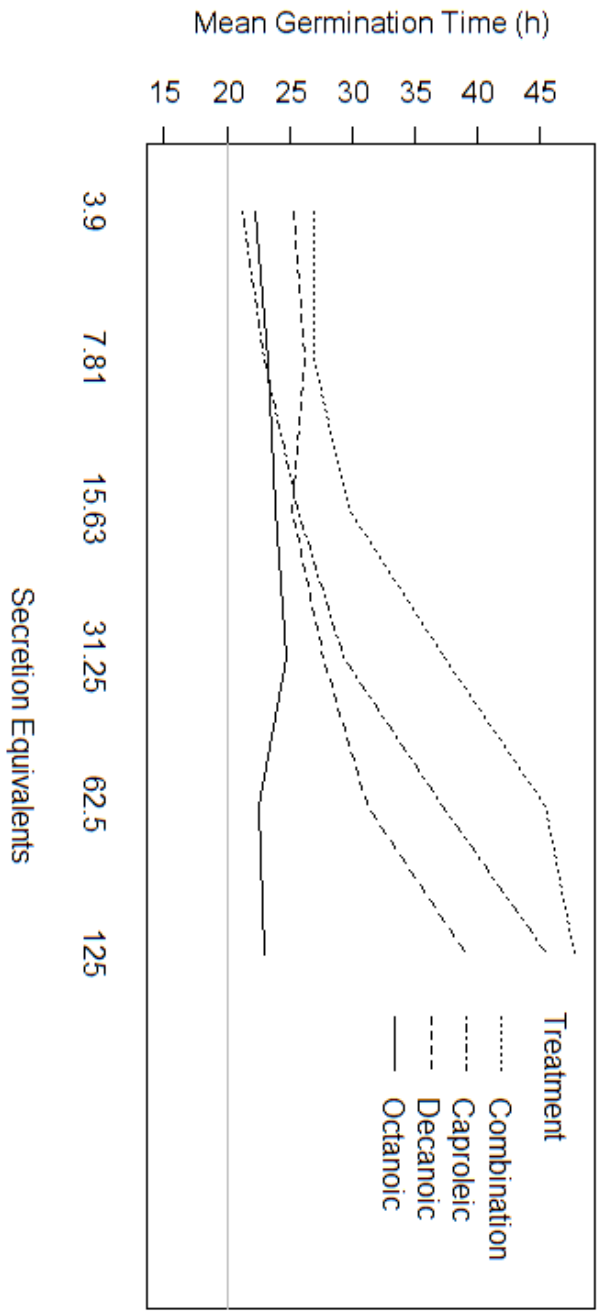
Mean Germination Time (h)
15
20
25
30
35
40
45
3.9
7.81
15.63
31.25
62.5
125
Secretion Equivalents
Treatment
Combination
Caproleic
Decanoic
Octanoic

Figure 4.3

Table 4.1

Concentration (SE) Acid	3.9	7.81	15.63	31.25	62.5	125
Caproleic	t = 1.181±0.8303 z = 1.423 p = 0.621	t = 2.994±0.8303 z = 3.606 p = 0.0019**	t = 5.509±0.8303 z = 6.636 p < 1e-4***	t = 9.213±0.8303 z = 11.10 p < 1e-4***	t = 17.20±0.8534 z = 20.15 p < 1e-4***	t = 25.55±0.85 z = 29.93 p < 1e-4***
Decanoic	t = 5.259±0.8303 z = 6.335 p = 1.51e-09***	t = 6.228±0.8303 z = 7.501 p < 1e-09***	t = 5.166±0.8303 z = 6.222 p = 3.00e-09***	t = 7.558±0.8303 z = 9.103 p < 1e-09***	t = 11.20±0.8303 z = 13.49 p < 1e-09***	t = 18.99±0.83 z = 22.88 p < 1e-09***
Octanoic	t = 2.213±0.8303 z = 2.665 p = 0.0446*	t = 3.181±0.8303 z = 3.832 p = 0.0008***	t = 3.775±0.8303 z = 4.547 p < 1e-04***	t = 4.697±0.8303 z = 5.657 p < 1e-04***	t = 2.447±0.8303 z = 2.947 p = 0.0189*	t = 2.963±0.83 z = 3.568 p = 0.0022**
Combination	t = 6.963±0.8303 z = 8.386 p < 1e10***	t = 6.916±0.8303 z = 8.330 p < 1e-10***	t = 9.619±0.8303 z = 11.59 p < 1e-10***	t = 17.24±0.8303 t = 20.77 p < 1e-10***	t = 25.40±0.8303 z = 30.59 p < 1e-10***	t = 27.70±0.83 z = 33.36 p < 1e-10***

Chapter 5

General Discussion and Conclusions

This thesis first presents a detailed literature review on the current knowledge of social insect pathogen defence and how studying the Australian *Acacia* thrips can aid in understanding the potential importance of this defence for the development of sociality. Three separate studies are then presented, which aim to increase our knowledge on how the group-living *Acacia* thrips defend their colonies from fungal attack. This chapter first provides a review of the main questions and results of each data chapter and their potential importance to the body of knowledge regarding pathogen defence in the Australian *Acacia* thrips. Finally, some potential future studies that should be considered are highlighted, followed by a brief conclusion statement.

Data Chapter summaries

Chapter two focuses on two main questions, how group-size and maturity can affect antifungal production in the *Kladothrips*, and if invading *Koptothrips* produce similar antifungal activity to their hosts. Here, we measured the effect of colony group size and maturity on the antifungal production of two closely related gall-inducing species (*Kladothrips arotrum* and *Kladothrips tepperi*) and one kleptoparasitic species (*Koptothrips dyskritus*). Within the gall-inducers, antifungal strength was dependant on both group size and maturity of the colony. Small, young groups with less than 50 individuals had varying antifungal activity, however, mature groups with over 100 individuals stopped germination of *Cordyceps bassiana* spores for the entire 48 hour sampling window in the majority of cases. Additionally, we observed that young colonies and foundresses alone produce similar antifungal effects, suggesting that the foundress produces the majority of antifungals for her colony while it is young, possibly to protect her immature brood.

At small group-sizes, *Koptothrips dyskritus* individuals produced more effective antifungals compared to the gall-inducers suggesting that this species produces its own microbial defence. This is an interesting result: even though the parasite is entering a colony already well defended against fungal pathogens, it still allocates resources to this end. This suggests that the parasite is contributing to this defence in accordance with the large group size and maturity of the host within the invaded gall.

Chapter three focuses on *Kladothrips sterni*, a species that produces a non-dispersing caste which does not serve a defensive role. This chapter asks if caste-specific antifungal defence developed before the morphology for macro defence and therefore may be the initial altruistic defence in the *Kladothrips*. Here, the first description for antifungal activity in *Kladothrips sterni* foundresses, gall-morphs, and larvae is presented as a preliminary study to determine if caste-specific antifungal defences are present in this species. At the maximum concentration tested, both foundress and gall-morph extractions produced significant antifungal activity. Overall however, foundress extractions produced significantly more antifungal activity compared to gall-morphs suggesting that the foundress effectively sterilizes the interior of the gall, protecting her brood from pathogens. The significantly lower antifungal production of gall-morphs could indicate that these individuals invest more heavily into reproductive potential than gall defence. Larvae extractions were found to contain effective antifungals but, due to the experimental design, could not be directly compared to foundress or gall-morph samples. The larval investment in microbial defence in *K. sterni* could be a product of the increased time spent within the gall or the high population density within a colony and the need to offset the increased threat of pathogen infection caused by these traits. However, due to the relatively small sample size presented in this study, the results presented require more in depth sampling and analysis to substantiate the claims stated.

In Chapter four, I ask what are the potential source and structure of *Kladothrips* antifungal compounds. This is investigated by testing for antifungal activity in the anal secretion and the compounds which it is primarily composed of. The anal secretion was found to have significant antifungal activity, suggesting that the source of *Kladothrips* antifungals is likely the hindgut and/or associated glands. Extractions taken from individuals that had their secretions previously removed exhibited significantly less antifungal activity than extractions taken from unaltered individuals, further strengthening our argument that the anal secretion is a major source of the antifungal activity described in full body washes. Finally, the three carboxylic acids present in the *K. intermedius* secretion showed significant antifungal activity at biologically relevant concentrations. The antifungal activity produced by the anal secretion is therefore likely due to the reduction in pH caused by these carboxylic acids.

Discussion of results

This thesis provides evidence that *Acacia* thrips colonies are likely protected against fungal invasion through the secretion of carboxylic acids by adults and some immature instars. With the exception of middens produced by *Dunatothrips aneurae* (Gilbert and Simpson 2013), no specialized antimicrobial behaviours have been described in the *Acacia* thrips. Therefore, we can only speculate on how colonies distribute antifungal secretions. The high population density present in most *Kladothrips* colonies would imply that any liquid secreted by an individual would likely coat both the interior gall wall and the cuticles of kin. These antifungals are presumably used to create an inhospitable environment for fungal pathogens, likely through lowering the pH within the gall, effectively inhibiting spore germination, a similar strategy also employed by leaf-cutter ants (Ortius-Lechner et al. 2000; Bot et al. 2002). This would afford antifungal protection to the whole colony and is arguably evidence that a social immune system is present in thrips societies.

The antifungal production of a colony varies depending on its maturity and the individuals which comprise it. Foundresses (Coates et al. 2017; Chapter three) and soldiers (Turnbull et al. 2012b) are able to increase their individual antifungal production but, as presented in Chapter two, full colonies may also increase their antifungal output when the population density surpasses a certain point. This variable defence may be a result of a possible group-size threshold as a response to changing conditions within the gall, preparation for dispersing or, drawing on the information presented in Alteen 2012, could be related to the time necessary for the majority of individuals within the group to culture beneficial *Streptomyces* species. In the gall-inducers, a high antifungal defence seems to be associated with young colonies and mature, high-density colonies. These two points are presumably the times when pathogen threat to the colony would be the highest, suggesting that the upregulation in antifungal production is linked to this threat.

The production of carboxylic acids to defend the colony is not unique among social insects. Ants (Bot et al. 2002) and termites (Rosengaus et al. 2004) also produce various carboxylic acids which are secreted externally to produce a barrier defence against pathogens. As stated in Chapter four, this finding suggests that the production of carboxylic acids for pathogen defence may be common in social insect lineages. Chapter four also determined that the anal secretion is not the only source of antifungals in the *Kladothrips*. This residual antifungal activity could be produced by the thrips having multiple antimicrobial secretions, symbiotic *Streptomyces* (Alteen 2012), or the presence of secretion-born carboxylic acids on the cuticle, similar to that of other social insect groups (Fernández-Marín et al. 2006; Rosengaus et al. 1998). Due to the defensive nature of its release, it is likely that the anal secretion functions both as a predator deterrent and as an antifungal, similar to the dual-function of venom in bees and wasps (Baracchi

et al. 2011, 2012). The anal secretion is therefore an excellent starting point for future studies regarding the antifungal defences of *Kladothrips* societies.

Future directions

The *Acacia* thrips remain an excellent study lineage for the comparative analysis of traits necessary for the development of eusociality (Chapman et al. 2008; Turnbull et al. 2011, 2012a, b). However, in terms of how antimicrobial production is tied to the development of eusociality, much more needs to be done before conclusive evidence can be presented. The studies presented in Chapters two and three show that antifungal production varies across the life-cycle of the colony, making a comparative analysis between species difficult unless all species are sampled at the same life-stage. The discovery of the source of antifungals presented in Chapter four is therefore much more useful. By measuring the differing levels of carboxylic acids across castes and species, a more precise measure of antifungal activity can be observed. A comparative analysis of secretion chemistry in species with differing social complexity would therefore be an interesting area for future research in attempting to link microbial defence to the development of eusociality in this lineage.

The production of antifungals by *Koptothrips dyskritus* opens up a new avenue for host-parasite dynamics. Depending on species, *Koptothrips* invade galls with differing maturities. For example, *Ko*. *flavocornis* invades several eusocial species while the gall is very young, presumably to invade before the eclosion of soldiers (Chapman et al. 2006), but *Ko*. *dyskritus,* which targets sub-social species, invade mature and even abandoned galls (Crespi and Abbott 1999). These differing invasion times could also select for differing antimicrobial production in the invaders. Early invaders would capitalize on the sterilization produced by the gall-inducer foundress in eusocial species. Late invaders of sub-social species would capitalize on the residual

antimicrobials produced by the mature colony. Invading a previously abandoned gall would convey no advantage and may even increase the chances of pathogen infection due to the gall being open to the environment. Therefore, a complementary study involving several kleptoparasitic species, both early and late invading, could provide interesting information on the effect invasion time and host antimicrobial investment have on parasite antimicrobial production.

In chapter three, the need for a follow-up study regarding *K. sterni* antifungals is described in detail. However, this species possesses several unique characteristics, all of which may affect colony investment in antimicrobial defences. The lobed structure of *K. sterni* galls is an additional characteristic that could affect antimicrobial investment. The physical structure of a social insect nest can effectively alter the ability of a pathogen to spread between individuals (Pie et al. 2004; Naug and Camazine 2002). This has already been shown in ants (Hughes et al. 2008), termites (Chouvenc et al. 2013), and is most prevalent in the bees, where the individual combs are a physical structure which inhibits pathogen infection of brood (Evans and Spivak 2010). The partitions inside the *K. sterni* gall may be the first step for increasing nest complexity in the *Kladothrips* lineage, possibly driven by pathogenic selection. Determining if the lobed structure is effective in slowing pathogen spread through a colony would require extensive field collection to find previously infected galls or could be tested through field inoculations and observation.

Longevity and pathogen defence

The relationship between colony longevity, pathogen threat and antimicrobial defence may be an important area of study in the *Acacia* thrips. As colony longevity increases, so too does the chance of that colony eventually becoming infected. We would therefore expect to see antimicrobial investment to increase in species that remain in a colony for longer periods of time. Therefore, the relationship between colony longevity and antimicrobial strength with respect to

the *Acacia* thrips phylogeny may be an interesting area of study. The gall-inducing thrips phylogeny has been fully mapped (Buckman et al. 2013; Morris et al. 2002; McLeish et al. 2013) and colony longevity varies dramatically between species (from several weeks to over two years; Crespi et al. 2004; Mound Pers. Comm.) making this lineage ideal for the phylogenetic comparative analysis of longevity and antimicrobial strength. Colony longevity is thought to be heavily influenced by kleptoparasite pressure (Crespi et al. 2004), however selection by microbial invaders have likely also had an effect. If a correlation between colony longevity and antimicrobial production exists, this suggests that microbial defence is an important factor allowing groups to stay together longer, thus producing the ideal conditions for the development of helping behavior and altruism.

Native pathogens

This thesis exclusively tested for defences against the *Cordyceps bassiana*, a common fungal entomopathogen, however, many species of bacteria and fungi infect social insect colonies (Schmid-Hempel 1998) and could pose a threat to *Kladothrips* colonies. Carrying out similar tests to those detailed here against gram-negative, and gram-positive bacteria, as well as, additional fungal species, would help to uncover what pathogens *Kladothrips* have had to develop defences against. The increased effectiveness of antimicrobials against one of these groups would suggest it has been naturally selected for, providing information on which groups are likely native pathogens to *Kladothrips* colonies. Although anecdotal evidence exists of thrips colonies being found covered in what is assumed to be fungal hyphae (Chapman et al. 2006, Coates pers. Obs.), no studies have attempted to culture the natural pathogens which target *Kladothrips* colonies. The culture of native pathogens could enable a more exact measure of antimicrobial defences for use in comparative studies of antimicrobial defence.

Pharmaceutical development from social insects

In addition to helping understand how social living developed, studying the antimicrobial production of lesser studied social insects may also be a source of novel antibiotics for use in medicine. Antibiotic prospecting in these societies would be highly useful where, through evolutionary time, specialized compounds have been developed to combat common pathogens. Some compounds have already been patented from ant immunity peptides and metapleural gland secretions as potential pharmaceuticals (Macintosh et al. 1998; Patent: WO1999000139 A1, 1999). Taking into account the sheer number of social insect species and their intimate relationship with Actinomycetes, bioprospecting in these societies for new antibiotics could be highly productive (Baltz 2008). The study of social insect antimicrobial defences therefore provides both practical and theoretical research opportunities.

Conclusion

The antifungal defences of the Australian *Acacia* thrips are an interesting area for continued research into how group-living and social insects defend their colonies against harmful pathogens. This thesis provides insights into which species produce antifungal defences and how this differs with respect to colony maturity, group-size, and caste. It further provides the likely source and structure of the *Kladothrips* antifungals, which will be highly useful for future studies. The research presented here provides a substantial base of information for future research to fully explore how thrips societies in this lineage have developed specialized defences against fungal entomopathogens. It is the authours' hope that these studies spur on future research into the *Kladothrips* and the possible link between their antimicrobial defences and the development of complex sociality in this lineage.

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Appendix:

Fifty years displaced from Hamilton's Rule: The search for altruism genes

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In partial fulfillment of the PhD comprehensive exam requirements

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Introduction

The evolution of altruism has baffled biologists since Charles Darwin recognized the propagation of an altruistic caste in social insects to be a potential caveat to his hypothesis of natural selection (Darwin 1859). Even now, 154 years later the question of how altruism came about is still unanswered. Although Darwin, unknowingly, hinted at a genetic explanation for altruism, it wasn't until W.D. Hamilton published his seminal pair of papers (1964 a; 1964b) describing the genetics of relatedness in relation to altruistic behaviour, that a genetic mechanism

for altruism was hypothesized. In celebration of the 50th anniversary of Hamilton's rule, this document will first provide a short history of the field followed by a review and critique of the contentious area of research surrounding the search for altruism genes, both within insects and the microbes. To conclude, insights on the direction of the field will be presented followed by some potential future directions.

Definition

Biological altruism will be the focus of this paper and can be defined as: An individual carrying out a behaviour that reduces its own reproductive fitness but increases the reproductive fitness of another individual (Okasha 2013). Biological altruism does not require any conscious decision to act altruistically, only the innate predisposition to do so. In fact, all species discussed here are not capable of making conscious decisions as they lack the necessary neural structures. How and why this predisposition for altruistic behaviour evolved are the major questions this paper addresses.

In insects, altruism is found within five different orders: Hymenoptera (ants, bees, and wasps, Isoptera (Termites), Thysanoptera (thrips), Coleoptera (beetles) and the aphids within the Hemiptera. The most striking examples of altruism are found in the social hymenoptera, where

in some taxa, individuals will live out their entire lives in service to one reproductively active individual, reducing their direct reproductive fitness to zero. I focus primarily on the social hymenoptera and isoptera in this document because of the large amount of research dedicated to understanding their highly derived form of altruism. In addition to the social insects, altruism among microbial species has also been the target of much research and will also be discussed here (Bourke and Franks, 1995).

Microbial altruism may seem significantly different than that seen in multicellular organisms, however, many of the same social factors that are expressed by social insects are found within microbes as well. The division of labour, kin selection, complex communication and cooperation have all been observed and described within differing microbial species (Crespi, 2001). In times when nutrients are limiting individual cells in some microbial species will sacrifice their direct fitness by forming a non-reproducing stalk which aids in the reproduction of other individuals. The biology of microbes allows for unique genetic studies to be carried out which would be impossible in social insects such as the silencing of genes associated with altruistic behaviour.

Outline

I will first give a brief historical review of the key research on the evolution of altruism Second, I will attempt to compare and contrast recent research on both insect and microbial altruism. Third I will discuss and provide some insights into possible future directions regarding the field as a whole.

Altruism's historical background

Altruism has been the focus of evolutionary biology even since Charles Darwin noted that the existence of the sterile worker caste in social insects could be fatal to his theory of the origin of

species via natural selection (Darwin, 1859). Although several researchers were beginning to theorize a genetic tie to altruism outlined in Hamilton (1963), it was not until Hamilton published his seminal pair of papers in 1964 that a sound theory for the evolution of altruism was presented: The evolution of altruism via kin selection (Hamilton 1964a; 1964b). Hamilton's rule: **rb > c** suggests that if the reproductive benefit to kin produced by helping behaviour (b), multiplied by relatedness to those kin (r) by the helper is higher than the cost of reproducing oneself (c), the individual would benefit more by helping its kin than reproducing itself. Hamilton's rule became easily applicable to the social hymenoptera, as being haplodiploid, they satisfy the conditions of Hamilton's rule.

Haplodiploidy is a genetic system in which females are produced via fertilized egg and thus have a diploid genome whereas eggs destined to be male, remain unfertilized and are, therefore, haploid. This life history trait of hymenoptera results in sisters being related by r = 0.75; possessing 100% of their father's genes and 50% of their mother's. The social implication of this mode of reproduction is if workers reproduced on their own they would be related to their own offspring by only r = 0.5. Therefore, according to Hamilton's rule, helping is more advantageous than reproducing. It was this ¾ relatedness which lead researchers to believe that kin selection and inclusive fitness were the driving forces behind the evolution of altruism (Bourke and Franks, 1995

In the early 1970's, Hamilton's rule began losing favor among researchers. Studies on the social hymenoptera uncovered several factors which lowered relatedness within a colony such as multiply mated queens, multiple queens, selfish worker reproduction, and the joining of non-related individuals to a colony. Lowered relatedness meant that, in some cases, helping was not favored over self-reproduction (Lin and Michener, 1972). Critiques of kin selection began to emerge around this time (Kurland and Gaulin, 1979; Grant 1978), although understanding of

the theory was often minimal prompting Dawkins (1979) to provided simple explanations for common misunderstandings regarding kin selection. Nonetheless, new concepts pertaining to the genetic tie to altruism were expanding rapidly, as well as, the importance of mutualism and parental manipulation as the drivers of altruism were entering the literature (Michener and Brothers, 1974; Eberhard, 1975).

Examination of the genetic link to altruism was becoming possible through the development of molecular technology such as allozyme starch gel and polyacrylamide electrophoresis to differentiate polymorphic loci in social insects (Ross and Fletcher, 1985). Using polymorphic loci, it was possible to quantify the relatedness within social insect colonies allowing researchers to establish if kin selection should be taking place. The late 1980's and early 90's brought more sophisticated genetic analysis tools such as polymerase chain reaction (Saiki et al., 1988) and the use of microsatellites (Estoup et al. 1993) allowing for a more in depth genetic analysis of altruism, specifically to measure relatedness within social insect colonies (Choudhary et al., 1993). Microsatellite analysis was the standard for genetic studies on genetic diversity and relatedness (Herbers and Mouser 1998), the occurrence of multiple mating in queens (Fjerdingstad and Boomsma, 1998; Strassmann, 2001), and the frequency of selfish worker reproduction (Montegue and Oldroyd, 1998; Hastings et al., 1998) for more than a decade.

The advent of modern sequencing allowed for an organisms whole genome to be quickly sequenced and initiated the discovery of completely new genetic structures, such as, the fire ant social chromosome (Wang et al., 2013) and the ability for hymenopterans to methylate their DNA (Kronforst et al., 2008). Initially it was thought that social insects would not be taxa good for the purposes of gene discovery.

"The animals mentioned above [social hymenoptera] which are used as model behavioural systems, will never rival fruit flies and mice as engines of gene discovery…, at least in terms of elucidating basic molecular functions."

Robinson and Shahar, 2002.

Four years after Robinson and Shahar published their somewhat short-sighted review, the honeybee genome was sequenced and many previously unknown genetic structures, and gene regulatory processes were discovered, many of which not present in other insect model organisms (Honeybee Genomic Sequencing Consortium, 2006). This was a huge step forward in the field of sociobiology, incorporating genetic studies involving 'non-model' social organisms for the purpose of gene discovery such as the honeybee (*Apis mellferia*) (Honeybee Genomic Sequencing Consortium, 2006), fire ant (*Solenopsis invicta*) (Wurm et al., 2011), and the altruistic slime mold (*Dictyostelium discoideum*) (Eichinger et al., 2005)

Within a period of 50 years, our understanding has gone from Hamilton's rule, to the ability to explore the detailed molecular basis of altruism. In this regard, Thompson and colleagues (2013) give a detailed description of seven testable hypotheses and studies that would independently provide insights on the presence of genes for altruism. They suggest that genes for altruism should: 1) satisfy Hamilton's rule, 2) be environmentally sensitive, 3) increase in number and complexity with increases in social and behavioural complexity, 4) coevolve with, or depend on the previous evolution of genes for kin recognition, 5) reside in regions of low recombination, exhibit co-expression and show molecular genetic architecture, 6) should be at least partially additive, and 7) exhibit strong pleiotrophy. Altruism remains a contentious area of study, with researchers divided between its evolution potentially caused by strictly genetic or a mixture of environmental, social and genetic factors (Linksvayer et al 2009a; 2009b).

Altruism within insects

The genetic differences between reproducing and non-reproducing individuals including genes associated with caste determination are of particular attention to studies exploring the evolution of altruism. Caste determination is the process in which an immature individual's development is altered by environmental and/or genetic factors eventually leading to either the reproductive phenotype (i.e. queen) or the altruistic phenotype (soldier or worker). The differences in gene expression between these two castes can provide information on what genetic structures are necessary for altruistic behaviour in that species.

The Honeybee

Intro to Honeybees

Bees are arguably the best model organism for the study of altruism. Of the more than 16,000 species of bees (Danforth et al., 2006) the honeybee (*Apis mellferia*) has been the most heavily researched. The initiative (and funding) to study honeybees stems from their economic ($220 CAD billion annually) value as pollinators (Gallai et al., 2009). From a social biology standpoint, the bees are important because they constitute an evolutionary lineage with extant fully eusocial, semi social and solitary species (Bourke, 2001). Different extant social complexities allow for comparative behavioural and genetic studies focusing on the evolution of altruism over evolutionary time, although more genetic comparisons between social structures need to be completed (Thompson et al., 2013). As the first social insect to have its genome fully sequenced, the honey bee is currently regarded as the model organism for the genetic study of the evolution of altruism. For example, many of the honeybee genes occur in A+T rich domains possibly due to the methylation of CpG sites opening the possibility of an epigenetic tie to gene regulation, a process that up until this point was not thought to occur in bees (The Honeybee Genome Sequencing Consortium, 2006).

Genes and pathways

The recent use of RNA interference (RNAi) has allowed several altruism candidate genes to be analyzed for their effect on social insects and microbes (Zhou et al., 2006; Patel et al., 2007; Xue and Hou, 2012). RNAi, also known as post transcriptional gene silencing, has been widely used in gene discovery since the early 2000's, (Hannon, 2002). In short, RNAi effectively silences genes after transcription has taken place by destroying specific mRNA molecules, allowing the effect of a gene to be analyzed. Within the honeybee for example, target of rapamycin (*am*TOR) was experimentally knocked out in queen destined larvae wherein they subsequently developed into workers, a result suggesting the *am*TOR pathway was necessary for the evolution of diphenic castes (Patel et al., 2007). TOR is linked to both juvenile hormone and the insulin/insulin-like signaling, where differences in expression effect growth and development associated with phenotypic differences between reproductive and worker individuals

As a regulator of gene expression, microRNA (miRNA) has been used in conjunction with RNAi to determine gene function(s) in relation to altruism. MiRNA's are non-coding, conserved segments that are involved in the genetic regulation of a particular gene. Behavioural differences have been linked to changes in miRNA in the brain tissue of queens and workers. For example, several miRNAs are associated with the change of behaviour from a nurse (young worker) to a forager (old worker) potentially presenting a genetic tie to the behavioural plasticity associated with the aging of workers (Greenberg et al., 2012; Behura and Whitfield, 2010). This form of gene regulation happens without direct alteration of the organisms DNA allowing for plasticity in phenotype.

The honeybee epigenome

Epigenetic factors are thought to play a role gene regulation associated with altruistic

behaviours. Epigenetics explores changes in gene regulation that are not caused by changes in the genomic sequence. An epigenetic system is a heritable, self-perpetuating and reversible regulatory system that includes DNA methylation, histone modification, nucleosome location or noncoding RNA (for the purpose of this document I'll focus on methylation) as a mode of altering gene expression (Riddihough and Zahn, 2010). With the publication of the honeybee genome, the presence of methylation sites (CpG sites) and a functioning methylation system was uncovered, thus opening the possibility of assessing their role in regulating social behaviours (Wang et al., 2006; The honeybee genome sequencing consortium, 2006). Post honeybee, the study of gene regulation and modification within certain groups of insects has caught much attention (Callinan and Feinberg, 2006; Glastad et al., 2011) where new research is increasing our knowledge of this previously little studied process and its effect on the field of sociobiology.

A key aspect of epigenetics is the methylation of DNA which primarily occurs at CpG sites in the genome. A CpG site is the occurrence of a cytosine directly followed by guanine with a phosphate (p) connecting the two nucleotides. Given the correct 'molecular machinery', methylation of the cytosine producing 5-methycytosine can occur, effectively turning off the production of the gene associated with a particular segment of DNA (Callinan and Feinburg 2006). Deoxycytosine methyltransferases or DNMTs for short are present in the honeybee, DNMTs being a common enzyme which carries out the methylation of DNA (Wang et al.,2006). Given this discovery and the knowledge that caste-specific genes occur in areas with a high CpG density (more prone to epigenetic modulation) in the brain tissue of the honeybee suggests a possible connection to behavioural differences among castes (Table 1); Elango et al., 2009; Lyko et al., 2010).

Phenotypic differences between castes such as reduced ovary development in workers and

increased body size in queens have also been linked to differing methylation of genes (Maleszka, 2008). A recent study, for example, found 550 genes expressed in brain tissue with significant methylation differences between queen and worker castes. Among differently expressed genes TOR signalling was exclusively methylated in worker brains reinforcing the view that this pathway is heavily involved in caste determination. Methylations are abundantly found in exons and play a role in splicing new elements in before translation occurs, effectively altering the product and affecting the phenotype of individuals (Lyko et al., 2010). Moreover, two additional studies suggest that the high nutritional input gained from royal jelly, a larval food produced by nurse bees, triggers diet-induced methylation altering caste-specific gene expression (Kucharski et al., 2008; Shi et al., 2012). Therefore, individuals fed royal jelly have their transcriptomes modified and experience different gene expression levels which ultimately alter if they will take up a helping or reproductive phenotype and behaviour (Shi et al., 2012).

The studies presented above suggest that the epigenome is the structure being subjected to evolutionary pressure meaning that the environment plays a crucial role on the genes expressed. The gene therefore might not be the driver of evolution, rather, the epigenome and its interface with the environment that is necessary for the development of complex social structures. Thompson and colleagues (2013) fail to mention this influential field of study, an omission possibly due to the necessity of environmental factors influencing the genome an area which seems to be a contentious issue in the area of sociobiology.

Table 1: Caste-specific genes found to be associated with high CpG areas in the honeybee. Modified from Elango et al., 2009)

Gene/gene family	Function	Caste-biased expression	$CpG_{O/E}$ class
AmIF-2$_{mt}$ translation initiation factor	Translation of mitochondrial-encoded mRNAs	Higher in queen larvae	0/1 high-CpG
AmILP-2 insulin-like peptide	Regulation of growth/metabolism	Higher in workers than queens from second instar onward	1/1 high-CpG
AmInR putative insulin-like peptide receptor family	Regulation of growth/metabolism	Higher in worker adults	2/2 high-CpG
amTOR (target of rapamycin)	Regulation of growth/metabolism	Higher in queen 3rd instar larvae, but not 5th instar larvae (RNAi linked to worker fate)	0/1 high-CpG
Hexamerin family	Storage of amino acids for use in metamorphosis or by adults	Either more highly expressed in queen or worker larvae (based on 2 empirically analyzed genes)	3/4 high-CpG
vitellogenin	Yolk protein	Higher in queen adults	1/1 high-CpG
Yellow/major royal jelly protein family	Sex-specific reproductive maturity among other functions	Primarily more highly expressed in workers, but some more highly expressed in queens (diverse tissue-dependent expression patterns)	16/18 high-CpG

An important consideration in sociobiology is whether caste differentiation is a completely genetic driven process, versus whether the social environment an individual experiences may also play a role in caste determination (Linksvayer et al., 2009a). Honeybees for example have many candidate genes associated with caste determination (Linksvayer et al., 2009b; Barchuk et al., 2007) but the social environment (the provisioning of royal jelly by other workers) is ultimately what determines caste. This effect on gene expression caused by the social environment has been termed the sociogenome (Linksvayer et al., 2009a). Nurse bees innately produce royal jelly and control the nutrition provided to larvae, ultimately relating back to the gene regulation presented earlier within the nurse bee brain.

The opinions of Linksvayer et al (2009a) regarding environmental and social factors playing a role in caste determination seem to be gaining favour among sociobiologists. A review by Schwander at al. (2010) argues that very little evidence is available for strict environmental caste determination across different taxa; rather the environmental importance for caste determination varies. For example environmental factors are more important in honeybees than in harvester ants (*Pogonomyrmex spp*.) where genes are more involved in caste determination (Schwander at al., 2010). The real insight that can be gained from these studies is that a universal, singular, genetic structure coding for altruism is unlikely to exist even just within one lineage, rather, it is likely a combination of environmental, social and genetic factors that allowed for altruism to first evolve and then persist over evolutionary time. Some of the biggest names in sociobiology including Laurent Keller, Benjamin Oldroyde being co-authours on Schwander et al., (2010), and Timothy Linksvayer (Linksvayer et al., 2009a;b) all agree this is where the field of researching the evolution of altruism is headed in the near future.

Ants: The purely eusocial hymenoptera

Many of the phenotypes and behaviours associated with altruism are not viewed as simply dictated by the genome of a species. For example in the early 2000's caste determination was still seen as an almost purely environmental driven process in the hymenoptera (Crozier, 1977; Volny and Gordon, 2002). In 2002 a breakthrough study on the harvester ant (*Pogonomyrmex barbatus*) using microsatellites found that a specific locus within this species determines the caste of an individual. Individuals homozygous or missing this "caste locus" developed as queens, and workers developed from the heterozygous (Volny and Gordon 2002). Caste determination was hypothesized to have evolved through the independent evolution of two genotypes requiring a multiply mated queen. Although the factors listed above are not universal to all ants, Volny and Gordon (2002) set the stage for a more genetic focus on caste determination within the ants. Multiple ant species were then found to have a genetic tie to caste determination rather than just environmental factors (Hughes et al., 2003; Ross et al., 2003).

Seven ant genomes have now been fully sequenced (Smith et al., 2011a; 2011b; Bonasio et al., 2010; Wurm et al., 2011; Suen et al., 2011; Nygaard et al., 2011) allowing for comparisons to be easily made between species and over evolutionary time. As described in the honeybee, methylation of DNA has also been discovered in ants and is associated with caste determination (Kronforst et al., 2008; Gadau et al., 2012; Bonasio et al., 2012). The very recent sequencing of the ant genomes means only a handful of epigenetic studies have been carried out but already differences between bees and ants caste determination via methylation have been found. For instance the presence of non-CpG methylation sites have been found in adult ants but not bees (Bonasio et al., 2012).

Candidate genes to the altruism chromosome

The fire ant (*Solenopsis invicta*) is an aggressively invasive species characterized by differing

social structures. Monogyne (one queen) and polygyne (multiple queen) colonies are both common and each displays differing colony behaviour and queen morphology. The willingness to accept more queens is a characteristic behaviour of workers where monogyne colonies will not accept more than one queen while polygyne colonies will (Wang et al. 2013). These differences have been an area of particular interest in the study of the evolution of altruism since the early 90's when the pgm-3 allele was found via electrophoresis to have differing frequency between colony forms. This specific allele codes for the pmg-3 gene and has an influence on social structure and reproduction (Ross, 1992; Keller and Ross 1993). Pmg-3 is tightly linked to the odorant-binding protein gene GP-9 which is a better predictor of the acceptance of multiple queens (Ross and Keller, 1998).

The sequencing of the fire ant genome by Wurm and colleagues (2011) has provided a new library of information allowing for a closer look into the genomic structure of individuals with the goal of determining the genetic link to these differing social structures. Since an odorant-binding protein alone cannot account for the many different behaviours and morphologies present in the fire ant, it was hypothesized that this gene must be tightly linked to others which can account for these differences (Wang et al., 2013). Using restriction-site- associated DNA tag sequencing, Wang and colleagues (2013) located GP-9 in an area of the genome with highly reduced recombination (commonly associated with supergenes). The cause of this reduced recombination was determined to be a chromosomal inversion, using bacterial artificial chromosome fluorescent in situ hybridization (BAC-FISH) the GP-9 gene was visualized as part of this inversion. The large chromosome inversion of over 9.3 Mb long is present in GP-9b creating what the authors termed a social chromosome, which displays many of the same attributes associated with the Y chromosome in humans.

Therefore, colonies can have two variants of the social chromosome: Social B and social b (SB

and sb) with homozygous SB/SB only accepting one queen and SB/Sb possessing the chromosome inversion supergene and forming polygyne colonies. Social complexity in the fire ant is a completely genetic derived trait that influences the social environment and subsequently queen number. Nipitwattanaphon and colleagues (2013) have since expanded on this study by detailing the gene expression differences in SB/SB vs SB/Sb queens of differing ages. Many of the genes within the supergene are differently expressed between castes, ages, and sexes suggesting that these genes are highly involved in regulation of social organization (Nipitwattanaphon et al., 2013).

What began as studies describing a possible candidate gene for social structure, turned into a research program eventually leading to the discovery of a supergene now accepted as the social chromosome within *S. invicta*. In the literature today there are hundreds of papers outlining candidate genes for many different social behaviours and morphologies not unlike the original pmg-3 gene noted in 1992. In terms of the evolution of altruism genes, the fire ant social chromosome is an example of how many tightly linked genes are able to persist without recombination, altering behaviour and social structure. The increasing number of social insect genomes being published now are allowing other research groups to follow suit and potentially find and describe similar genetic structures in other focal species.

Termites

Termites are atypical social insects for a number of reasons; a colony contains a king and queen mating pair, workers can be female or male, all individuals are diploid and carry out hemimetabolis (incomplete metamorphosis) development. Workers within the lower termites are facultative altruists, due to their development immature individuals act as workers but since they have not completed their terminal molt retain the ability to reproduce (Korb and Heinze,

2008). The molt of a worker into a soldier however defines a transition from facultative to obligate altruism as soldiers are unable to reproduce.

A genetic tie to polyphenism has been discovered within the termites. Two hexamerin genes (Hex-1 and -2) carry out caste regulatory functions by supressing workers from carrying out their terminal molt keeping them in their immature stage and allowing them to continue to act as workers (Zhou et al., 2006). Queen controlled reproductive suppression is therefore necessary for altruism to take place and has been linked, using RNAi and behavioural experiments, to the overexpressed *Neofem2* gene in queens (Korb et al., 2009). Manipulation of brood by the main reproductive pair is therefore an important factor in maintaining social behaviour and reducing worker reproduction within the termites.

Contrary to the research presented above, some termites are found to have very strict genetic caste determination. In the absence of environmental cues, the caste determination of *Reticulitermes speratus* is suggested to be sex-linked (X,Y), one-locus-two-allele model (Figure 1) by Hayashi and colleagues (2007). Worker destined offspring are wk^{AB} and $wk^{A}Y$ and those destined to be reproductive offspring (nymphs) are $wk^{B}Y$ and wk^{AA} (Hayashi et al., 2007; Kitade et al., 2011). This genetic predisposition is not only present within this species, in 2011 Kitade and colleagues found that the same genetic caste determination is present in two other species within the *Reticulitermes* genus, suggesting that this genetic predisposition is the ancestral state. Termites as an atypical social insect also have a unique genetic and behavioural link to caste determination and reproductive division of labour (Korb and Heinze, 2008). Altruism in this species is therefore 100% dependent on genetic predisposition. An interesting future direction might be to compare and contrast the fire ant social chromosome research with *Reticulitermes speratus*, although this would first require a full genomic sequence.

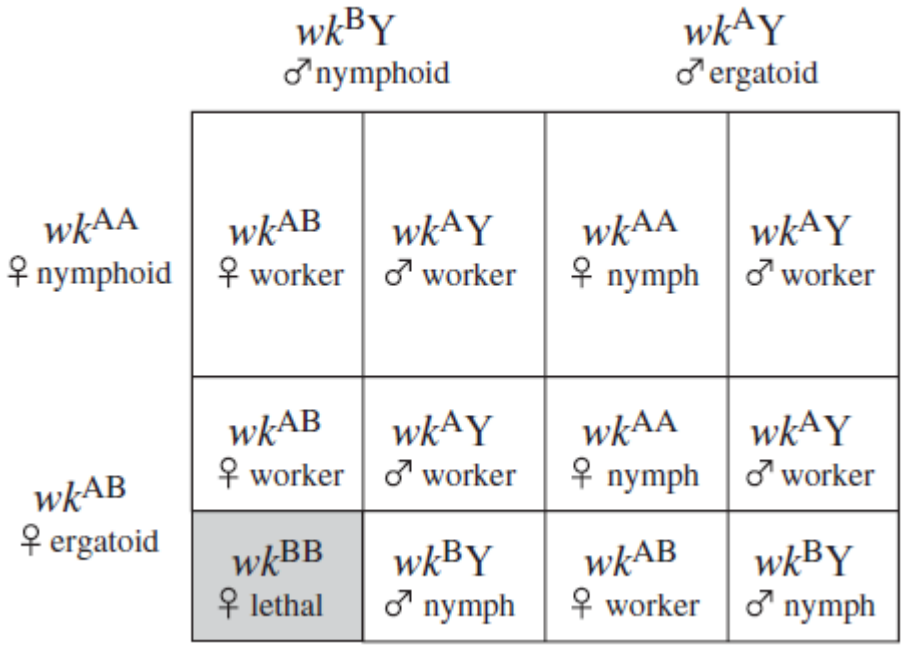

Figure 1: The sex-linked one-locus-two-allele model of *Reticulitermes speratus* caste determination. Reproduction via parthenogenesis not shown. Modified from Kitade et al., 2011

Microbial altruism

When considering altruistic behaviour, the social insects are the taxa that draw the most attention; however, there has also been increasing interest in understanding altruistic behaviour among certain microbes. Microbial altruism is mainly manifested by the formation of fruiting bodies like that seen in *D. discoideum* (Strassmann et al., 2000) and *Myxococcus xanthus*, (Velicer and Vos, 2009), or the production of biofilms as seen in many bacteria including *Pseudomonas aeruginosa*. Both fruiting body and biofilm production require that some individuals sacrifice their own reproduction and resources for others and therefore fits the definition of altruism. A real advantage of studying altruism within microbes rather than insects is the short generation time and ease of genetic manipulation, and, therefore the ability to track genetic and social behaviour changes throughout evolutionary time allowing studies to be completed that would otherwise be impossible in other model organisms (Velicer et al., 1998).

Cheaters

In order to gain knowledge on the social aspects of microbes it is advantageous to study individuals that do not carry out normal altruistic behaviour and look for changes in the genes they express. One factor which is studied in depth with microbial altruists is the presence of cheater strains. Cheating is characterized as an overabundance of a strain in reproductive tissues opposed to that seen in altruistic tissues, or a strain taking advantage of beneficial extracellular factors without contributing themselves. The study of cheaters is important because the mutations in the genome which bring about selfish behaviour would under normal circumstances be involved with the upkeep of altruism before the mutation took place. Mutants are not only used to determine candidate genes for altruism but can also test if the existence of specialized social behaviours such as kin selection taking place (Strassmann et al., 2000).

Biofilms

Bacterial social complexity can be seen in the formation of biofilms: a complex structure comprised of an extracellular matrix and a colony of bacteria. The biofilm serves several different purposes for the bacteria inside including protection from harmful substances such as antibiotics (Boyle et al., 2013; Costerton et al., 1999) and providing access to higher oxygen and nutrient concentrations (Xavier and Foster, 2007). The matrix or extracellular factors are an example of a 'public good' that is placed outside of the cell at the cost of the individual for communal uses (Parsek and Greenberg, 2005). Understanding the way in which bacteria communicate the need to form these biofilms is an important step towards understanding how social complexity and altruism is initiated within single-celled organisms. Communication among cells is initiated via quorum-sensing; a type of cell-to-cell communication in which signal molecules are sent from individual cells to and induce the production of 'public goods' such as the extracellular matrix in biofilms, as well as, more signal molecules. Communication through quorum-sensing effectively coordinates group behaviours for the production of a multicellular community when cell density is high enough (Rumbaugh et al., 2012; Fuqua et al., 1994; Darch et al., 2012). The coordination of the gene expression associated with this social behaviour can then be regarded as possible candidate genes for altruism in microbes (Miller and Bassler, 2001).

Biofilms are produced by a high density community of bacteria which can be clonal (relatedness = 1) or a chimera of different lineages (relatedness < 1). Clonal colonies that are genetically identical follow the more favorable preconception that altruism occurs when individuals contain a high level of relatedness. There is positive evidence for kin selection within pathogenic bacteria (Rumbaugh et al., 2012). *Pseudomonas Aeruginosa*, for example, has better growth rates when densities are high enough to allow quorum-sensing. Moreover, high relatedness among colonies favoured this communication providing evidence for increased reproductive

potential through the altruistic behaviour of kin (Rumbaugh et al., 2012). Within the chimeric colonies altruism could also favor strains which carry out selfish behaviour such as not aiding in biofilm production, therefore, exploiting the group (Nadell et al., 2008). One simulation study has predicted that biofilm producing bacterial strains are outcompeted by non-altruistic strains initially but as they secrete the biofilm non-altruistic strains are selected against by a reduction in oxygen and nutrients caused by the biofilm producing bacteria (Figure 2). In this way helping is selected for and altruism cannot be invaded by cheaters (Xavier and Foster, 2007).

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Figure 2: Simulation of bacterial growth under no oxygen concentration gradients at 0(a), 4(b) and 9(c) hour increments and with oxygen gradients (e – g). Growth rates with no oxygen gradient (d) and oxygen gradient (h) of both EPS+ and EPS-. EPS+: biofilm producing strain, EPS-: biofilm non-producing strain, EPS: Extracellular polymer structure (biofilm). Note the structural difference when oxygen gradient is present selecting for altruistic strains. Modified from Xavier and Foster, 2006.

Slime mold cheats, kin selection, altruistic genes

The slime molds represent a well-known group of microbial organisms in which altruism has been well studied. Specifically, the species *Dictyostelium discoideum* has received much of this attention. In times when food is readily abundant, *D. discoideum* live as solitary amoebas in the soil, however, when under nutritional stress individuals will join together into one multicellular organism. The initial multicellular stage is called a "Slug" in which all individuals move uniformly together until the signal to form a fruiting body is given. The newly formed fruiting body consists of a stalk topped by a spore body (sporus), lifting the sporus off the ground allowing for better dispersal of the spores. Only the cells that differentiate into spores will ultimately survive to form new colonies when nutritional stress is removed, the cells with stalk fate die. It is the altruistic behaviour of the stalk cells which has made this organism the model for social microbial research (Li and Purugganan, 2011).

Understanding both the pathways involved and the genes associated with communication in the slime molds elucidates the genetic mechanisms controlling altruistic behaviour. The release of cyclic AMP (cAMP) by individuals under nutritional stress is the signal that communicates the need to group together. Groups can be clonal (100% related) or chimeras with ranging relatedness; the presence of different strains within the group opens the possibility of cheating to take place. In *D. discoideum*, cheater strains are identified when they are present at a higher proportion within the spores of the fruiting body than the altruistically acting stalk cells suggesting that the genes silenced that led to this cheating behaviour are normally associated with altruistic behaviours (Strassmann et al., 2000).

The csA gene, involved in cell adhesion is a possible communicator of altruistic behaviour; individuals will actively avoid forming groups with strains not expressing csA (Queller et al., 2003). However, the lack of csA may cause a strain to be unable to form groups simply due to a

lack of cell adhesion proteins, for this reason and the fact that csA mutants are not a naturally occurring strain in populations its effect as a communicator of altruism is questionable (Queller et al., 2003; Li and Purugganan 2011). Recently however, strains of *D. discoideum* with the chtB gene knocked out exhibit selfish behaviour and are over represented in the sporus suggesting that when this gene is normally expressed it tied to altruistic behaviour (Santorelli et al., 2013). Selfish behaviour has also been tied to environmental cues where individual cells that experience nutritional starvation first are less likely to be present in stalk cells (Kuzdzal-Fick et al., 2010). It is likely that starved cells initiate pre-spore stage first and in doing so release stalk differentiation inducing factor causing other cells around it to form pre- stalk cells, effectively exploiting these individuals (Kudzel-Fick et al., 2010).

Both genetic and environmental factors can have effects on the social order in the slime molds. Cheaters strains are produced in labs and have been proven to exploit wild type colonies. Although, within naturally occurring populations of *D. discoideum* clonal groups were found much more often than chimeras and no strains within chimeras were found to be cheaters. High relatedness between clonal lines and low distribution helps to maintain altruism and lower the effectiveness of cheaters in natural populations (Gilbert et al., 2007).

The production of an altruistic stalk and fruiting body is not limited to the slime molds, the bacteria *Myxococcus xanthus* also produce these specialized structures. Group living is advantageous for a number of reasons including coordinated hunting of prey, protection against stressors, increased motility and the increase of dispersal when forming fruiting bodies (Velicer and Vos, 2009). Social living in *M. xanthus* involves the production of vesicle chains which interconnect cells and facilitate communication. These interconnections play a pivotal role in the formation of fruiting bodies and the altruistic behaviour associated therein (Remis et al. 2013).

Cheater strains have been known to occur in *M. xanthus* (Velicer et al., 1998). When faced with a cheater strain *M. xanthus* lines rapidly evolve a defence to the cheater by both increasing their own productivity but also suppressing the productivity of the cheater. Thispolicing by evolved lines is a selfish trait directly benefiting the lineage but has indirect positive effects on cooperative lines (Manhes and Velicer 2011). The changes in gene expression between cheaters and wild type populations provide information on genes normally associated with social behaviours. Kadam et al (2008) did just that and found over 100 genes differently expressed many of which are suggested as being involved in social behaviours.

Concluding remarks

Insights and opinions

Altruistic behaviours have complex regulating mechanisms. Many different pathways, mechanisms, and regulatory factors are all acting together to produce the characteristic behaviours seen in the altruistic caste. The quickly advancing technology associated with genetic testing allows researchers to scrutinize altruistic organisms with ever increasing detail since the formulation of Hamilton's rule. The advent of quick and cheap genome sequencing has produced full social organism genomes to become available allowing for comparison between taxa.

The field of epigenetics is becoming a major field in the study of altruism. Methylation of DNA, a mechanism not even known to exist in hymenoptera as of 2006, is present in all taxa studied to date and also includes termites and some social microbes. This commonality between social organisms suggests that altruism may require environmental input for gene regulation. Researchers today are beginning to realize that the gene might not be the driver of evolution as it once was thought to be.

The relative importance of environmental verses genetic factors is different between taxa adding to the difficulty associated with determining singular genetic factors associated with altruism. Social environment is also a key factor influencing the gene regulation of an individual and the colony as a whole which is seen in both insects and microbes alike.

Future directions

Some general future directions to consider include:

-Compare other ant species genomes to the social chromosome supergene in the fire ant.

-Determine if the ability to methylate DNA is present other altruistic species such as gall-inducing thrips.

-A better understanding of cooperation and coordination within biofilms paying particular attention to communication.

-Closer study of cheater strains in slime molds with emphasis on natural populations.

-Detailed studies of genetic differences observed between closely related social and non-social species.

Conclusion

The genetic tie to altruism has been an area of study hotly contested since Hamilton first described kin selection. Advances in technology have allowed researchers to get closer to defining specific genetic structures associated with the evolution of altruism in recent years in species ranging from single celled bacteria to large super colonies of ants and bees. Many different structures have been implicated in changing behaviour and phenotype towards that of an altruistic individual. However, the potential of a singular genomic element coding for altruistic behaviour in any organism is unlikely, just as unlikely as altruism forming strictly through environmental cues. The truth likely lies somewhere in the middle where altruism evolved through complex environmental and social cues while underlying genes and gene

regulation mechanisms made it likely for this life history to develop.

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Appendix II: Papers published during candidature

Pages 194-213 of this thesis have been removed as they contain published material. Please refer to the following citation for details of the articles contained in these pages.

Coates, P. J., Stow, A., Turnbull, C., Beattie, A., Hammill, C. F., & Chapman, T. W. (2017). High density brood of Australian gall-inducing Acacia thrips aid in fungal control. *Evolutionary Ecology*, 31, 119-130.
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