The Dynamics of Reproductive Dominance in Dinosaur Ants

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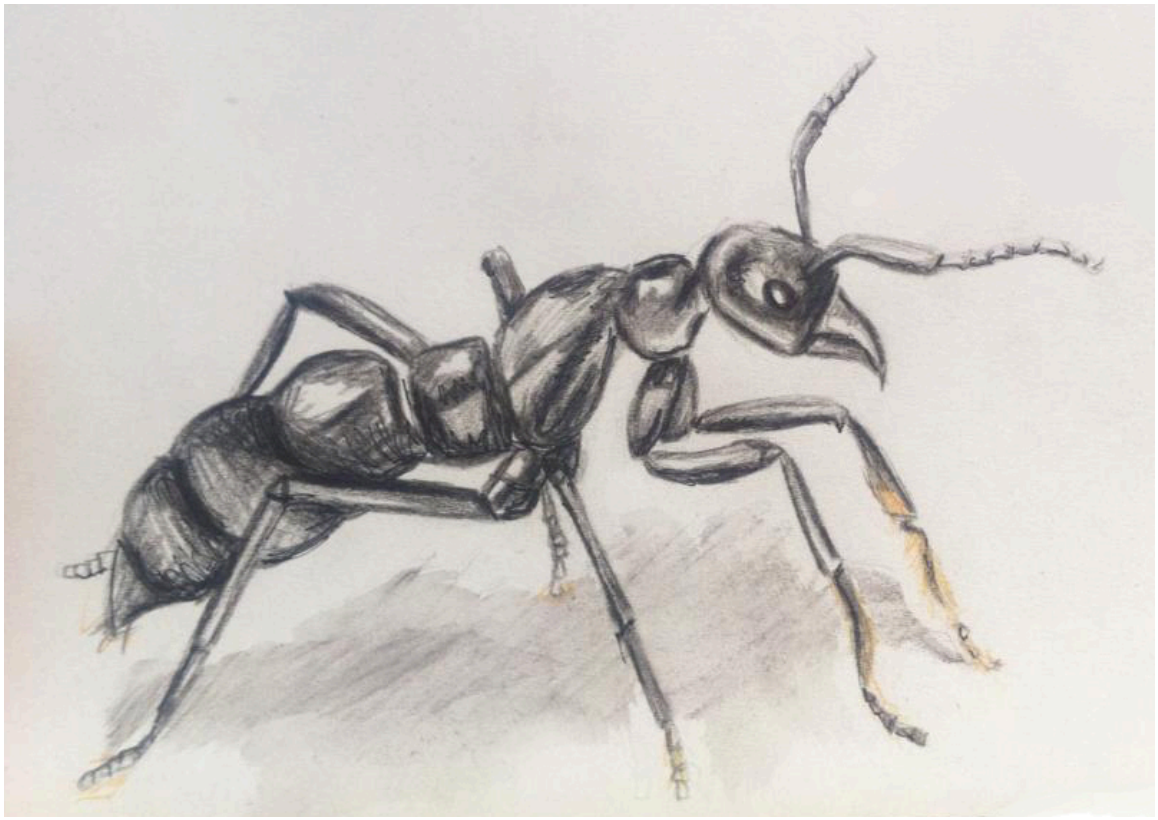
Submitted in accordance with the requirements for the degree of

Doctor of Philosophy

The University of Leeds

School of Biology

September 2013



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The candidate confirms that the work submitted is her own and that appropriate credit has been given within the thesis where reference has been made to the work of others.

The contribution of the candidate and the other authors to this work has been explicitly indicated below.

Acknowledgements

Professional Acknowledgements

I would like to thank the Natural Environment Research Council (NERC) (NE/G012121/1) and the Institute of Zoology, for funding this research. I also thank the Brazilian Government for their help in facilitating the collection and transport of colonies to the UK (transported under permits 10BR004553/DF and 11BR006471/DF from the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais).

The work for several chapters of this thesis includes collaborations with Dr Jose O Dantas (JOD), Dr Aline Andrade (AA), Natália Dantas (ND), Luzicleia Sousa (LS), Rafael Figueredo (RF), Benjamin White (BW), Dr Afsaneh Maleki (AfM), Dr Heinz Himmelbaur (HH), Dr Anna Ferrer Salvador (AFS), Dr André Minoche (AnM), Dr Francisco Câmara Ferreira (FCF), Dr Pedro Ferreira (PF) and Dr Anna Vlasova (AV). Their contributions are explicitly detailed below.

Chapter Two: JOD assisted in locating and collecting ant colonies in Aracaju. AA, ND, LS and RF assisted in field observations of foraging behaviour, ND also assisted in collection of colonies in Campo Formoso.
Chapters Three and Four: JOD assisted in collecting ant colonies in Aracaju.
Chapter Five: BW performed some behavioural observations and collected video data.
Chapters Six and Seven: HH and AF sequenced DNA and RNA samples, HH, AM and FCF assembled the genome, AM assembled and normalised the transcriptome data. AM and AV performed functional annotation of the transcriptome.

My own contributions, fully and explicitly indicated in the thesis, have been as follows. Collecting and transporting ant colonies from Brazil to the UK, as well as set-up and daily care of ant colonies in the UK (all chapters). Nest ecology measurements and behavioural observations of natural foraging in Campo Formoso (chapter two), behavioural observations of aggression (all chapters) and division of labour (chapter three and six) in the laboratory in Aracaju, Leeds and London, as well as statistical

analyses of these data. All egg policing experiments (chapter five), all RFID set-up and data analysis including developing R code for this task (chapters two and six). Behavourial observations of allogrooming, and statistical analysis of data generated by myself and BW (chapter four). DNA and RNA extractions, including protocol optimisation (chapter six and seven). Statistical analysis of the transcriptome data, BLAST analyses against the *Apis mellifera* genome, correlational analyses of foraging activity and gene expression (chapter six). Generation and analysis of toolkit list (chapter seven).

My supervisors Prof William Hughes and Dr Seirian Sumner have provided feedback on experimental design, data analysis and drafts of all chapters. As a collaborator on this project, Dr Fabio Nascimento provided invaluable assistance during field trips to Brazil. My assessors Dr Lesley Morrell and Dr Keith Hamer gave useful feedback during my 9-month and 2-year vivas. I would like to thank the members of the Hughes and Sumner labs, past and present, for their support, comments and advice throughout my PhD. In no particular order: Rowena Mitchell, Pete Graystock, Dr Crystal Frost, Dr Sophie Evison, Dr Adam Smith, Dr Kat Roberts, Dr Judith Slaa, Dr Paula Chappell, Henry Fergusson-Gow, Thibault Lengronne, Emily Bell, Dr Eli Leadbeater, Dr Inti Pedroso and Dr Ian Warren. In addition, thanks to Dr Kate Cibrowski for her tireless support with optimising laboratory protocols for RNA and DNA extraction. Several informal meetings have also provided feedback on experimental design and data interpretation including the London Social Insect group, and the Behavioural and Population Ecology (BPE) and Evolutionary and Molecular Ecology (EME) theme groups at the Institute of Zoology.

Personal Acknowledgements

A massive thank you to my family, and particularly my parents, Nick and Jane, for their tireless support, both financial and emotional, throughout my PhD and before. I also owe a huge thank-you to my friends at the Universidade Federal De Sergipe, in particular Fernanda Veira, Hans Kelstrup and Becky Matter and Tiago Santos da Silva, who went out of their way to accommodate me during my fieldwork. I owe a special thanks to Fernanda and her family, who gave me a place to stay and a friend when I

needed one most and who helped me navigate the logistics of fieldwork in Brazil. Likewise, Aline Andrade provided me with a place to stay in Campo Formoso, welcomed me into her home and went way over and above to make sure my trip went well. And Natália Dantas, who stayed with me in a remote field site, dealt with appalling weather and access issues, helped make sure my field work ran as smoothly as possible, and most of all provided companionship during my visit. I also want to thank some of my friends at the University of Leeds and the Institute of Zoology, who I have not already mentioned, for friendship, support and laughter throughout my PhD, in particular Katie Arundel, Clare Duncan, Terri Freemantle, Liz Elliot, Helen Beeston and James Lloyd. I would also like to thank Craig Roscoe, my A-level biology teacher at Newbury College, who inspired me to study biology at university. Finally, some of my oldest friends, for their support and understanding during my fieldwork and PhD as a whole, for skyping with me when I was in the middle of a field, for long chats, and also for putting up with me being a terrible friend during the more stressful parts of this project, thanks to Lynne Shone and Gemma Dodd. You are the best friends anybody could ask for. I would also like to thank Dian Thompson for the front cover image of this thesis.

Abstract

Social insects represent one of the pinnacles of social evolution, and their huge ecological success may be attributable to the sophisticated division of labour and conflict resolution observed in their societies. Eusocial societies exist along a continuum from facultative and primitive (simple) societies in which subordinates retain reproductive totipotency into adulthood, to advanced societies in which the sterile worker caste are committed to their subordinate role. Queenless ponerine ants are unusual, however, exhibiting a simple social structure but having recently diverged from an advanced ancestor. They therefore represent a powerful model system for understanding the roles of evolutionary history, ecology and sociality on behavioural and physiological division of labour. Here, I investigate the influence of reproductive dominance on division of labour and social cohesion in the queenless dinosaur ant, *Dinoponera quadriceps*. I also present the first description of their natural foraging and nesting ecology. Finally, I investigate the physiological control of division of labour and behavioural plasticity, and explore the relative contribution of conserved and novel genes in the evolution of simple society in this species. Dinosaur ants exhibit remarkable behavioural plasticity despite their advanced ancestry; individual behaviour is strongly influenced by future reproductive prospects and learned aspects of the social environment. They exhibit a discontinuous social hierarchy, in which the reproductive female is transcriptionally distinct from her subordinates, with the largest expressional differences observed in relation to reproductive physiology. Their advanced ancestry is evident both behaviourally and transcriptionally; they exhibit few differences in gene expression within the ancestral worker caste as well as advanced behaviours such as allogrooming, which has been co-opted for a role in social cohesion since their reversion to simple society. Dinosaur ants reveal the relative influences of social behaviour and evolutionary history in shaping the behavioural and physiological characteristics of eusocial societies.

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Chapter 1

Introduction

1.1 Overview

The evolution of eusociality was one of the major transitions in evolution (Maynard Smith and Szathmary 1995). Eusociality is characterised by three key traits: reproductive division of labour, cooperative care of young, and an overlap of at least two generations, so that offspring assist their parents (Wilson 1974, 2000). Superficially, the occurrence of a sterile worker caste, and the dramatic acts of self-sacrifice often exhibited by this caste, appears to be at odds with the theory of natural selection, as genes causing sterility in the worker caste could not be passed on to the next generation directly. Darwin attempted to resolve this problem by proposing the idea of colony-level selection (Darwin 1859), but it has since been better explained by inclusive fitness theory (Hamilton 1964; Trivers 1971). Inclusive fitness theory, as elegantly expressed in Hamilton's Rule, showed that altruism could be favoured by evolution if the recipient was sufficiently closely related to the altruist, and the benefit was sufficiently high relative to the cost (Hamilton 1964). Formally, altruism should be expected whenever:

$$rB > C$$

(where r = coefficient of relatedness, B = benefit to recipient, and C = cost to altruist)(Hamilton 1964)

The relatedness of colony members is therefore a key influence on altruistic behaviour, and monogamy is thought to have been a key characteristic necessary to favour the evolution of sociality (Boomsma 2009; Hughes *et al.* 2008). Although some researchers have recently questioned the validity and applicability of inclusive fitness theory (Nowak *et al.* 2010), it remains a powerful tool that can provide both explanatory and predictive power (Abbot *et al.* 2104; Boomsma *et al.* 2011; Bourke 2011; Strassmann *et al.* 2011). Following the emergence of reproductive division of labour, morphological adaptations to caste increased colony productivity, enabling them to

grow larger and more complex, which may in turn have favoured increasingly complex caste systems (Bourke 1999).

Along with the evolution of eukaryotes and of multicellularity, the evolution of eusociality was a major transition in evolution, representing the advent of higher-order organisation and cooperation between previously separate entities (Maynard Smith and Szathmary 1995; Queller 2000). Both multicellularity and eusociality involved the aggregation of related individuals, making these transitions fraternal in nature, and many commonalities can be identified between these two seemingly disparate evolutionary innovations (Bourke 2011; Patalano *et al.* 2012; Queller 2000). Understanding how eusociality evolved, and how complexity developed and was maintained, or lost, during the evolutionary history of social insects, is key to a more in-depth understanding of social behaviour, cooperation and altruism throughout the animal kingdom. New technologies, including more advanced tracking systems, improvements in analytical and statistical modelling techniques and next-generation sequencing, are allowing biologists to ask new questions about sociality, and to exploit new approaches to better answer old ones (Ament *et al.* 2012; Ferreira *et al.* 2013; Gill *et al.* 2012; Sumner *et al.* 2007). For the first time, we are beginning to gain deep insights into individual behaviour in vast colonies, and the genetic, epigenetic and physiological control mechanisms that underpin sociality.

Here, I investigate the social lives of Brazilian dinosaur ants, *Dinoponera quadriceps,* a powerful model system for investigating behavioural plasticity and social evolution. I use behavioural observations, RFID monitoring and next generation molecular techniques to examine foraging and nesting ecology, division of labour, reproductive dominance and conflict resolution in a species which has recently undergone an evolutionary reversion from a complex to socially simple condition.

1.2 The Spectrum of Eusociality

Eusociality is most commonly observed in the Hymenoptera (~15,000 ant spp., ~1900 bee spp. and ~900 wasp spp.) but is also found in around 2800 species of termites, about 50 species of aphid, 7 thrips, 6 snapping shrimps, one species of beetle, and

within mammals, in two species of mole-rat (Crespi 1992; Duffy 1996; Honeycutt 1992; Ito 1989; Jarvis and Bennett 1993; Kent and Simpson 1992; Wilson 2000). Eusociality is believed to have evolved independently at least 34 times, of which only 9 occurred within the Hymenoptera (Hughes *et al.* 2008). Therefore, eusociality represents a case of convergent evolution.

1.2.1 The Eusociality Continuum

Table 1.1 The Eusociality Continuum

Characteristics of societies at different points on the spectrum of eusociality, including examples of genera that exhibit these characteristics.

	Facultative Simple Society	**Obligate Simple Society**	**Complex Society**
Group Living	Sometimes	Always	Always
Sterile Worker Caste	No	No	Yes
Morphologically Adapted Queen	No	No (sometimes in secondarily primitive sp.)	Sometimes
Examples	**Halictid Bees** (*Augochlora, Augochlorella*, *Halictus* and *Lasioglossum*) **Hover Wasps** (*Liostenogaster*)	**Paper Wasps** (*Polistes*) **Ponerine Ants** (*Dinoponera*, *Harpegnathos*, *Diacamma*, *Ophthalmopone*) **Mole Rats** (*Heterocephalus*, *Fukomys*)	**Honeybees** (*Apis*) **Leaf-Cutter Ants** (*Atta*, *Acromyrmex*)

Across species exhibiting eusociality, there are marked differences in the extent of sociality in terms of the degree of caste-commitment (Crespi and Yanega 1995). Advanced eusociality, defined as complete, physiological commitment of the worker-caste to sterility, occurs only in insects, and primarily in members of the order Hymenoptera (bees, wasps and ants) (Crespi and Yanega 1995). However, the Hymenoptera exhibit a diverse range of social behaviour ranging from facultatively social species (e.g. halictid bees, Danforth 2002; stenogastrine hover wasps, Bell and Sumner 2013), through primitively eusocial invertebrates and cooperatively breeding

vertebrates, in which workers retain reproductive totipotency (although they generally refrain from reproduction), to advanced eusocial societies such as those seen in honeybees and leaf-cutter ants (Sherman *et al.* 1995)(*table 1.1*).

1.2.2 Reversions in Sociality

Commitment of the worker caste to sterility and the emergence of complex society is thought to be a key step in social evolution; a threshold from which it is difficult to return (Boomsma 2009). However, around 100 species of ponerine ant have undergone an evolutionary reversion to a socially simple state; worker sterility has been lost (Monnin and Peeters 2008; Peeters 1991). In some species, a single mated worker or 'gamergate' (Peeters and Crewe 1984) is responsible for all of the colony reproduction, whilst in other species several gamergates reproduce simultaneously (Peeters and Crewe 1984), and in some species, colonies contain both a queen and gamergates (Monnin and Peeters 2008). Recent molecular phylogenies of the ants, and of ponerine ants in particular have confirmed that the poneroid clade is paraphyletic (Brady *et al* 2006), however there is strong support for the monophyly of the subfamily Ponerinae (Schmidt 2013). The occurrence of gamergates across the phylogeny of this subfamily indicates that this condition has evolved secondarily multiple times (Schmidt 2009). *Dinoponera*, one of the few queenless ponerine ants, is part of the genus group *Pachycondyla*, containing predominantly advanced, queenright ponerine ants lacking gamergates (Schmidt 2009). This strongly suggests that the occurrence of gamergates, and of the queenless condition, in *Dinoponera* is a secondarily derived trait (Schmidt 2009).

1.2.3 Queenless Ants

While the majority of research into social insects has focussed on highly eusocial species such as the honeybee, primitively eusocial species offer the opportunity to gain insight into the early evolution of sociality. Primitively eusocial species have workers who retain their reproductive totipotency into adulthood, and consequently exhibit little or no differentiation between 'queen'- and 'worker'-forms (Wilson 2000). Despite reproductive totipotency, subordinates in primitively eusocial species generally refrain from reproduction (Crespi and Yanega 1995; Sherman *et al.* 1995),

which is instead dominated by one or a few dominant female(s), with subordinates often forming dominance hierarchies to determine future reproductives (Bang and Gadagkar 2012; Monnin and Ratnieks 1999). This social system has evolved convergently in both primitively eusocial species (solitary ancestor) and secondarily primitive species (advanced ancestor). Primitively eusocial species are largely descended from solitary species, and so their social organisation and the social, environmental and genetic regulators of behaviour which govern their colonies are likely to be similar to those exhibited in the early evolution of eusociality. Comparisons of secondarily derived primitively eusocial families, such as *Dinoponera* and *Harpegnathos*, with ancestrally primitive species such as *Polistes*, have the potential to yield powerful insights into the early evolution of eusociality.

1.3 Division of Labour in Social Insects

One of the key features of eusocial societies is the division of labour between colony members (Beshers and Fewell 2001; Robinson 1992), and it may have been a key contributor to the enormous success of ant societies (Wilson 1985).

1.3.1 The Evolution of Caste

The most important form of division of labour exhibited by social insects is reproductive (Wilson 2000), whereby a relatively small number of individuals in the colony are responsible for reproduction, while the majority of colony members (workers) assist in rearing young, foraging, and maintaining and defending the nest (Robinson 1992). In advanced societies, caste determination is often based on larval nutrition (Wilson 2000; Winston 1987), however in species with more flexible castes the reproductive role is more plastic and may depend upon abiotic or social conditions (Peeters 1991; Tibbetts *et al.* 2011). Several physiological controls of caste determination have now been identified. JH influences queen-worker caste differentiation in honeybee larvae (Watson 1985); *Vitellogenin* is a yolk precursor protein (Tian *et al.* 2004) that also plays a role in caste differentiation between workers (Amdam et al. 2004; Guidugli et al. 2005; Nelson et al. 2007); *Major royal jelly* proteins are glycoproteins involved in nutritional caste-determination in several

eusocial species (Thompson *et al.* 2006; Tian *et al.* 2004). Together these proteins interact to generate caste differentiation in a range of eusocial taxa.

1.3.2 Division of Labour: A Hallmark of Eusociality

In many eusocial species, the worker caste is further divided into individuals who specialise in different tasks for at least part of their adult life (Robinson 1992; Wilson 1974, 2000). While it does not increase the number or complexity of behaviours performed by members of the colony, division of labour offers the major advantage of allowing all behaviours to be performed concurrently, enabling the colony to deal with all important contingencies simultaneously (Oster and Wilson 1978). Thus, understanding how and why division of labour evolves is an important question in social evolution.

Division of labour is defined as stable variation within a colony in the tasks that individuals perform (Beshers and Fewell 2001). There are two main types of division of labour in insect societies; temporal polyethism and morphological polyethism (Beshers and Fewell 2001) or alloethism. In temporal polyethism, worker task changes with age, often with younger workers performing tasks within the nest, such as brood care, and older workers performing tasks outside the nest, such as foraging or defence (Robinson 1992). Alloethism occurs when task choice is dependent on specific morphological characteristics of the worker (Beshers and Fewell 2001), for example solider ants, which are physically adapted for defence (Wilson 2000). Alloethism is almost totally absent in bees and wasps, and relatively rare even amongst ants (Oster and Wilson 1978).

1.3.3 Flexible Organisation and Phenotypic Plasticity

One important aspect of the division of colony labour is that individual behaviour is still flexible, and individuals can switch to new behaviours according to colony requirements (Beshers and Fewell 2001). The flexibility of division of labour in insect societies may be one of its most important features (Robinson 1992), and the physiological, genetic and epigenetic mechanisms underlying this flexibility have been the focus of intense research (Chittka *et al.* 2012; Fischman *et al.* 2011; Gadagkar

2011; Patalano *et al.* 2012). The organisation and flexibility of division of labour gives the appearance of central control, however no such control system has been found (Robinson 1992). One of the proposed mechanisms of self-organisation in social insects is individual variation in internal response thresholds to certain stimuli (Beshers and Fewell 2001); a worker will only perform a task if stimuli relating to that task exceed its intrinsic threshold (Bonabeau *et al.* 1996). Individual response thresholds to stimuli could be fixed (Bonabeau *et al.* 1996, 1998), or could vary over time (Beshers and Fewell 2001; Robinson 1992), and temporal variation could explain the emergence of temporal polyethism in many social insects (Robinson *et al.* 1994). Response thresholds may be reinforced by social cues, increasing flexibility in task allocation in response to demographic changes (Beshers *et al.* 2001; Huang and Robinson 1992). Known as the 'activator-inhibitor' model, this theory is consistent with precocious foraging and reversions to nursing observed in colonies whose demography has been experimentally manipulated (Huang and Robinson 1992). The activator in this model is likely to be a physiological mechanism by which response thresholds or task preferences change with changing gene expression patterns of genes encoding key hormones or other proteins (Huang and Robinson 1992; Naug and Gadagkar 1999).

With improvements in the speed and cost of molecular technologies such as qPCR and next-generation sequencing, we are getting a glimpse of the physiological, genetic and epigenetic mechanisms that control division of labour and phenotypic plasticity in social insects. One of the first identified physiological correlates of behavioural maturation was *juvenile hormone* (*JH*), which is associated with the move from brood care to foraging (Whitfield *et al.* 2006). *JH* influences foraging behaviour in paper wasps (Giray *et al.* 2005; Hunt *et al.* 2011; Shorter and Tibbetts 2009), and termites (Weil *et al.* 2007). Changes in *JH* levels during behavioural maturation are regulated by a mutually inhibitory relationship with the yolk-precursor protein, vitellogenin (Guidugli *et al.* 2005; Simola *et al.* 2013; Sullivan *et al.* 2000), which is linked to caste determination and phenotypic plasticity (Amdam et al. 2004; Ferreira et al. 2013; Guidugli et al. 2005; Nelson et al. 2007; Weil et al. 2009). The *foraging* (*for*) gene encodes a cGMP-dependent protein kinase, PKG, which is associated with foraging behaviour in honeybees (Ben-Shahar *et al.* 2002), ants (Ingram *et al.* 2005) and

bumblebees (Kodaira *et al.* 2009; Tobback *et al.* 2010). *For* also influences behaviour in fruit flies, where allelic variation affects activity levels (Sokolowski 1980). Finally, the insulin / insulin-like growth factor signalling pathway has also been found to regulate foraging behaviour in honeybees (Ament *et al.* 2008; Wolschin *et al.* 2011).

1.3.4 The Evolution of Division of Labour

The type and complexity of division of labour and colony organisation in social insects depends on a number of intrinsic and environmental factors. However, it has been suggested that colony size is a key factor in determining social complexity (Bourke 1999, 2011). The strong influence of colony size on social complexity stems from the change in reproductive potential of workers as colony size increases (Bourke 1999). Increases in colony size sharply decrease an individual's prospect of reproduction, leading to selection to maximise indirect fitness through specialisation (Bourke 1999). This may have been a key force driving the evolution of both reproductive and non-reproductive division of labour (Bourke 2011). In the absence of morphological differences between workers, division of labour may relate to the relative costs and benefits of certain tasks to different workers; temporal polyethism often results in riskier tasks (e.g. foraging) being performed by older workers, whose loss has a smaller effect on colony productivity (Cant and Field 2005; Field *et al.* 2006). Mathematical modelling has identified two important factors affecting division of labour; the number of tasks needed to maintain colony function ('task number'), and the amount of work available relative to the size of the workforce ('demand') (Jeanson *et al.* 2007).

1.3.5 Technological Developments in Behavioural Research

One of the key problems with behavioural studies of social insects has been the need to identify individuals (Streit *et al.* 2003), from colonies of up to millions of individuals (Wilson 1974), in order to satisfactorily answer many of the interesting questions about division of labour and reproductive conflict. The classic solution to this problem is to use paint markers or numbered tags applied to the abdomen, to allow the identification of individuals on video recordings, although this is an extremely time-consuming task, necessarily involves significant disruption of the nest, and is only practical with relatively small samples (Streit *et al.* 2003).

A major development in behavioural studies of social insects is the design of tracking technologies that permit continuous, long-term monitoring of entire colonies (Robinson *et al.* 2009). One such technology is passive radio-frequency identification tagging (RFID); RFID tags do not require a battery source, receiving power from the reader when passed through an antenna, and are therefore lightweight and have an essentially unlimited life-span (Streit *et al.* 2003). RFID tagging has now been successfully utilised in studies of ants (Robinson *et al.* 2009), wasps (Sumner *et al.* 2007), bumblebees (Molet *et al.* 2008) and honeybees (Gill *et al.* 2012), and promises to revolutionise the study of social insect behaviour in future studies.

1.4 Conflict in Eusocial Societies

Although social insects may superficially appear to represent the pinnacle of cooperative behaviour, they are in fact characterised by many conflicts (Ratnieks *et al.* 2006). These conflicts arise because individuals within a colony are not genetically identical (Ratnieks *et al.* 2006). Hamilton's rule specified the conditions under which we would expect cooperative behaviour to evolve, but it also highlighted occasions when cooperative behaviour should not be expected (Hamilton 1964), leading to the prediction of a number of conflicts which were later discovered in the social Hymenoptera (Ratnieks *et al.* 2001). Hymenoptera are haplodiploid, meaning that females are diploid and develop from fertilised eggs, whilst males are haploid, developing from unfertilised eggs, and this has a major impact on relatedness between different individuals within a colony (Trivers and Hare 1976).

1.4.1 Why Conflict Arises

Conflicts that may be expected in social insect colonies include conflict over worker reproduction, conflict over the sex-ratio of queen-laid eggs and conflict over caste determination (Ratnieks *et al.* 2006). The extent to which each, or any of these conflicts is observed in different Hymenopteran species often depends on the specifics of relatedness within the colony (Ratnieks *et al.* 2006), as well as the resources

available to different parties involved in the conflict (Ratnieks *et al.* 2006; Trivers and Hare 1976).

In general, conflicts are expected to occur when individual workers, or the worker caste as a whole, differ from the queen in their reproductive optima (Ratnieks *et al.* 2006). For example, in haplodiploid insect colonies with a single, monogamous queen, there is potential conflict over her relative investment in the production of male and female offspring (Ratnieks *et al.* 2006). Female workers are more closely related to sisters than to brothers, so should favour a sex ratio (female: male) of 3:1, whilst the queen would prefer a 1:1 ratio (Ratnieks *et al.* 2006; Trivers and Hare 1976). A ratio close to the worker optimum of 3:1 was found across 21 species of monogynous ant, supporting the existence of this conflict in insect societies (Trivers and Hare 1976). However, later studies have found that sex ratios are in fact intermediate between the two optima (Boomsma 1989), suggesting that neither caste has total control.

1.4.2 Conflict Resolution

1.4.2.1 Honest Signals

Potential conflict may not be realised for various reasons. Conflict resolution may occur if one of the conflicting parties has insufficient power or information to take the necessary actions to reach their optimum (Ratnieks *et al.* 2006; Trivers and Hare 1976). Information in the context of conflict generally comes in the form of cuticular hydrocarbons (CHCs), which have been shown to signal a wealth of information about identity, including nest membership or relatedness (Soro *et al.* 2011), dominance (Mitra *et al.* 2011) and fertility (D'Ettorre *et al.* 2004; Izzo *et al.* 2010; Liebig *et al.* 2000; Monnin 2006; Smith *et al.* 2012). CHCs generally provide honest signals, for example certain hydrocarbons appear to be inextricably linked to ovary development and fertility (D'Ettorre *et al.* 2006), providing a means of identifying queens as well as illicitly reproductive individuals (Liebig *et al.* 2000). CHCs are also transferred to eggs and can be used to discriminate between eggs laid by queens and workers (D'Ettorre *et al.* 2006). Sex differences are not so clearly signalled, however, and there is little evidence that hymenopteran workers can identify the sex of brood prior to the larval or pupal stage (Nonacs and Carlin 1990; Passera and Aron 1996).

1.4.2.2 Social Policing

In some cases, the reproductive optimum for an individual worker may differ from that of the worker caste as a whole, and this may lead to conflict resolution by worker policing. Consequently, although the majority of hymenopteran workers are unable to reproduce sexually, they are often capable of producing unfertilised eggs that will develop into males (Hart and Ratnieks 2005). In some colonies workers may attempt to cheat and lay unfertilised, male eggs (Ratnieks and Visscher 1989). In many species, workers should oppose this and may police the behaviour either by attacking the cheater or by eating her eggs (worker policing) (Ratnieks and Visscher 1989). Queen policing could also potentially resolve conflicts in insect societies, however this is unlikely to be effective in large colonies, because there are too many workers for the queen(s) to police (Ratnieks *et al.* 2006; Trivers and Hare 1976). In species with simpler societies, however, where colony size tends to be lower, queen policing is an effective mechanism to deter subordinate reproduction (Fletcher and Ross 1985; Kikuta and Tsuji 1999; Spradbery 1991)

Worker reproduction is a widespread source of conflict in eusocial societies, and policing of worker reproduction has been documented in numerous species including bees (Pirk *et al.* 2003; Ratnieks and Visscher 1989; Wenseleers and Ratnieks 2006), wasps (Foster and Ratnieks 2001a; Wenseleers and Ratnieks 2006; Wenseleers *et al.* 2005) and ants (D'Ettorre *et al.* 2004; Monnin and Peeters 1997). The occurrence of worker policing can be explained in some species on relatedness grounds alone; workers in polygynous or polyandrous societies are more closely related to sons of the queen than sons of other workers, favouring worker policing (Ratnieks 1988). However, policing has been documented in a number of species despite an absence of these relatedness benefits (D'Ettorre *et al.* 2004; Foster and Ratnieks 2001a; Kikuta and Tsuji 1999; Saigo and Tsuchida 2004). Policing in these species may be favoured if widespread worker reproduction has a detrimental effect on colony productivity (Hartmann *et al.* 2003), or if conflicts over sex allocation combined with an error-prone sex discrimination mean that policing is an effective mechanism for removing male eggs (Foster and Ratnieks 2001b; Mehdiabadi *et al.* 2003).

1.4.3 Conflict in Simple Societies

In primitively eusocial species, where workers are reproductively totipotent, the potential for conflict is much greater (Hart and Ratnieks 2005). In some species, subordinates are able to found nests independently, however in other species low fecundity necessitates group nest founding and in other cases precludes nest foundation as a reproductive strategy for subordinates. Workers in these colonies have several options for reproduction: overthrow the current reproductive, wait and hope to supersede her after she dies, or illicitly produce males within the natal nest (Cant *et al.* 2006; Hart and Ratnieks 2005). There is therefore significant potential conflict over worker reproduction, in particular over the timing of breeder replacement, as well as over subordinate production of males (Hart and Monnin 2006; Hart and Ratnieks 2005; Tsuchida and Suzuki 2006).

1.4.4 The Molecular Basis of Sociality

The complex, plastic phenotypes of caste we observe across the spectrum of eusociality are underpinned by an equally complex network of physiological control, involving epigenetic modifications and transcriptional regulation of gene expression. Sociogenomics, the study of how social behaviour is influenced by genes and their expression patterns (Robinson *et al.* 2005), is yielding insights into the evolution of sociality. Several recent studies have investigated transcriptome profiles of different castes in honeybees (Cardoen *et al.* 2011; Grozinger *et al.* 2007), bumblebees (Colgan *et al.* 2011), paper wasps (Ferreira *et al.* 2013), harvester ants (Bonasio *et al.* 2012), and a ponerine ant (Bonasio *et al.* 2012). Research has also focussed on the epigenetic regulation that generates these expressional differences (Greenberg *et al.* 2012; Lockett *et al.* 2011; Lyko *et al.* 2010; Shi *et al.* 2013; Simola *et al.* 2013).

Between 12% and 39% of genes in honeybees have been shown to exhibit caste-bias (Cardoen *et al.* 2011; Grozinger *et al.* 2007), while in paper wasps, between 7% and 12% of genes show expression bias in relation to caste (Ferreira *et al.* 2013; Toth *et al.* 2010). A comparison between sterile and mutant reproductively active honeybee workers revealed a greater number of genes up-regulated in the sterile worker-caste

(Thompson *et al.* 2006), and a similar pattern has been observed in paper wasps (Ferreira *et al.* 2013). Across bee lineages, gene expression differences have been shown to relate to numerous caste-related differences including maturation, foraging and aggression (Chandrasekaran *et al.* 2011; Colgan *et al.* 2011; Zayed and Robinson 2012).

1.4.5 A Toolkit for Sociality

Division of labour decouples behaviours that were previously observed in a single, solitary individual (Johnson *et al.* 2010; West-Eberhard 1987). In many solitary species, reproduction and provisioning behaviours are temporally decoupled, and genes regulating this cycle may have been readily co-opted for division of labour during the evolution of eusociality (Ament *et al.* 2010; Ihle *et al.* 2010; Tibbetts *et al.* 2011; Toth *et al.* 2007; West-Eberhard 1987). Thus, it is possible that, much like the homeobox genes in multicellular evolution, a set of 'toolkit' genes may have underpinned the convergent evolution of eusociality across lineages (Johnson *et al.* 2010; Toth and Robinson 2007).

There is now substantial evidence from a range of different social insects that the foraging gene plays a crucial role in caste-specific behaviours, in both temporal and morphological caste species. Along with *JH*, *vitellogenin* and IIS, the foraging gene provides evidence that the evolution of eusociality has proceeded through changes in the regulation and expression patterns of ancestral genes, rather than through the evolution of novel genes (Robinson and Ben-Shahar 2002). However, caste differentiation cannot be mediated purely by the action of such a small number of genes. Toolkit genes may have played a role in the evolution of eusociality, however novel gene families and regulatory pathways must also have been important.

1.4.6 Genetic Innovation in the Evolution of Eusociality

Genome-wide studies are increasingly revealing a large contribution of novel genes to polyphenism in social insects. Across the honeybee genome, 696 genes (6%) are found only in insects; 182 of these genes are found exclusively in the honeybee, and these genes tend to be associated with phenotypes unique to eusociality (Johnson and

Tsutsui 2011). Furthermore, only 6% of genes showing recent, rapid evolution are conserved across 3 independent origins of eusociality in bees, with greater novelty in primitively eusocial species (Woodard *et al.* 2011). A similar pattern has been observed in two closely related species of termite, where novel genes appear to have contributed substantially to eusocial evolution over a relatively short evolutionary timescale (Weil *et al.* 2009). Novel genes appear to have been of greater importance in the emergence of reproductive than non-reproductive division of labour (Johnson and Tsutsui 2011; Toth *et al.* 2010). It seems likely that both toolkit genes and novel genes have contributed to the multiple convergent evolutionary origins of eusociality.

1.4.7 Epigenetic Control of Division of Labour and Caste

Although the relative importance of toolkit genes to sociality remains uncertain, a transcriptional toolkit is becoming increasingly apparent. Caste-biased DNA methylation patterns have been observed in honeybees (Bonasio *et al.* 2012; Lockett *et al.* 2011). MicroRNAs and chromatin modifications are also important epigenetic regulators and have been found to show caste bias in several eusocial species (Behura and Whitfield 2010; Greenberg *et al.* 2012), and a conserved set of transcription factors has been found to regulate caste-biased gene expression across taxa (Ament *et al.* 2012; Chandrasekaran *et al.* 2011; Zayed and Robinson 2012).

1.5 Dynamics of Reproductive Dominance

1.5.1 Aims and Hypotheses

The overall objective of this project is to gain a greater understanding of the organization, sociogenomics and dominance dynamics in societies with simple sociality, using the ponerine ant *Dinoponera quadriceps* as a model system. Dinosaur ants have an unusual system of colony organization; workers are all morphologically identical and physically capable of sexual reproduction, unlike more advanced social insects where the queen is morphologically distinct. This provides a rare opportunity to investigate how cooperative societies are maintained in the absence of sophisticated morphological adaptations, and therefore can offer insight into how cooperation may have been maintained during early social evolution.

In addition to describing some aspects of the basic biology and ecology of the study species (chapter two), the project aims to address four outstanding hypotheses in social behaviour and evolution. Firstly, I aim to investigate how reproductive dominance influences the behaviour of individual dinosaur ants, in particular in relation to division of labour and risk aversion (chapter three), and explore the role of non-aggressive interactions in maintaining social cohesion (chapter four). Secondly, I test the role of learning and experience in conflict resolution in the form of social policing (chapter five). Finally, I investigate how gene expression relates to dominance rank, reproductive physiology and provisioning behaviour (chapter six), and provide further test of the toolkit hypothesis in a secondarily simple society (chapter seven). By combining behavioural observations and radio-frequency identification tracking with next generation sequencing methods, I aim to investigate the social and transcriptional controls of behavioural plasticity in a secondarily primitive eusocial insect

1.5.2 Study System

One of the best studied species of queenless ant is *Dinoponera quadriceps*, a species with a single gamergate (Monnin and Peeters 1998) followed by a short, linear hierarchy of workers (Monnin and Peeters 1999). Workers within this hierarchy will take her place if she dies (Monnin and Peeters 1998) or her fertility is reduced (Monnin and Peeters 1999). When a gamergate is replaced, she will generally be replaced by the beta worker (Monnin and Peeters 1998, 1999), who will then venture a short distance outside the nest to mate (Peeters 1991). Males are attracted to colonies containing a virgin gamergate, and will meet her just outside her natal nest (Peeters 1991). Workers have functional spermatheca (De Araujo *et al.* 1990), and so are capable of sexual reproduction. However they generally do not have fully developed ovaries (Monnin and Peeters 1997), and are not attractive to males (Monnin and Peeters 1998), so only the new gamergate will mate.

Ponerine ants are a good model organism for the study of division of labour and reproductive conflict in social insects because all females have equal reproductive potential (Monnin and Ratnieks 1999). Ponerine ants, unlike other primitively eusocial

species such as polistine wasps, are also easy to keep in the laboratory, where they will behave naturally and even mate (Monnin and Ratnieks 1999). In general, aggression is low within queenless colonies, primarily occurring between the gamergate and high-ranking workers (Monnin and Peeters 1999). The presence and fertility of the gamergate is probably signalled chemically, and her cuticular hydrocarbon profile is distinct from all other workers (Peeters *et al.* 1999). One aggressive interaction: gaster rubbing, in which a worker grasps one antennae of another worker, and rubs it against their abdomen (Monnin and Peeters 1999) (*figure 1.1*), is likely to facilitate the transfer of cuticular hydrocarbons, and thus inform the target worker of their rank. There is good evidence that the cuticular hydrocarbon profile is an honest signal of the fertility and dominance rank of the gamergate (Peeters *et al.* 1999).

Figure 1.1 Gaster Rubbing in *D.quadriceps* (Monnin and Peeters 1999)

High-ranking subordinates, whose high-rank will only be maintained for a short time (Ratnieks *et al.* 2001), should favour overthrowing the gamergate instead of allowing another individual to supersede her later (Hart and Ratnieks 2005). However, this is opposed by the rest of the workers in the colony, and low-ranking workers will cooperate with the gamergate to prevent premature overthrow (Monnin and Peeters 1999; Monnin *et al.* 2002). Once the gamergate's fertility drops below 75%, workers no longer cooperate with her to prevent overthrow and may even immobilise her to allow replacement to occur (Monnin and Ratnieks 2001). High-ranking subordinate dinosaur ants should benefit from high phenotypic plasticity and access to information about the fertility and reproductive status of her nestmates.

1.5.3 Concluding Remarks

Social insects live in complex, dynamic societies, which bear striking resemblances to other cooperative units including chromosomes and multicellular organisms, as well as to other social animals including humans. Understanding the behavioural and physiological mechanisms that underpin their societies will provide insights into social behaviour, cooperation and conflict across living organisms. With their unusual social structure and evolutionary history, the Ponerine ants have the potential to disentangle the complex relationship between reproductive dominance, physiology and behaviour in simple societies, and answer novel questions about how eusocial societies are maintained.

Chapter 2

Ecological and Social Effects on Nesting, Foraging and Circadian Activity

2.1 Abstract

Ants are of major importance both ecologically and as models in evolutionary biology, and queenless ponerine ants are of particular interest for understanding the behaviour and evolution of simple societies. Most studies with queenless ponerine ants have been focused on investigations of social dynamics using laboratory colonies, but information about the basic ecology and biology of model species is also important for understanding their life-histories. Here, I investigate the foraging behaviour, circadian rhythms and nest ecology of the dinosaur ant, *Dinoponera quadriceps,* using behavioural observations of field and laboratory colonies, and radio-frequency identification tagging. Field observations of marked foragers revealed an individual, opportunistic foraging strategy, informed by short reconnaissance trips to gauge abiotic conditions. The diet of *D. quadriceps* is composed mainly of live insect prey, supplemented by significant amounts of fruit and seeds which suggest the species has an important role in seed dispersal. Foraging activity was influenced by time of day, temperature and humidity, with peaks in foraging activity occurring at dawn and dusk. Tandem running was observed occasionally after dark, and four out of seven field colonies exhibited nest drifting by workers. Remarkably, this nest drifting was over considerable distances (12 m on average) and involved the drifting workers transporting food into the non-natal nest, making it unlikely to be accidental or due to polydomy. A greater understanding of the natural foraging biology and nesting dynamics of ponerine ants is needed to understand their social behaviour and evolution.

2.2 Introduction

Ants (Hymenoptera, Formicidae) are among the most abundant and ecologically successful species on Earth. There are around 15,000 species worldwide (Antweb 2012), which exhibit a huge range of different life history strategies, diets, nesting

habits and foraging behaviour, in adaptation to an enormous variety of different climatic and habitat conditions. They perform vital ecosystem services as major predators, scavengers and mutualists and play a key role in symbiotic interactions, soil aeration and nutrient cycling. They are important as indicators of biodiversity, playing a key role in monitoring the impact of global change on ecosystems (Folgarait 1998; Majer 1983; Peck *et al.* 1998). They are also of great interest because of their complex eusocial societies, which exhibit high levels of altruism and phenotypic plasticity, representing one of the pinnacles of social evolution (Holldobler and Wilson 1990; Wilson 1974). Many ant species can be easily kept under laboratory conditions, facilitating more detailed experimental investigations of their social behaviour. However, an understanding of their foraging and nesting behaviour in the wild provides an important backdrop in which hypotheses and data interpretation must be framed.

2.2.1 Foraging Behaviour and Circadian Rhythms in Ants

A number of different abiotic variables are known to influence foraging activity and behaviour among ants (Carroll and Janzen 1973). Across habitats and latitudes, temperature constrains and influences foraging activity. For some species temperature may set an upper and / or lower limit on foraging activity (Fowler and Roberts 1980; Gamboa 1976; Ibm 2003; Jayatilaka *et al.* 2011; Meisel 2006), while for others it plays a smaller, but still significant role, influencing variation in foraging activity (Duncan and Crewe 1994; Fourcassie and Oliveira 2002; Kuate *et al.* 2008; Oudenhove *et al.* 2011; Yamamoto and Del-Claro 2008). Temperature may also influence foraging strategies, as pheromone trails decay more rapidly at higher temperatures and individual foraging in many species may represent an adaptation to high soil temperatures (Oudenhove *et al.* 2011; Ruano *et al.* 2000). Another abiotic influence on foraging activity may be humidity (Kuate *et al.* 2008; Yamamoto and Del-Claro 2008), although evidence for its importance is less well documented. Other climatic variables such as rain can also prevent activity (Fowler and Roberts 1980; Gamboa 1976; Gobin *et al.* 1997).

Endogenous (built in) factors such as circadian influences may also be important in influencing foraging behaviour. Both nocturnal (Fowler and Roberts 1980; Jayatilaka *et*

al. 2011) and diurnal (Dejean and Lachaud 1994; Duncan and Crewe 1994; Fourcassie and Oliveira 2002; Ibm 2003; Jayatilaka *et al.* 2011; Pie 2004) foraging patterns are known in the ants, as well as more complex rhythmic activity patterns (Lewis et al. 1974) and specialised forager sub-castes working on different circadian rhythms (Orr and Charles 1994). It may be difficult to discern an effect of time of day on foraging behaviour because it is often tightly correlated with temperature. In some species, time of day appears to be a crucial factor controlling foraging behaviour (Jayatilaka *et al.* 2011), while in others these effects disappear when temperature is controlled for (Ibm 2003).

2.2.2 Ponerine Ants

The Ponerinae are a diverse and widely distributed subfamily of tropical ants (Antweb 2012; Bolton 2006; Paiva and Brandao 1995). They have been of particular interest to studies of evolutionary biology because of their unusual social structure; in these species the queen has been partially or completely replaced by reproductively active workers (Peeters 1991). The dynamic mechanisms for maintaining reproductive skew observed in these species have made them a focus of research into social evolution and phenotypic plasticity (Asher *et al.* 2013; Monnin *et al.* 2002; Peeters 1993; Peeters *et al.* 1999). Further, ponerine ants have an unusual evolutionary history, being descended from an advanced ancestor and having secondarily lost the queen caste in the last 70 million years (Peeters 1991; Schmidt 2009). Ponerine ants are therefore powerful model systems for investigating reproductive dominance, behavioural plasticity and polyphenisms, and conflict resolution in eusocial systems.

Several species of ponerine ant have been extensively studied in laboratory conditions (Cuvillier-Hot *et al.* 2002; Fukumoto and Abe 1983; Monnin and Peeters 1997; Tsuji *et al.* 1998), and foraging behaviour in the field has been reported for *Hagensia havilandi* and *Brachyponera senaarensis* in Africa (Dejean and Lachaud 1994; Duncan and Crewe 1994), *Gnamptogenys menadensi* in Asia and *Ectatomma opaciventre*, *Dinoponera gigantea* and *Dinoponera quadriceps* Santschi in South America (Araujo and Rodrigues 2006; Fourcassie and Oliveira 2002; Pie 2004). Ponerine ants are generally diurnal foragers (Dejean and Lachaud 1994; Duncan and Crewe 1994; Fourcassie and Oliveira

2002; Pie 2004), however some species are active throughout the day (Gobin *et al.* 1997). Nest architecture has been described for several species of ponerine ant; *Ectatomma edentatum* (Antanialli 2001), *Ectatomma vizottoi* (Vieira *et al.* 2007), *Dinoponera roger* (Paiva and Brandao 1995), and *Dinoponera quadriceps* Santschi (De Araujo *et al.* 1990). However, the external characteristics of the nests have rarely been investigated.

Dinosaur ants are members of the subfamily *Dinoponera*, found in Northern and Central South America, an area encompassing a wide variety of habitats including moist broadleaf forest, coniferous forest, grassland, savannah, shrubland and xeric shrubland (Olson *et al.* 2001), and covering a range of different climatic conditions. Even within single species, populations exist in a variety of different habitat types, indicating adaptability to a variety of conditions. *Dinoponera quadriceps* is one of few species whose range covers both coastal and inland habitats (Paiva and Brandao 1995), which differ greatly in the extent of their seasonality and climatic conditions. Dinosaur ants, and *D. quadriceps* in particular, appear to be adaptable to a variety of different environments, and variability in their nesting and foraging behaviour might be expected across these environments.

2.2.3 Aims and Hypotheses

In this study I investigate the environmental, ecological and social determinants of foraging behaviour in colonies of the Brazilian ponerine ant, *D. quadriceps*, across ecological and abiotic gradients. I first investigate nest ecology across abiotic conditions, comparing between nests in Caatinga and Atlantic Forest biomes (aim 1). Following this, I use behavioural observations of foraging behaviour in wild colonies, combined with continuous monitoring under laboratory conditions, to provide the most accurate study yet of foraging behaviour in the dinosaur ant *D. quadriceps*. Using this data, I report an investigation of diet in this species (aim 2) and investigate the role of several abiotic factors including temperature, humidity and rainfall on foraging activity in field colonies of *D. quadriceps* (aim 3). I then look at the influence of time of day on foraging behaviour, measuring the diurnal foraging patterns of field colonies,

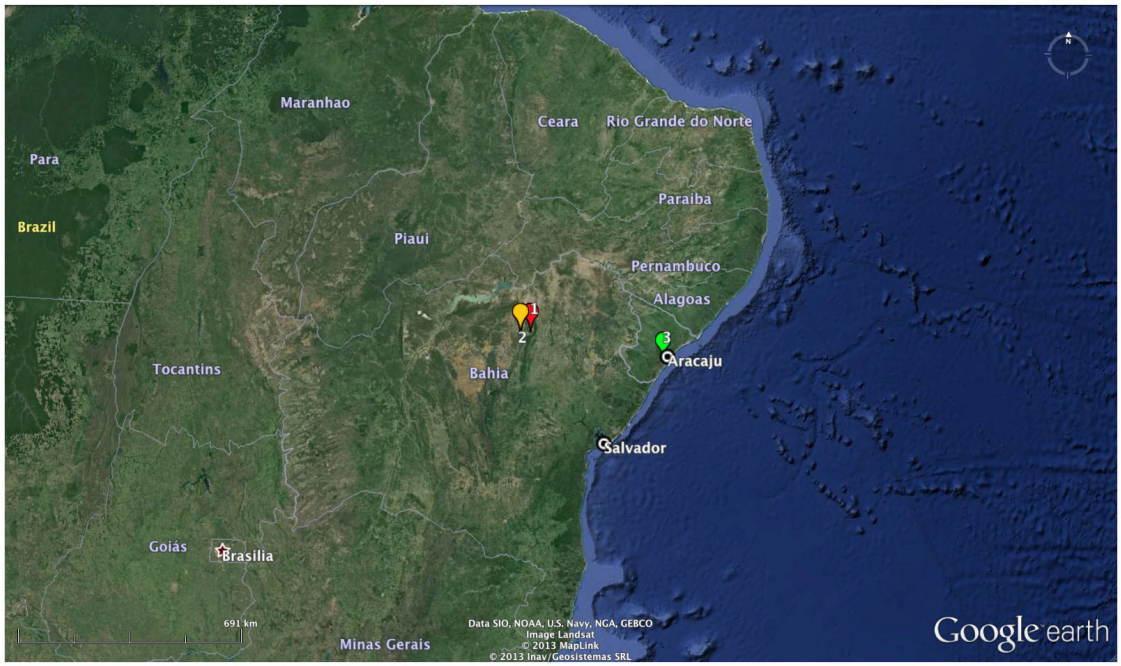
and entire circadian foraging pattern for laboratory colonies (aim 4). I also investigate social determinants of foraging through mechanisms of nestmate recruitment (aim 5).

2.3 Methods

2.3.1 Field Sites

Colonies of *D. quadriceps* were located at two sites near Campo Formoso, Bahia and a third near São Cristavão, Sergipe in Brazil between 2009 and 2011 (*table 2.1*). In total, 46 nests were located, 17 in woodland and 16 in a fruit plantation in Campo Formoso, and a further 13 nests in closed scrubland near São Cristavão (*figure 2.1*; *appendix A1.1*). The two sites in Campo Formoso are categorised as part of the Caatinga biome (Velloso 2010), characterised by a semi-arid environment with thorny-shrubs and stunted trees (Galindo-Leal and Camara 2003) (*figure 2.1b*). The woodland site (site one) was primarily composed of broadleaf semi-deciduous trees and shrubs, including the following plant families; Melastomataceae, Apocynaceae. The fruit plantation (site two) had been mostly cleared of natural vegetation and replaced by flowering plants from the Fabaceae, Euphorbiacea, Rubiaceae, Bromeliaceae, Myrtaceae, Rutaceae and Moraceae. The scrubland site (site three) is part of the Atlantic forest biome (Velloso 2010) which runs along the North east coast of Bahia and Sergipe. Sites one and two show greater seasonality (difference between min and max temperatures) and lower rainfall than site three (*table 2.1*)(Hijmans *et al.* 2005).

a



b

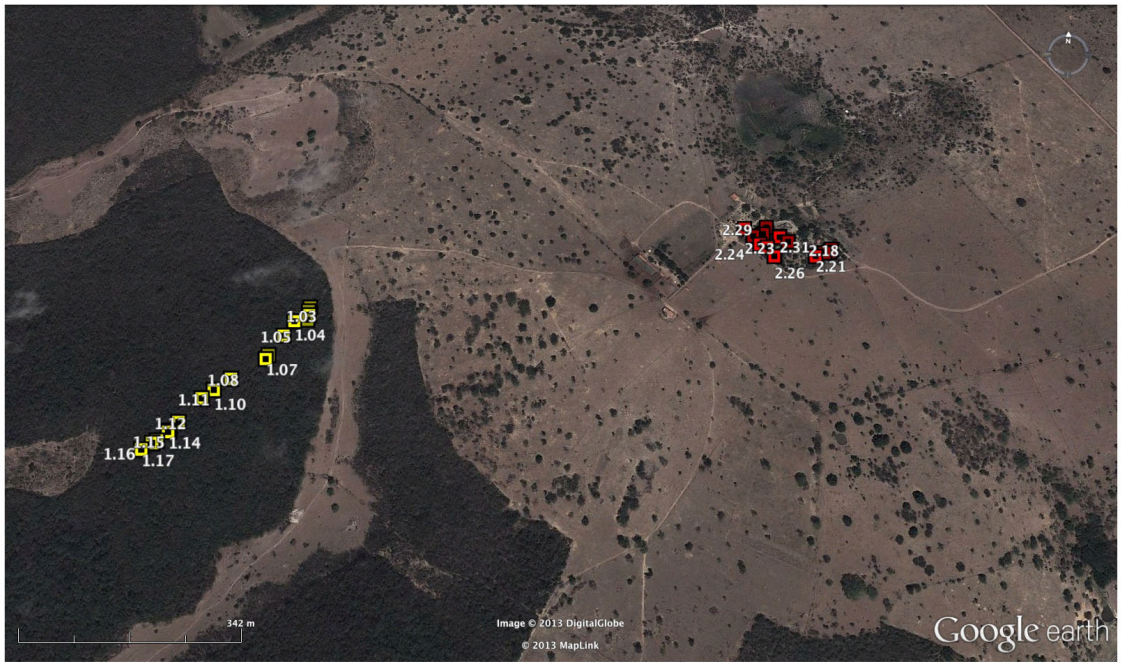


Figure 2.1 Field Sites in Brazil

Maps / satellite images showing the location of colonies studied. a) Map of Northeast Brazil showing three sites – site one (yellow), site two (red) and site three (green). b) Map of colonies located in Campo Formoso at the woodland site (site 1, yellow) and the fruit plantation (site 2, red).

Table 2.1 Characteristics of Field Sites

Location and description of each field site. Climatic data for annual precipitation, max temperature, mean temperature and min temperature from WorldClim (Hijmans *et al.* 2005). Data for max and min observed temperature for site one collected using EasyLog USB data logger across duration of foraging behaviour study.

	Site One "Woodland Site"	**Site Two "Fruit Plantation"**	**Site Three "Scrubland"**
Location	Campo Formoso, Bahia	Campo Formoso, Bahia	São Cristavão, Sergipe
Exact Location	10°27'1.66"S 40°20'47.72"W	10°26'52.94"S 40°20'18.22"W	11° 1'24.37"S 37°12'6.23"W
Biome	Caatinga	Caatinga	Atlantic Forest
Major Plant Families	Melastomataceae, Apocynaceae	Fabaceae, Euphorbiacea, Rubiaceae, Bromeliaceae, Myrtaceae, Rutaceae, Moraceae	
Annual Precipitation (mm)	716	706	1349
Min Monthly Precipitation (mm)	18	18	45
Max Monthly Precipitation (mm)	98	97	246
Max Temperature (°C)	29.3	29.1	30.2
Mean Temperature (°C)	21.8	21.6	25.4
Min Temperature (°C)	14.4	14.2	20.7
Max Temp Observed (°C)	34.5	NA	NA
Min Temp Observed (°C)	23	NA	NA

2.3.2 Field Observations

2.3.2.1 Nest-Site Ecology

In order to address aim 1, I measured a number of characteristics for 35 *D. quadriceps* nests located at three different locations in 2011 (*appendix A1.1)*. GPS coordinates were recorded, as well as the diameter of the nest mound, proximity of the nearest plant to the nest entrance, number of plants or branches within a 0.5m radius of the nest entrance, and the number and direction of the nest entrances. The entrances of some nests were partially or completely surrounded by small twigs placed in a regular pattern (*figure 2.2a*), so for each nest a visual estimate of the percentage coverage

around the entrance of twigs was made. A total of 28 colonies were excavated, 9 at the woodland site (site one), 6 at the fruit plantation (site two) and 13 at the scrubland site (site three). During excavations, care was taken to ensure the entire nest had been excavated; visible changes in soil colour, along with the discovery of a large, densely populated brood chamber, were taken as indications that the edge of the nest had been reached. The number of ants in each excavated colony was recorded.

2.3.2.2 Foraging Ecology

To address aims 2 – 5, observations of foraging behaviour of marked individuals were performed for seven randomly selected colonies at site one. Individuals exiting and entering the nest were collected and given a unique paint mark on the thorax to facilitate individual recognition. They were then released in close proximity to the nest, where they were collected, and allowed at least 24 hours to recover before observations began. Foraging observations were performed over 3 consecutive days between 9am and 5pm. Colonies were observed for 30-minute intervals (mean 13.14 ± 1.38 observations per nest), during which time the identity of any individuals entering or exiting the nest was recorded. All unmarked ants seen entering or exiting the nest during an observation period were immediately collected and paint marked, and the numbers of unmarked individuals observed decreased rapidly with time indicating that all or most foraging individuals had been marked. The order in which colonies were observed during the day was randomised to ensure that each colony was observed at least once for each hour between 09:00 and 17:00. To investigate the diet of *D. quadriceps* (aim 2), the type of food was recorded to order level (where possible) for all individuals returning with prey or forage. Larval stages of insects could not be identified to this level, and were recorded simply as 'insect larvae'. Likewise, fruit was recorded as a single category. Temperature and relative humidity (aim 3) was recorded at the woodland site for the entire duration of the study using an EasyLog USB data logger (site wide air temperature) and 7 DS1921G Thermochron iButtons placed at each nest (local ground temperature). Course-level weather classifications (aim 3) were also recorded for each 30-minute observation period. Weather was classified as cloud, light rain, rain, fog, sun.

2.3.3 Laboratory Monitoring

To complete aim 4, I also monitored foraging behaviour in laboratory populations, using both behavioural observations and RFID tagging.

2.3.3.1 Housing and Husbandry

Twenty-one colonies were transferred to a control temperature laboratory at first at the Universidade Federal de Sergipe in Aracaju, and later in the UK (University of Leeds / Institute of Zoology). Individuals were marked with a unique number tag immediately after transfer to the laboratory. Colonies were kept in the laboratory at 23-30°C and 70-85% relative humidity on a diet of *Tenebrio* mealworms and banana three times a week, corned beef or live cockroaches once a month, and water *ad libitum*. Colonies were kept either in two plastic containers (40cm x 30 cm x 5cm; one dark nest box and one open foraging box) connected by a plastic tube, or in a plastic nest box (33cm x 19cm x 11cm; dark and divided into six chambers with cardboard) within a larger box (38cm x 58cm x 18cm).

2.3.3.2 Laboratory Monitoring of Foraging

I monitored changes in extranidal activity over a 24 hr period using a combination of behavioural observations (n=11 colonies), or RFID (n=10). In 2010, 11 colonies were monitored at 30-minute intervals covering three 24-hour periods at the University of Leeds. During these observations the location of individually marked ants (inside or outside the nest) was recorded. Failures in the CT lighting system during the behavioural observation period meant that for some observations (288 out of 1579, 18.4%) light and time of day were decoupled. This enabled me to investigate the roles of time of day and temperature independently.

Radio-frequency identification tagging was used to track the movement of all ants in 10 colonies in the laboratory in either Aracaju (n = 6) or London (n = 4). Individual ants were subdued by chilling and RFID tags (passive RFID, 16 bit programming mode [GiS TS-Q5Bee Tags], 18 mg, 6 x 3 x2 mm) encoded with unique 4-digit identification

numbers, were affixed to the thorax using superglue (Loctite)(Sumner *et al.* 2007). A small unique number tag (E.H. Thorne Ltd) was also glued to each RFID tag to facilitate individual identification during behavioural observations. Two circular RFID antennae (3 cm diameter, GiS TS-A37) were placed around the entrance tube of each nest box, spaced at least 7cm apart in order to prevent simultaneous detection of the same ant by both antennae. Using two antennae enables not just the time but also direction of movement to be determined, since the relative timing of records from each antenna can be compared. RFID colonies were monitored continuously 24 hrs a day for a minimum of 5 days for each colony (mean 11.98 ± 0.069 days per colony).

2.3.4 Statistical Analyses

Statistical analyses were performed using a linear mixed effects modelling approach in R. The duration of foraging bouts was calculated, either as an absolute time (mins) (e.g. in cases where the individual exited and returned to the nest within a continuous observation), or as a minimum duration (e.g. in cases where were the individual was only recorded once during the observation period). Minimum durations were calculated based on the time since the beginning of the observation period, in the case of foraging bouts for which only the return was observed, or time until the end of the observation period in the case of bouts for which only the exit was observed. Foraging bout data was pooled into 60-minute intervals, and average temperature and relative humidity was calculated for each interval. Behavioural observations conducted in Leeds were used to calculate colony-wide foraging patterns, and the effect of time of day, temperature, humidity and lighting on the proportion of the colony outside the nest was investigated using an LME modelling approach in R. RFID data manipulation and analyses were performed in R. Records from each antennae were aligned and assigned to either inside or outside the nest. Statistical analyses were performed using a linear mixed effects modelling approach in R using the following response variables: trip duration and proportion of time spent outside per day (mean for each ant). The number of trips and the time spent outside for each ant at 60-minute intervals throughout the day were used as additional response variables. For each colony, the proportion of the colony outside the nest was calculated for each 60-minute interval and used as a response variable for a colony-level analysis. In all models, colony was

included as a random effect, and ant ID was included as a random effect in all models except the colony-level analysis in order to account for repeated measures. Models were simplified by removing the term with the largest non-significant p-value in succession to reach a minimum adequate model, for which an R-squared value was calculated. Several of the response variables were not normally distributed, and so transformation was used to improve model fit; trip duration was log transformed whilst number of trips and the time spent outside were square root transformed. Model check plots indicated that the fit of all minimum adequate models was good.

2.4 Results

2.4.1 Nest Ecology

The first aim of this study was to investigate differences in the nesting ecology of *D. quadriceps* between three sites, representing a selection of different habitat types and abiotic characteristics (*table 2.1*). A dense population of *D. quadriceps* was found in Campo Formoso, with nests located every meter or so, and colonies were particularly densely packed in the fruit plantation. The number of plants within a 0.5m radius of the nest entrance differed significantly between locations (woodland, fruit plantation, closed scrubland), with the greatest number of plants occurring around the nest entrances of nests in the scrubland (site three) (ANOVA $F_{32} = 39.8$, df = 2, $p < 0.001$). However, there was no significant difference between locations in the proximity of the nearest plant to the nest entrance (Kruskal-Wallis $\chi^2 = 3.29$, df = 2, p = 0.19), nest diameter (ANOVA, $F_{32} = 1.33$, p = 0.280) or number of entrances (Kruskal-Wallis $\chi^2 = 0.97$, df = 2, p = 0.615). Location also had a significant effect on the appearance of the nest entrance, with the greatest coverage of twigs around the nest entrance for nests in the fruit plantation (Kruskal-Wallis $\chi^2 = 10.6$, df = 2, p = 0.005). Both number of plants and proximity of the nearest plant showed strong positive correlations with coverage of twigs around the entrance (Spearman's rho r = 0.40, df = 34, p = 0.018; Spearman's rho r = 0.46, df = 34, p = 0.006, *figure 2.1a*). Colony size differed significantly between locations, being largest in the scrubland (site one: 73.5 ± 9.17, site two: 38.67 ± 6.14, site three: 88 ± 20; ANOVA $F_{2,34} = 5.19$, p = 0.021). There was no correlation between nest diameter and colony size (mean nest diameter: 51.7 ±

3.07cm; Pearson correlation r = 0.18, df = 34, p = 0.489). Nest entrance direction did not deviate significantly from random ($\chi^2_{3,0} = 5.11$, p = 0.164).

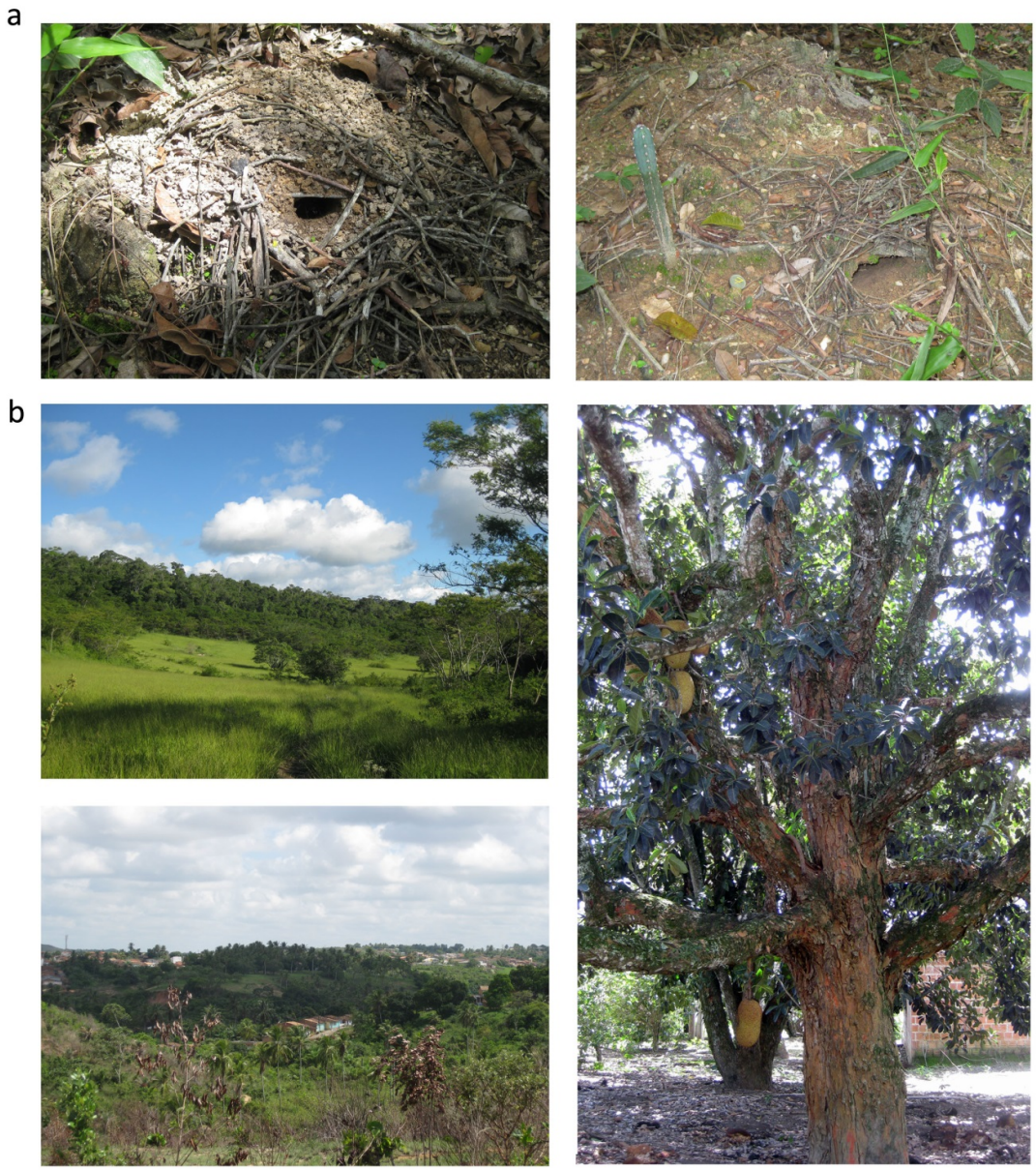


Figure 2.2 Field Sites and Nest Ecology in Brazil

Photographs of a) nest entrances surrounded by twigs, as observed in Campo Formoso, and b) a representation of each field site: site one (top left), site two (right), site three (bottom left).

2.4.2 Foraging Ecology in the Field

2.4.2.1 Diet

Of the 7 nests I observed in 2011, a total of 480 extranidal trips were recorded over a period of 3 days. Of these, 214 (44.58%) resulted in a worker returning to the nest with food during the observation period. The most common food items returned to the

nest were fruit (30%) and insect larvae (29%), with adult insects cumulatively representing a further 26%.

2.4.2.2 Abiotic Determinants of Foraging

Ground temperature ranged between 20.5°C and 39.5°C in this study, whilst air temperature ranged from 23°C to 34.5°C. Humidity ranged from 55% to 93%. There was a significant interaction between the effect of time of day, temperature and humidity on the duration of foraging trips in *D. quadriceps*. Across all trips outside the nest, both nearby ground temperature (local colony-specific environment) and air temperature (global site-wide environment; *figure 2.3a*) interacted with time of day (*figure 2.3d*) to influence foraging behaviour ($\chi^2_{35,480} = 20.07$, $p = 0.0054$; $\chi^2_{35,480} = 24.44$, $p = 0.001$). The minimum adequate model explained 37% of the variance in trip duration. For most times of day, the duration of foraging trips increased with temperature, between the extremes of 20.5°C and 39.5°C observed in this study. However, between 12:00 and 14:00 this relationship was no longer detectable, and foraging activity at this time remained stable regardless of temperature. There was also a significant relationship between trip duration and humidity (*figure 2.3b*), again in interaction with time of day. Humidity shows a negative relationship with trip duration, except between 12:00 and 14:00, where no relationship is detectable. For 'foraging' trips (> 2 minutes) only, an additional significant interaction was detected between the effect of air temperature and humidity on trip duration ($\chi^2_{20,363} = 10.73$, $p = 0.001$), and these two ecological variables were also found to correlate strongly with one another (correlation coefficient -0.66). Interestingly, ground temperature at the nest entrance did not influence trip duration for trips longer than 2 minutes ($\chi^2_{28,363} = 0.8965$, $p = 0.344$). For 'reconnaissance' trips (< 2 minutes), only humidity had a significant effect on trip duration ($\chi^2_{4,117} = 6.7332$, $R^2 = 0.32$, $p = 0.009$). Weather as measured in this study (cloud, sun, light rain, heavy rain, fog), had no influence on trip duration when all trips are included ($\chi^2_{6,480} = 6.41$, $p = 0.09$), however, this effect was significant for 'foraging trips' (> 2 minutes) ($\chi^2_{6,363} = 9.72$, $R^2 = 0.16$, $p = 0.002$; *figure 2.3c*). Foraging trips were longest under cloudy conditions (15.67 ± 9.139 mins) and shortest in sunshine (11.59 ± 7.48 mins). The proportion of the colony that was outside

the nest was only significantly influenced by time of day ($\chi^2_{10,7}$ = 0.15.882, R^2 = 0.59, p = 0.027).

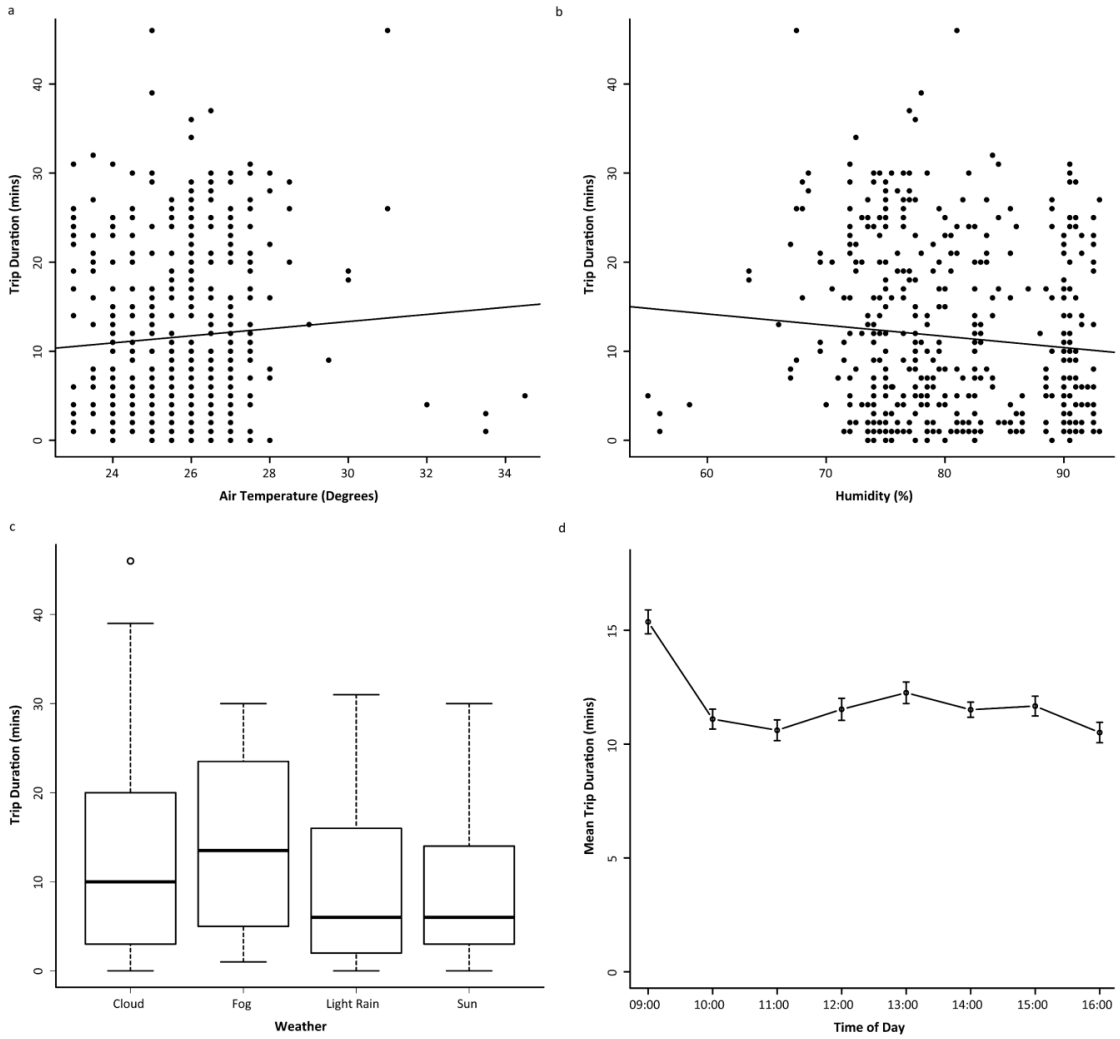


Figure 2.3 Abiotic Influences on Natural Foraging Behaviour

The relationship between trip duration and a) temperature, b) humidity, c) weather and d) time of day for field observed colonies (n = 7). The equations for the lines of best fit in a) and b) are y = 0.4x + 1.33 and y = -0.1256x + 21.72, respectively. Data in (a) and (c) are presented as the means (thick line), 25% and 75% quartiles (box), and outliers as determined by the interquartile range (IQR) rule (bars), with more extreme values represented as individual circles. Error bars in d) represent 1 se.

2.4.2.3 Nest Drifting

During field observations of foraging, on several occasions ants paint marked from one colony were recorded entering the nest of a different colony, and in several cases carrying food. These 'drifting' events were relatively rare, representing just 3.1% of foraging trips (15 drifting events in 3 days), however apparently widespread, occurring in 4 out of the 7 nests under observation. Nest drifting occurred over large distances

(mean drifting distance 11.97m ± 2.12m), and most drifting occurred between colony 2 and 3 (7.92m) with 11 drifting events performed by 9 different individuals

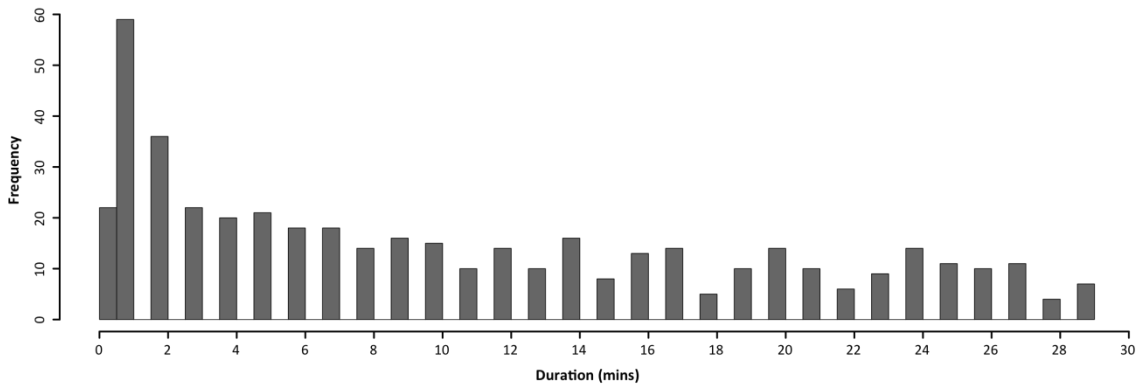
2.4.2.4 Nestmate Recruitment

I also investigated nestmate recruitment in *D. quadriceps* (aim 5). All foraging observed during this study was performed individually and no recruitment of other workers to discovered prey or food items was directly observed during the day-time foraging observation periods. However, tandem-running behaviour was observed daily around the premises of the field station, particularly in the evenings. Usually a single pair would be observed, walking nose to abdomen for extended periods of time (minutes or hours). Further, dinosaur ants were frequently observed leaving and returning to the nest after a very short period of time (< 2 minutes)(*figure 2.4a*), often prior to a longer foraging bout. These putative reconnaissance trips represented 24.38% of trips outside the nest (117 out of 480 trips). Thus, separate analyses were performed on all trips together, reconnaissance trips and foraging trips. The mean length of all trips outside the nest was 11 minutes 36 seconds (± 27 seconds), where as mean length of reconnaissance trips was 1 minute 7 seconds (± 4 seconds) and mean foraging trip length was 14 minutes 58 seconds (± 5 seconds) long.

To investigate whether these short trips serve a role in reconnaissance in assessing the external abiotic conditions, I analysed the relationship between putative reconnaissance and foraging trips, at both the individual and colony levels. I compared the numbers of long-duration trips that an individual performed during the 60 minutes following a short-duration trip, to the number that occurred in the 60 minutes prior to the short-duration trip. In order to control for individual or colony differences in foraging effort, numbers of trips are reported relative to the baseline mean foraging effort for that individual or colony. A t-test revealed no significant difference in the number of long trips occurring before or after short trips ($t_{113} = 0.9499$, $p = 0.3442$), however there was a non-significant trend towards more frequent long-duration trips after short-duration trips than before (*figure 2.4b*). Further, I found a significant difference in the relative number of long trips performed by all colony members before and after a short trip, with a greater number of long-trips occurring before a

short trip than after (t210 = - 3.428, p = 0.0007). In order to test whether information about the abiotic environment gleaned during reconnaissance trips might be influencing foraging behaviour, I also investigated the effect of abiotic variables (temperature, humidity, weather) on the relative number of long trips before and after a short trip for both the individual-level and colony-level response, using a linear mixed effects modelling approach. Long-trips occurring before a short-trip were not significantly influenced by abiotic variables at the individual level, and only ground temperature ($\chi^2_{31,106}$ = 13.36, p = 0.0003) had an effect on the number of long-trips at the colony-level (*figure 2.5a*). Likewise, no abiotic influences had a significant impact on the individual-level response, however for the colony-level response, there was a significant interaction between temperature and humidity on the number of long trips following a short-trip ($\chi^2_{36,106}$ = 7.34, p = 0.0069) (*figure 2.5b*), as well as a significant influence of weather ($\chi^2_{36,33}$ = 19.87, p = 0.0002) (*figure 2.5c*).

a)



b)

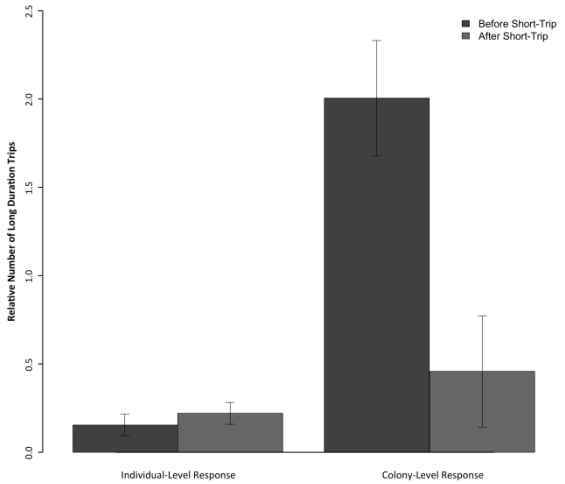


Figure 2.4 Reconnaissance Trips

a) A histogram of trip duration showing the frequency of foraging trips of different duration. b) The number of long-duration ('foraging') trips made within a 60-minute period before (dark grey) and after (light grey) an individual short-duration 'reconnaissance' trip by the individual, or the colony as a whole. Number of foraging trips is relative to the baseline as determined as the average number of long-duration trips made by the individual or colony in any given 60-minute period. A value of zero therefore represents the individual's or colony's average foraging behaviour, positive values indicate above average frequency of long-duration trips, negative values indicate lower than average.

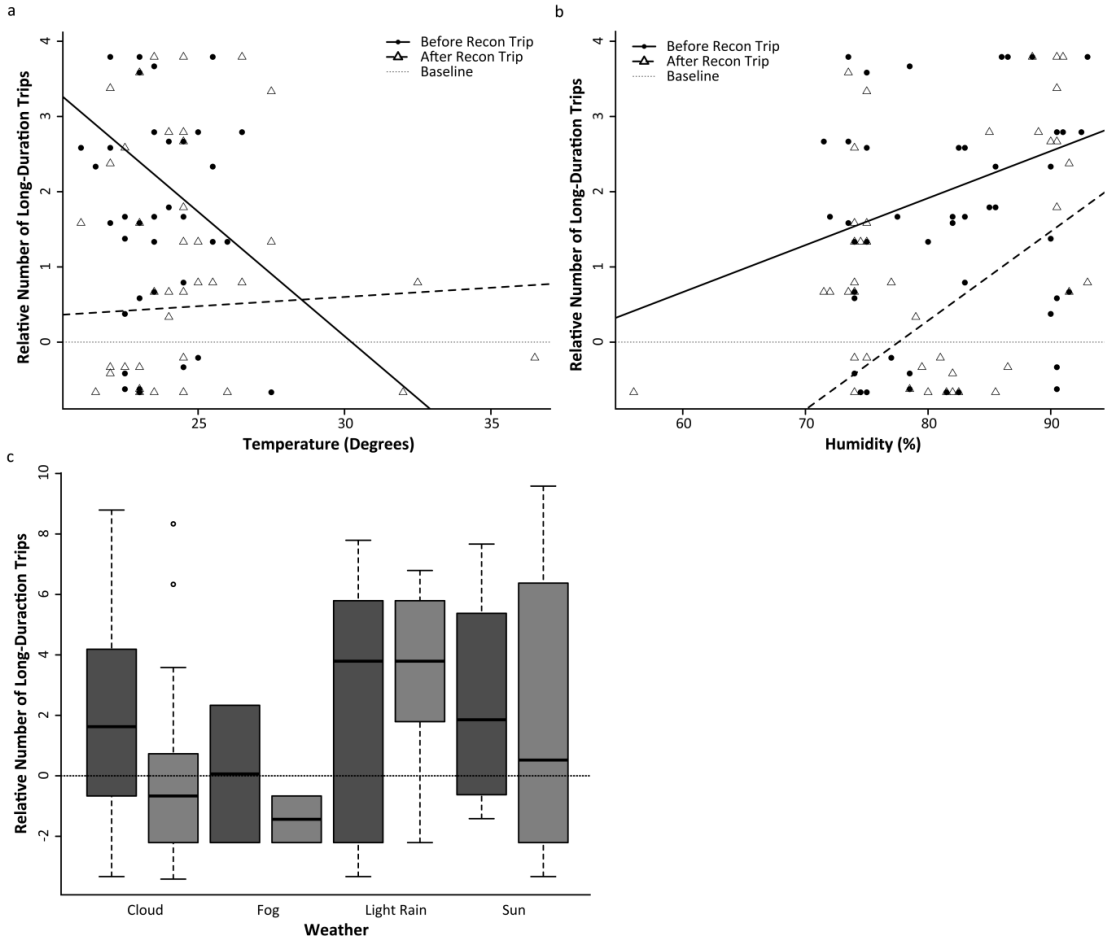


Figure 2.5 Do Dinosaur Ants Use Reconnaissance Trips to Judge Abiotic Conditions?

The effect of a) temperature, b) humidity and c) weather on changes in the number of long-trips made by all colony members in relation to a short 'reconnaissance' trip. Data are split into long-trips before or after a short-duration trip, and are presented relative to the baseline. Baseline is calculated as the mean number of long-duration trips made by the colony. The equations for the lines of best fit in a) and b) are y = -1.16 x + 38.54 (before recon trip), y = -0.26 x + 13.64 (after recon trip), and y = 0.36 x – 18.97 (before recon trip), y = -0.17 x + 20.93 (after recon trip), respectively. Data in (c) are relative number of trips before (dark grey bars) and after (light grey bars) a recon trip, presented as the means (thick line), 25% and 75% quartiles (box), and outliers as determined by the interquartile range (IQR) rule (bars), with more extreme values represented as individual circles.

2.4.3 Foraging Behaviour in the Laboratory

In the laboratory, 1531 extranidal trips were recorded for 329 ants in 9 colonies (mean colony size = 38.67 ± 9.14) using radio frequency identification (RFID). Seven (2.13%) RFID tag numbers could not be matched with available behavioural data, possibly due to RFID tag malfunction, reader failures or interference, and thus data for these individuals was not included in subsequent analyses. Mean duration of RFID monitoring was 6.27 ± 0.07 days per colony. The average length of an extranidal trip was 2.86 ± 0.08 hours. On

average, ants spent 3.19 ± 0.18 hours outside the nest per day during 1.18 ± 0.05 trips, amassing 51.59 % ± 0.02 of their time outside the nest.

2.4.3.1 Circadian Rhythms in the Laboratory

Using both behavioural observations and RFID monitoring, I explored circadian patterns of extranidal activity in the laboratory (aim 4). Time of day had a strong influence on extranidal activity as measured by average trip length ($\chi^2_{47,1531}$ = 104.03, R^2 = 0.42, p < 0.0001), number of trips ($\chi^2_{47,1531}$ = 377.19, R^2 = 0.63, p < 0.0001)(*figure 2.6a*) and time spent outside ($\chi^2_{47,1531}$= 170.64, R^2 = 0.68, p < 0.0001). Time of day also affected colony-wide foraging activity ($\chi^2_{26,9}$ = 72.583, R^2 = 0.82, p < 0.0001; *figure 2.6b*). Extranidal activity was highest between 18:00 (mean mins spent outside: 7.41 ± 0.63, mean no. trips: 0.39 ± 0.02) and 04:00 (mean mins spent outside: 7.31 ± 0.64, mean no. trips: 0.33 ± 0.021), with a peak in activity at 20:00 (mean mins spent outside: 9.55 ± 0.74, mean no. trips: 0.5 ± 0.03). Behavioural observations of extranidal activity showed no significant effect of time of day on the proportion of the colony outside the nest ($\chi^2_{118,11}$ = 46.24, p = 0.5042l *figure 2.7a*). However light levels (day or night settings) did show a significant effect ($\chi^2_{70,11}$ = 18.75, p < 0.0001; *figure 2.7b*).

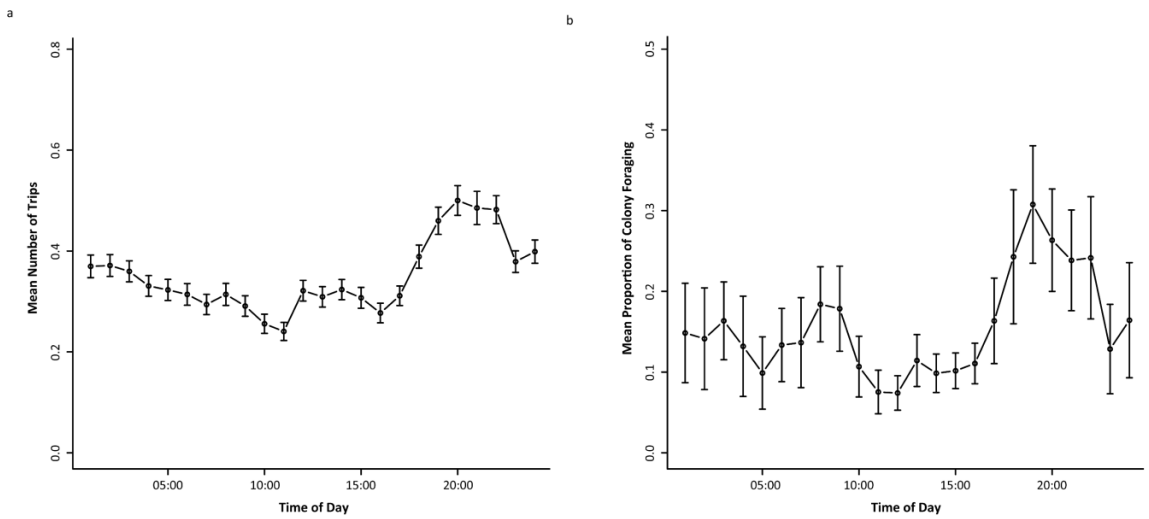


Figure 2.6 RFID Monitoring of Circadian Rhythms in the Laboratory

The effect of time of day on a) mean ± 1 se number of trips per individual and b) mean ± 1 se proportion of the colony foraging for ten RFID monitored *D. quadriceps* dinosaur ant colonies. Data are from 6.27 ± 0.07 days of monitoring per colony.

Although laboratory colonies were kept in controlled temperature rooms, some variability in temperature and humidity was measured (mean = 26.3°C ± 0.06; 82.8% ± 0.19, range: 24.9°C – 28.8°C; 77% - 90%). There was a marginally non-significant effect of temperature ($\chi^2_{70,69}$= 3.62, p = 0.0570), but no effect of humidity ($\chi^2_{71,70}$ = 0.78, p = 0.5042) on the proportion of the colony foraging during behavioural observations of laboratory colonies. Together, whilst controlling for effects of colony and date, temperature and lighting together explained 88.42% of the variance in the proportion of colony members foraging.

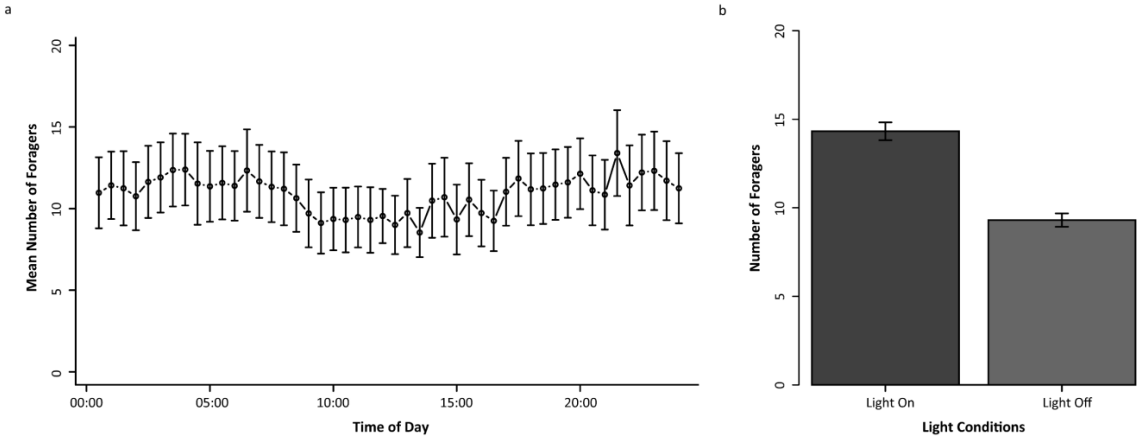


Figure 2.7 Behavioural Observations of Circadian Rhythms

a) mean number of foragers per colony at different times of day, and b) lighting status (lights on: dark grey, lights off: light grey), as measured using 1579 behavioural observations covering all times of day for 11 colonies.

2.5 Discussion

Here I present the most thorough investigation to date of foraging and nesting ecology in both the field and the laboratory for the queenless dinosaur ant, *Dinoponera quadriceps*. I show differences in the nesting ecology of *D. quadriceps* between sites with differing climatic conditions and habitat types. Foraging behaviour in both the field and the lab is influenced by abiotic conditions such as temperature and humidity, as well as by time of day and lighting conditions. I also present evidence for two types of extranidal trip: short-duration 'reconnaissance' trips and long-duration 'foraging trips', and report the occurrence of nest drifting over considerable distances. I discuss

these findings in the context of conservation, the foraging ecology of other ant species and the social ecology of *D. quadriceps* and other ponerine ants.

2.5.1 Nest Ecology

Nests in Campo Formoso occurred at high density, particularly in the fruit plantation, and this probably reflects the greater abundance of food at this site. Almost all nests were located at the base of trees and shrubs, consistent with reports for other ponerine ants living in similar habitats (Fourcassie and Oliveira 2002), and a likely adaptation to defend the nest entrance against predation and abiotic stressors. Vegetation was more dense in the scrubland site (site three), and lowest in the fruit plantation (site two), and correlated negatively with the coverage of twigs around the nest entrance. As these twigs appeared carefully arranged, and would be reassembled following disturbance, it seems likely that their occurrence is deliberate. Their use could be for protection against environmental or predatory threats, and may possibly help to disguise the nest entrance.

2.5.2 Ponerine Ants as Seed Dispersers

Ponerine ants are predominantly predators or scavengers of other insects (Duncan and Crewe 1994; Fourcassie and Oliveira 2002; Gobin *et al.* 1997; Pie 2004; Pratt 1994). Here, I show that *Dinoponera quadriceps* is an opportunistic omnivore, with fruit and insect larvae representing the majority of their diet (aim 2). The large contribution of fruit to the diet of *D. quadriceps* (30%) is consistent with data for some other ponerine ants (Araujo and Rodrigues 2006; Fourcassie and Oliveira 2002; Passos and Oliveira 2002). Nearly 5% of angiosperms show specific adaptations to seed dispersal by ants (Myrmecochory)(Lengyel *et al.* 2010), but it is also important to many tropical plant species adapted to dispersal by frugivorous mammals (Passos and Oliveira 2003) and birds (Passos and Oliveira 2002). Ponerine ants in general are excellent seed dispersers because they are able to transport large seeds back to the nest whole (Passos and Oliveira 2002; Pizo and Oliveira 1998), as *D. quadriceps* foragers were observed to do here. Germination in ant waste piles increases both germination speed and success (Levey and Byrne 1993; Passos and Oliveira 2002; Passos and Oliveira 2003; Pizo and

Oliveira 1998), thus seed dispersal by *D. quadriceps* may be important in influencing tropical flora.

2.5.3 Abiotic Influences on Foraging Behaviour

Both temperature and humidity influenced foraging behaviour in the field, with increasing activity as temperature increased and as humidity decreased. Temperature is a strong influence on foraging behaviour in many ant species (Duncan and Crewe 1994; Fowler and Roberts 1980; Ibm 2003; Jayatilaka *et al.* 2011; Oudenhove *et al.* 2011), and has been shown to influence on foraging activity in other ponerine ants, including *D. gigantea* (Fourcassie and Oliveira 2002). The influence of humidity has only rarely been investigated, however it was found not to influence foraging in *Ectatomma opaciventre* (Pie 2004), contrary to my results for *D. quadriceps*. Weather also had a significant influence, with the highest rates of foraging occurring in 'foggy' conditions. Field observations indicated a peak in foraging activity at the start of the observation period (09:00), and in the laboratory, two peaks in activity were observed, one in the morning around 08:00 – 10:00 and a second, larger peak between 18:00 and 21:00, outside the field observation period (*appendix A1.2*). In contrast to RFID monitoring, behavioural observations in the lab failed to detect a significant relationship between time of day and extranidal activity. However behavioural observations were able to distinguish between the effects of time of day and lighting, and revealed that the circadian rhythms in this species are likely caused by the day/night transition as opposed to time of day. A bimodal pattern of foraging activity is similar to reports of circadian rhythms in other ants (Dejean and Lachaud 1994; Duncan and Crewe 1994; Kuate *et al.* 2008; Pie 2004), including the closely-related Brazilian ponerine ant, *D. gigantea* (Fourcassie and Oliveira 2002). However, few studies have monitored the full circadian pattern of foraging in ponerine ants. Comparing between field and laboratory colonies, higher daytime extranidal activity was observed in the laboratory, suggesting *D. quadriceps* colonies may increase their foraging effort in response to a reduction in available intranidal tasks associated with a laboratory environment (*appendix A1.2*).

2.5.4 Nestmate Recruitment and Tandem Running

I report the occurrence of short-duration (< 2 mins) trips in *D. quadriceps*, which occur frequently and show different characteristics to longer-duration trips. Thus, I propose that extranidal trips by subordinates can be divided into two classes: reconnaissance and foraging trips. Reconnaissance trips may be used by the ants to gauge the external abiotic conditions before embarking on a longer, riskier foraging trip. Short trips such as these have also been observed in *D. gigantea*, in which they often involve waste or obstacle removal (Fourcassie and Oliveira 2002). This may explain some short extranidal trips in *D. quadriceps*, however in many instances, ants emerged for short trips without any waste, and trips may also be used as a means to assess external conditions before embarking on a longer foraging trip. In general, the occurrence of reconnaissance trips tended to reduce the number of long-duration trips in the following hour, suggesting that information about the external environment may be suppressing foraging activity. Consistent with this, temperature, humidity and weather all influenced the number of long-duration trips performed by the colony after a reconnaissance trip, but not before it. This preliminary evidence suggests that short-duration extranidal trips may indeed act as information-gathering exercises, which inform subsequent foraging decisions.

In the majority of ponerine ants, foraging is performed individually and recruitment of nestmates is rare (Araujo and Rodrigues 2006), although not unknown (Breed *et al.* 1987; Duncan and Crewe 1994; Pratt 1994). The lack of sophisticated nestmate recruitment in ponerine ants may be a result of their large size; ranging from 1cm to 4cm (Ito 1993). Most ponerine ants feed on food items substantially smaller than themselves, and thus have little difficulty in transporting them back to the nest alone. Further, ponerine ants feed predominantly on resources that are scattered in the environment, and their small colony sizes may therefore make recruitment unprofitable. No nestmate recruitment was seen during the daytime foraging observation, but tandem running was observed occasionally at night. The behaviour was observed daily, suggesting a role in nocturnal foraging rather than nest relocation or fission.

2.5.5 Nest Drifting

Movement of ants between nests was observed on several occasions during field observations of foragers. On 15 occasions, marked ants returned to a nest other than the one at which they were originally collected, and frequently they transported prey to a non-natal nest. This 'nest drifting' behaviour may be adaptive if individuals are drifting between related nests (Fourcassie and Oliveira 2002; Sumner *et al.* 2007). Alternatively, nest drifting could represent a form of social parasitism, if drifting individuals are reproducing in non-natal nests, as has previously been reported in several species of bee (Birmingham *et al.* 2004; Nanork *et al.* 2007). Nest drifting has been reported previously in another ponerine ant, *Gnamptogenys menadensi* (Gobin *et al.* 1997). *Dinoponera quadriceps*, like most queenless ponerine ants, can only reproduce by colony fission as gamergates are not sufficiently fecund to found colonies alone (Peeters 1991). Thus, neighbouring nests in this population are expected to be closely related, however to-date no molecular genetic investigation of the population dynamics of *D. quadriceps* has been completed. Data on the population structure of other ponerine ants, *Gnamptogenys striatula* and *Diacamma cyaneiventre*, which also reproduce by colony fission, indicates high population viscosity and strong isolation-by-distance effects at a small geographical scale (Doums *et al.* 2008; Giraud *et al.* 2000). It is therefore plausible that ants were drifting between nests to which they are highly related, one nest being the 'daughter' nest of the other. This could represent a deliberate tactic, however colony odours between recently fissioned nests would likely remain similar, and thus drifting could represent errors on the part of returning ants. *Dinoponera quadriceps* could also be considered to be polydomous (Debout *et al.* 2007), with the 'drifting' events observed in this study representing movement of members of a single colony between nests. However, movement between some nests was observed over distances of up to 12m, suggesting this behaviour is more likely to represent drifting than polydomy. Due to the small number of observed 'drifting' events, it is not clear whether individual 'drifters' show bias towards one particular nest (indicative of drifting) or spend equal time and energy in both nest sites (polydomy). Polydomy has previously been reported in another Brazilian ponerine ant, *Dinoponera gigantea*, although only over distances of less than

1m (Fourcassie and Oliveira 2002). Nest drifting over such large distances is unusual amongst the eusocial hymenoptera (Goerzen *et al.* 1995; Paar *et al.* 2002; Pfeiffer and Linsenmair 1998; Sumner *et al.* 2007), and represents a substantial distance relative to those over which they forage; in *D. giantea* foragers rarely travel more than 6m from their natal nest (Fourcassie and Oliveira 2002).

2.5.6 Final Remarks

Dinosaur ants are of great interest to studies of social evolution, and are also ecologically important, playing a role in seed dispersal in highly diverse tropical ecosystems of conservation importance. Their foraging and nesting behaviour, like their social behaviour, appears to be highly dynamic and adaptable to a variety of social and environmental conditions.

Chapter 3

Division of Labour and Risk Taking

3.1 Abstract

The success of social insects can be largely attributed to division of labour. In contrast to most social insects, many species with simple societies contain workers which are capable of sexual reproduction. Headed by one or a few reproductive individuals, subordinate workers form a dominance hierarchy, queuing to attain the reproductive role. In these species task allocation may be influenced by individual choice based on future reproductive prospects. Individuals with a better chance of inheriting the colony may be less likely to take risks and high-ranking workers that spend a greater amount of time in proximity to the brood may be able to increase the ability to police egg-laying by cheating subordinates. I investigated division of labour and risk taking in relation to dominance rank in the queenless ponerine ant, *Dinoponera quadriceps*, a species with relatively simple societies. Using behavioural observations, I show that high-ranking workers spend more time performing egg care, less time foraging and are less likely to defend the nest against attack. High-rankers also spent a greater amount of time guarding and inspecting eggs, behaviours which are likely to improve detection of egg laying by cheating subordinates. I also show that high-ranking workers spend a greater amount of time idle, which may help increase lifespan by reducing energy expenditure. My results suggest that both risk-taking and egg care behaviours are related to future reproductive prospects in *D. quadriceps*. This highlights a mechanism by which effective division of labour could have been achieved during the early stages of eusocial evolution.

3.2 Introduction

Social insects represent one of the pinnacles of social evolution, and the evolution of eusociality is considered to be one of the major transitions in evolution (Maynard Smith and Szathmary 1995). Understanding how such highly cooperative societies evolved has been a key area of interest for evolutionary biologists since Darwin, who

highlighted the apparent paradox of worker behaviour (Darwin 1859). One of the primary characteristics of eusocial societies is division of labour (Beshers and Fewell 2001; Robinson 1992) a stable variation within a colony in the tasks that individuals perform (Beshers and Fewell 2001). Division of labour is believed to have been a key factor in the success of social insects, increasing efficiency and maximising resource use (Page and Mitchell 1998; Wilson 1974, 1985). All social insect colonies show reproductive division of labour, where a relatively small number of individuals (queens) are responsible for reproduction, while the other colony members (workers) rear the young, forage and maintain the nest (Robinson 1992). In some species, the worker caste is further divided into individuals who specialise in certain tasks for at least part of their adult life (Robinson 1992; Wilson 1974, 2000). The majority of social insect species lack morphologically differentiated castes (Wilson 1974) and in these societies labour is often divided according to age, via temporal polyethism (Beshers and Fewell 2001). Most commonly, temporal polyethism involves younger workers performing tasks within the nest, and older workers leaving the nest to forage (Beshers and Fewell 2001). This results in individuals with a shorter life expectancy performing the more dangerous outdoor tasks (Kay and Rissing 2005; Moron *et al.* 2008).

In contrast to species with large, complex societies, species with small, relatively simple societies can sometimes contain workers who are physically capable of sexual reproduction, a state which is most likely ancestral in wasps and bees but which is secondarily derived in a number of ant species (Field *et al.* 2000; Peeters 1991). Reproduction is still dominated by one or a few individuals, however, and younger subordinate workers queue to take over the reproductive role (Field and Cant 2009; Peeters 1991; Shreeves and Field 2002).

Queuing for reproduction results in differences in the future reproductive prospects of different individuals in the colony (Cant and Field 2001; Field and Cant 2009). In these societies individual workers may select tasks based on the costs and benefits associated with them (Cant and Field 2001). Since high-ranking workers have a greater chance of future reproduction (Monnin and Peeters 1998, 1999; Pardi 1948) I might expect them to be less likely to engage in foraging and nest defence (Cant and Field

2001; Field and Cant 2009), tasks that are likely to have a high mortality rate (Field and Cant 2009; Visscher and Dukas 1997). High-ranking individuals may also increase their chances of future reproduction by spending a greater amount of time idle, or by increasing body condition, for example by increasing time spent grooming (Field and Cant 2009). However to my knowledge these hypotheses have not yet been thoroughly tested in a species in which worker reproduction is secondarily derived.

Although subordinate workers in species with simple societies may be physically capable of reproduction, there is in fact little opportunity for them to gain direct reproductive fitness because in some species (e.g. ants), males show no interest in mating with subordinates (Peeters 1991). However, unmated workers are capable of laying male eggs, enabling them to gain direct reproductive fitness (Monnin and Ratnieks 2001; Ratnieks and Visscher 1989). Widespread subordinate reproduction is likely to reduce colony productivity and in many species worker reproduction is deterred by policing (D'Ettorre *et al.* 2004; Liebig *et al.* 1999; Ratnieks 1988; Ratnieks and Visscher 1989). However, some individuals still attempt to cheat and lay male eggs, leading to conflict within the colony. By spending more time performing egg care, high-rankers could remain in close proximity to the egg pile, where most conflict is likely to occur.

Within the eusocial Hymenoptera, reproductive totipotency and the absence of a morphologically distinct queen caste occurs in four main groups; within ants in the subfamily Ponerinae (Peeters 1991), within bees in the subfamily Halictidae (sweat bees; (Danforth 2002), and within wasps in the subfamilies Stenogastrinae (hover wasps) and Polistinae (paper wasps; (Hines *et al.* 2007). Despite marked similarities in their social structure, primitively eusocial wasps, bees and ants differ in their evolutionary histories. The stenogastrine and polistine wasps, and halictine bees evolved independently from solitary ancestors (Danforth 2002; Hines *et al.* 2007). By contrast, queenless ponerine ants evolved from a highly eusocial ancestor with a morphologically distinct queen caste (Peeters and Crewe 1984). If workers in queenless ponerine ants are capable of modifying their behaviour according to their future reproductive prospects, the mechanisms underlying this must have evolved

along with or shortly after the loss of the sterile worker caste. Although rank has previously been found to affect risk-taking behaviour in two species of primitively eusocial wasp (Cant and Field 2001; Cronin and Field 2007; O'Donnell 1998), very little work has been done to investigate this phenomenon in species with secondarily derived worker reproductive totipotency.

The dinosaur ant, *Dinoponera quadriceps*, has small, simple, queenless societies, in which workers form a short, linear hierarchy behind the alpha (Monnin and Peeters 1999; Peeters and Crewe 1984). When the alpha dies she is replaced by a high-ranked subordinate, most commonly the beta, and high-ranking workers themselves are frequently replaced by newly emerged workers, resulting in age-based hierarchy in which low-ranking workers are the oldest colony members (Monnin and Peeters 1999). Observations suggest that foraging and nest maintenance may tend to be carried out by lower ranked individuals and brood care by higher ranked individuals (Monnin and Peeters 1999), in keeping with hierarchical position affecting division of labour, but the data in support of this is still quite limited. Here I carry out a detailed examination of the relationship between dominance rank and behaviour in *D. quadriceps*. Using observations of 24 behaviours and experimental stimulation of nest defence I test the hypothesis that individuals exhibit different behaviours in relation to their current and future reproductive prospects. The effects of age and rank are confounded in this species, therefore I do not attempt to identify the mechanism but merely whether behaviour is related to reproductive potential. Specifically, I predict that (1) high-ranking workers show lower energy expenditure and avoid dangerous tasks such as foraging and nest defence, thereby increasing their chances of future reproduction, and (2) high-rankers spend a greater amount of time performing egg care, maximizing their ability to prevent and detect cheating. I predict that the reproductive female (alpha) and the highest ranking subordinate (beta) should engage more in egg guarding and egg antennation, as a means of preventing and detecting cheating amongst other high-rankers.

3.3 Methods

3.3.1 Study Species

Dinoponera quadriceps are found in Northeast Brazil, where they live in colonies of between 40 and 100 workers (Monnin and Peeters 1998). They construct chambered nests, usually found at the base of trees, which extend up to 1 meter below ground (Paiva and Brandao 1995). They are both predators and scavengers, feeding on a mixture of other insects and fruit (Chapter 2). Fecundity in reproductives is relatively low and winged reproductives are only produced for the male sex, therefore new colonies are formed by fission of a small group (Bourke 1999; Monnin and Peeters 2008; Peeters 1991).

3.3.2 Collection, Housing and Husbandry

Colonies of *Dinoponera quadriceps* were collected from Altantic forest in Sergipe (S11°01'23,W37°12'9), Brazil in 2009 and 2010, and housed at 26-29°C, 70-90% relative humidity and a 12:12 light: dark cycle. Colonies were housed in plastic containers (38cm x 58cm x 18cm) containing a plastic nest chamber (33cm x 19cm x 11cm), divided into 6 compartments by a cardboard divider. Colonies were fed *Tenebrio* mealworms and banana three times a week, corned beef once a month, and provided with water *ad libitum*. To allow individual identification, all ants were tagged with a small unique number tag (E.H. Thorne Ltd). For each colony, a weekly census was performed to record the approximate number of eggs, larvae and pupae. Births and deaths were also monitored in order to maintain a record of the size of the colony.

3.3.3 Determining Dominance Rank

The dominance hierarchy in *D. quadriceps* is maintained by frequent ritualised aggressive interactions between high ranking workers (Monnin and Peeters 1999). These 'dominance interactions' have been categorised into 6 types: blocking, gaster rubbing, gaster curling, antennal boxing, immobilisation and leg biting (Monnin and Peeters 1999) (*table 3.1*). Blocking, where the actor stretches her antennae around the head of the recipient, is characteristic of interactions between the alpha and the beta

(Monnin and Peeters 1999). These 6 interactions can be reliably used to determine dominance rank, which is correlated with ovarian activity (Peeters *et al.* 1999). The aggressive interactions have been ranked, according to severity, by Monnin and Peeters 1999. Individuals who perform the greatest number of higher ranked interactions have a higher dominance rank.

Table 3.1 Description of all behaviours

All behaviours recorded in *D. quadriceps* during dominance observations, division of labour observations and nest defence experiment.

Behaviour	Description
	Aggressive (dominance) Interactions
Block	Actor stretches antennae on either side of the head of the recipient, which stands crouched. (Highest ranked dominance behaviour, characteristic of alpha – beta interactions)
Gaster Rub	Actor bites one antenna of the recipient and rubs it against her gaster (abdomen), which is curled forward.
Gaster Curl	Actor bites one antenna of the recipient, often pulling at it. The target often crouches, with her antennae folded against her head or stretched backward.
Antennal Box	Actor rapidly and repeatedly hits the head of the recipient with her antennae.
Immobilisation	One to six actors bite the recipients legs, antennae or mandibles and prevent her from moving, sometimes for up to several hours.
Leg Bite	A single actor bites the leg of a recipient worker, for 1 or 2 seconds. (Lowest ranked dominance behaviour).
	Risky Tasks
Forage	Actor moves around foraging area
Nest Defence	Actor attacks foreign object, or leaves nest box in response to foreign object.
	Brood Care
Egg Antennate	Actor touches eggs with tips of antennae, sometimes moving egg with them.
Egg Carry	Actor carries a single or a pile of eggs.
Egg Guard	Actor stands in close proximity to the egg pile, with antennae squarely around eggs.
Larva Antennate	Actor touches larva with tips of antennae
Larva Carry	Actor carries larva.
Larva Clean	Actor wraps mandibles around larva and licks surface of the larva.
Larva Feed	Actor places or arranges small food items on belly of larva to allow it to feed
Larva Guard	Actor stands in close proximity to the larva, with antennae squarely around it.
Pupation Help	Actor assists larva to pupate by biting or wrapping silk around the larva as it is produced.
Pupa Antennate	Actor touches pupa with tips of antennae.

Pupa Carry	Actor carries pupa.
Pupa Guard	Actor stands in close proximity to the pupa, with antennae squarely around it.
	Other Colony Tasks
Waste Removal	Actor carries remains of prey items, dead nest mates or other pieces of waste out of the nest and places them on the waste pile in the foraging area.
Self Groom (inside / outside)	Actor cleans self using legs or mandibles. Location recorded.
Allogroom	Actor cleans recipient's body using mandibles.
Nest Maintenance	Actor bites or moves parts of the nest (tissue paper or cardboard divider)
Carry Food	Actor carries a prey item into or around nest.
Process Food	Actor bites prey item into smaller pieces, without consuming them.
Idle (inside / outside)	Actor is completely still. Location recorded.

The identity of the alpha and high-ranking subordinates in six colonies (n = 142) was assessed using behavioural observations. Colonies were observed for 30-minute periods, during which the nature of any aggressive interactions was recorded. For each aggressive interaction observed, the type of interaction and the identity of the actor and recipient were recorded. Repeat occurrences of the same interaction between the same pair of individuals during one observation session were not recorded. Colonies were observed for a total of 18 hours 45 minutes (mean per colony 3:07 ± 0:54). Dominance hierarchies were then constructed for each colony. It was only possible using this method to assign precise linear ranks to high-ranking individuals, because of the rarity of aggressive interactions in medium- and low-rank classes. The remaining colony members were assigned to coarse-scale hierarchical categories; medium- and low-rank. Individuals were assigned to these categories based upon both the frequency and intensity of aggressive interactions observed (Monnin and Peeters 1999). High-ranking individuals are frequently involved in high intensity interactions (e.g. 'blocking' and 'gaster rubbing'), whilst medium-ranked workers are only rarely involved in aggression and usually of a low intensity (e.g. 'immobilisation' and 'antennal boxing'). Low-ranked workers are involved in aggressive interactions only extremely infrequently; when aggression does occur low-ranked workers are the recipients rather than the actors, and interactions are of low intensity.

3.3.4 Division of Labour

The non-aggressive behaviours of all colony members (N = 142, mean colony size = 23.7 ± 4.6) were recorded during 100 spot-samples between 6th July and 7th September 2010. In total, 24 different behaviours were recorded (*table 3.1*). Additionally, in order to control for the overrepresentation of low- and medium-ranking workers in *D. quadriceps* colonies, focal observations were performed with 9 individuals (3 of each rank) from 3 colonies, for six 10-minute periods each. In these observations, it was possible to differentiate idle individuals from individuals moving around the nest but with no clear task (henceforth termed as 'walking').

3.3.5 Nest Defence

To investigate nest defence behaviour, a two-phase nest defence experiment was performed. During the first phase, colonies were disturbed by repeatedly performing sharp taps in the foraging area with a pair of forceps. Individuals attacking the forceps during this phase were removed in sequence until there was no further response to tapping for 60 seconds. The second phase then began, during which the forceps were inserted into the nest entrance and removed again, repeatedly. Individuals attacking the tweezers or exiting the nest to defend were collected in sequence until no further response was generated for 60 seconds, at which point the trial ended. This was repeated for 10 trials with each of 6 colonies.

3.3.6 Statistical Analysis

Statistical analysis of the division of labour data was performed using a generalised linear mixed effects model approach in SPSS. For each individual, the percentage of time spent engaging in each different activity was calculated. Each behaviour was tested against rank, with colony included as a random effect, using a poisson error distribution. The division of labour focal observation data was analysed using a repeated measures generalised linear mixed effects model with colony included as a random effect. For each 10-minute observation, percentage of time spent performing each behaviour was calculated and each behaviour was analysed against rank. The nest defence experiment was analysed using a proportional hazards regression survival

analysis and a pair-wise Kaplan-Meier survival analysis using the Breslow statistic, both performed in SPSS with defence as the event of interest.

3.4 Results

3.4.1 Dominance Ranks

The dominance rank of a total of 142 individuals across 6 colonies was determined (mean colony size = 23.6 ± 4.6). Each colony contained a single alpha, with subordinate workers being composed, on average, of 7% ± 0.7 high-rankers, 37% ± 4 medium-rankers and 51% ± 4 low-rankers.

3.4.2 Risk Taking

I predicted that high-ranking workers should spend less time foraging, removing waste and maintaining the nest, and be less likely to defend against attack. I found a significant effect of rank on proportion of time spent foraging ($F_{2,566}$ = 60.5, $p < 0.001$). Low-rankers spent the most time foraging (15.5 % ± 2.3), followed by medium-rankers (7.7% ± 1.8), with high-rankers foraging less than 1% (0.4% ± 0.2) of the time (*figure 3.1a*). There was no significant effect of rank on time spent removing waste from the nest ($F_{2,566}$ = 1.94, p = 0.148, *figure 3.1b*). Nest maintenance was also significantly affected by rank ($F_{2,566}$ = 8.96, $p < 0.001$), with medium-rankers engaging in this behaviour most often (*figure 3.1c*). There was a significant effect of rank on nest defence behaviour (Cox regression, $p < 0.001$). Low-rankers were the most likely to defend the nest, followed by medium-rankers, with high-rankers the least likely to defend (*figure 3.2*). Defence behaviour was also significantly affected by an individuals' location at the beginning of the defence trial (Cox regression, $p < 0.001$), however the effect of rank was still highly significant when starting location was controlled for (LMER, $F_{2,566}$ = 34.686, $p < 0.001$). The effect of rank was significant for all pairwise combinations (Kaplan-Meier, $p < 0.001$).

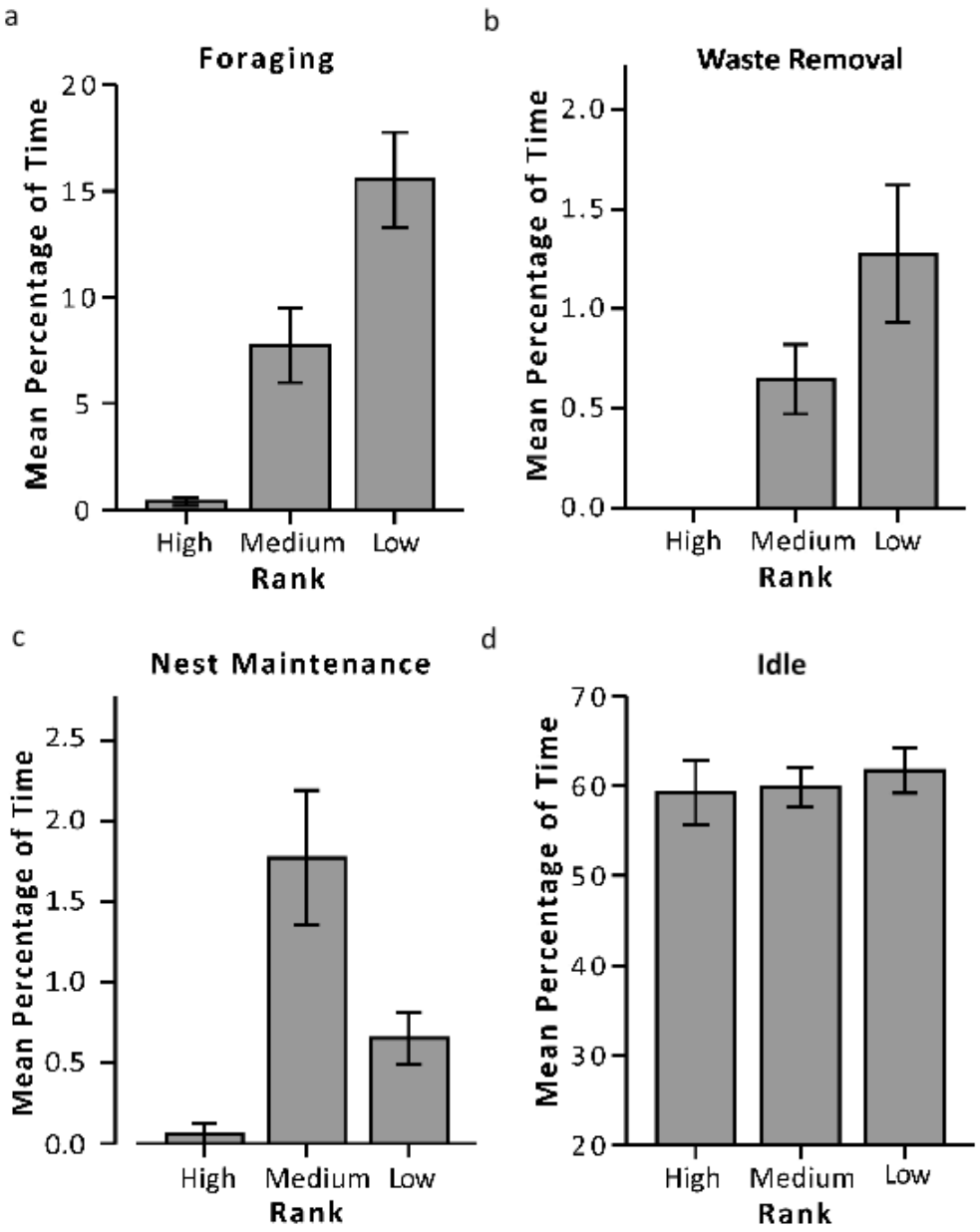


Figure 3.1 Division of Labour in Dinosaur Ants

Mean ± 1SE percentage of time spent performing a) foraging, b) waste removal, c) nest maintenance and d) idle for 142 *D. quadriceps* ants of high, medium or low rank.

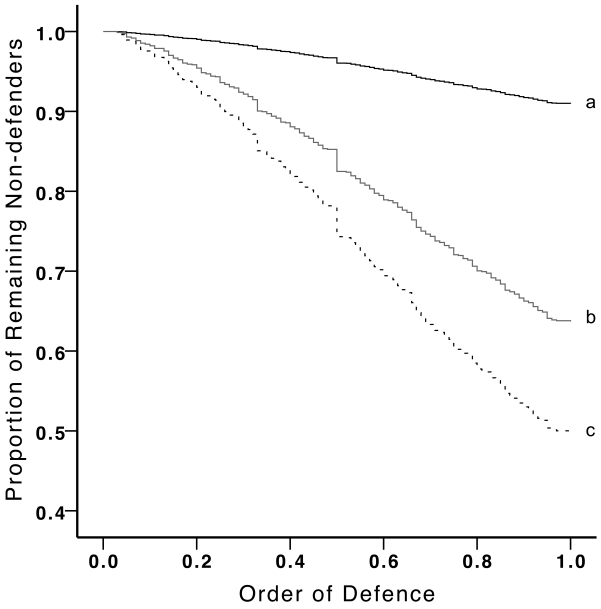


Figure 3.2 Nest Defence in Dinosaur Ants

Cox regression curve for order of nest defence behaviour, for individuals of different rank. Solid black line = high rank, solid grey line = medium rank, dotted line = low rank. Order ranges from 0 for individuals who defended the nest first, to 1 for individuals who never defended. Letters indicate groups that were significantly different in Kaplan-Meier analysis

3.4.3 Activity Levels

I hypothesised that high-ranking individuals would spend more time idle and self grooming in order to maximise lifespan. All individuals spent the majority of their time idle (60.8 % ± 1.8). The most common active behaviours were self-grooming, foraging and brood care (table 3.2). There was a significant effect of rank on the proportion of time spent idle outside the nest ($F_{2,566} = 16.8$, $p < 0.001$) but the effect was not significant for time spent idle inside the nest ($F_{2,566} = 0.47$, $p = 0.629$). However, data from the focal observations showed a significant effect of rank on time spent idle inside the nest, ($F_{2,68} = 67.8$, $p < 0.001$). These conflicting results were due to the fact that during focal observations walking was differentiated from being completely idle, which was not possible during spot observations.

For grooming behaviour, there was a significant effect of rank on the proportion of time spent self-grooming inside ($F_{2,566} = 3.38$, $p = 0.038$), with medium-rankers spending the greatest time performing this behaviour. Time spent self-grooming outside the nest was also significantly correlated with rank ($F_{2,566} = 8.56$, $p < 0.001$), being primarily performed by low-ranking workers. This is consistent with a role of self-grooming in reducing pathogen load, since low-ranking foragers are likely to be exposed to the greatest number of pathogens. The effect of rank was also significant for time spent allogrooming others ($F_{2,566} = 4.75$, $p = 0.001$). High-and medium-rankers

spent more time allogrooming other individuals than low-rankers did (0.6% ± 0.2 compared to 0.5 % ± 0.1 and 0.2 % ± 0.06). This is contrary to my expectation that low-rankers should perform the most allogrooming to improve the health of high-ranking colony members. Low-rankers may refrain from grooming and in general minimise contact with other workers in order to minimise the opportunity for the transfer of potentially harmful pathogens which they may have obtained whilst foraging. High-rankers spent only a very small proportion of their time being aggressive towards other workers (2.9 % ± 0.9) and aggressive behaviour was almost completely absent in medium- (0.4 % ± 0.01) and low-rankers (0.08 % ± 0.4). High-rankers spent significantly more time eating ($F_{2,566}$ = 3.47, p = 0.034) and less time drinking ($F_{2,566}$ = 3.76, p = 0.026). The effect of rank on all other behaviours investigated was non-significant. Results from focal observations were consistent with those from spot observations presented so far; the only exception being that time spent idle inside the nest was significantly affected by rank in the focal observations but not in the spot observations.

Table 3.2 Division of Labour in Relation to Rank

Mean percentage of time spent performing different tasks for each rank. n = 142 individuals (6 colonies).

Behaviour	Mean			
	All	High-Rank	Medium-Rank	Low-Rank
Aggression	< 1 %	2.9 % ± 0.9	0.4 % ± 0.1	0.08 % ± 0.04
Allogrooming	< 1 %	0.6 % ± 0.2	0.5 % ± 0.1	0.2 % ± 0.06
Carrying Food	1.3 %	0.7 % ± 0.5	1.2 % ± 0.2	1.5 % ± 0.2
Drinking	< 1 %	0.2 % ± 0.1	0.7 % ± 0.1	1.2% ± 0.2
Eating	1.8 %	2.3 % ± 0.6	2.1 % ± 0.2	1.5% ± 0.2
Egg Care	4.6 %	14.1 % ± 2.4	5.0 % ± 0.9	2.4 % ± 0.6
Foraging	11.2 %	0.4 % ± 0.2	7.7 % ± 1.8	15.5 % ± 2.3
Idle	60.8 %	59.3 % ± 3.6	59.8 % ± 2.2	61.7 % ± 2.6
Larva Care	2.6 %	3.5 % ± 0.9	4.0 % ± 0.7	1.5 % ± 0.3
Nest Maintenance	1 %	0.06 % ± 0.06	1.7 % ± 0.4	0.7 % ± 0.2
Pupa Care	1.9 %	1.1 % ± 0.4	2.8 % ± 0.5	1.5 % ± 0.3
Processing Food	1.4 %	0.9 % ± 0.3	1.6 % ± 0.2	1.3 % ± 0.2
Self Grooming	8.5 %	6.9 % ± 0.7	9.0 % ± 0.6	8.5 % ± 0.5
Waste Removal	< 1 %	0 %	0.7 % ± 0.2	1.3 % ± 0.3

3.4.4 Brood Care

Hypothesis two predicted that high-ranking individuals would perform more brood care, specifically egg care, enabling them to remain in close proximity to the site of potential cheating. Rank had a significant effect on all types of egg care behaviour, with high-rankers performing more egg care than other ranks (*figure 3.3a*). There was a significant effect of rank on proportion of time spent egg antennating ($F_{2,566} = 30.87$, $p < 0.001$), egg carrying ($F_{2,566} = 110.46$, $p < 0.001$) and egg guarding ($F_{2,566} = 112.69$, $p < 0.001$). Within high-rankers, there was a significant effect of rank on proportion of time spent guarding eggs ($F_{4,91} = 11.24$, $p = 0.001$) and egg carrying ($F_{4,91} = 5.17$, $p = 0.008$), with the alpha and beta performing more of each behaviour than other high-rankers. There was also a significant difference between high-rankers in the time spent egg antennating ($F_{4,91} = 4.44$, $p = 0.0014$). The beta performed significantly more antennation than the alpha ($t = -2.326$, $df = 15$, LSD $p = 0.034$, *figure 3.3b*), whilst all other pairwise comparisons were non-significant. There was a significant effect of rank on proportion of time spent larva antennating ($F_{2,566} = 9.27$, $p < 0.001$), larva cleaning ($F_{2,566} = 5.10$, $p = 0.007$), larva guarding ($F_{2,566} = 11.50$, $p < 0.001$) and larva carrying ($F_{2,566} = 3.60$, $p = 0.03$). Medium-rankers performed the most antennating and cleaning, whilst high-rankers were responsible for the most larvae guarding behaviour. The effect of rank was not significant for larva feeding ($F_{2,566} = 1.50$, $p = 0.227$). There was no significant difference between larva care behaviour within high-rankers for any of the behaviours recorded. The effect of rank was not significant for pupa antennating ($F_{2,566} = 1.05$, $p = 0.353$), pupa guarding ($F_{2,566} = 1.22$, $p = 0.299$) or helping a larva to pupate ($F_{2,566} = 2.136$, $p = 0.122$). However there was a marginally non-significant effect of rank for pupa carrying ($F_{2,566} = 2.94$, $p = 0.056$), with medium-rankers performing this task most often.

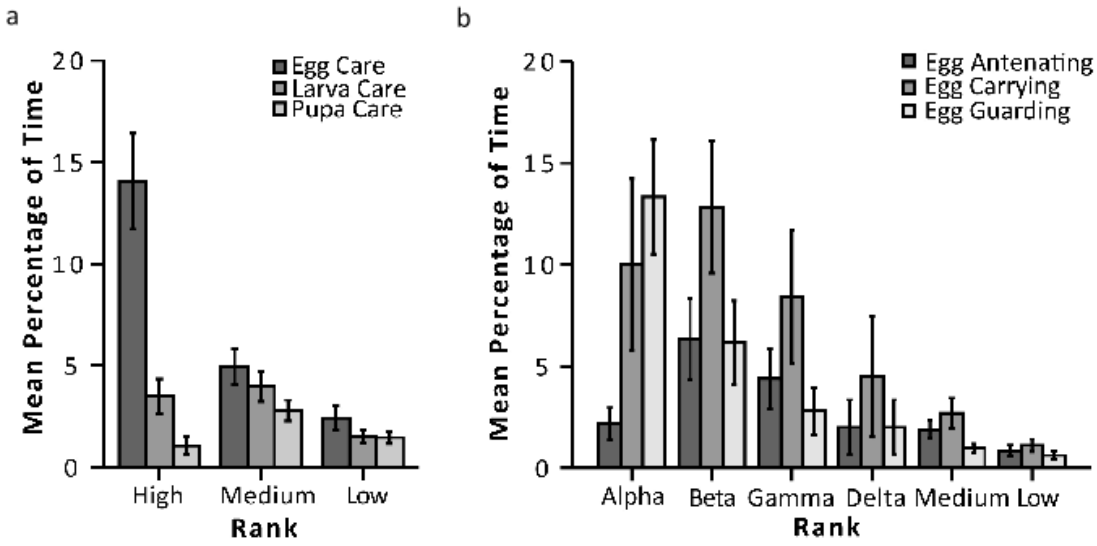


Figure 3.3 Brood Care in Dinosaur Ants

Mean ± 1SE percentage of time spent performing brood care activities for individuals of different rank. a) Egg care, larva care and pupa care for high-, medium- and low-rank individuals. b) Egg antennation, egg guarding and egg carrying for each of the four top-ranking individuals, and medium- and low-rankers.

3.5 Discussion

My results show a strong relationship between rank and behaviour in *D. quadriceps.* This is consistent with individual behaviour relating to future reproductive prospects, and suggests that the autonomy of queenless ponerine ant workers has increased since the divergence from their recent highly eusocial ancestor. My results are consistent with previous studies of division of labour and risk taking in other species (Franks and Scovell 1983; Monnin and Peeters 1999), which have shown a tendency for subordinate colony members to take more risks.

While my data show that behaviour is related to reproductive potential in dinosaur ants, the results do not allow us to distinguish whether the relationship is driven by age or by age-independent effects of rank. Effects of age on behaviour are well known from across the social insects (Hurd *et al.* 2007; Johnson 2008; Naug and Gadagkar 1998; Seid and Traniello 2006; Winston 1987) and dominance rank is likely to be correlated with age in this species because newly emerged workers tend to enter near the top of the hierarchy (Monnin and Peeters 1999). Thus both age and rank are expected to be correlated with reproductive potential and direct fitness, and age may

then be a useful criterion for an individual to estimate reproductive potential if the correlation is reasonably strong. However, the correlation between age and reproductive potential is unlikely to be perfect in species such as *D. quadriceps*, especially if many new workers emerge simultaneously, and age-independent mechanisms of assessing reproductive potential are therefore likely to be advantageous. Individual variation in fertility and the effects of this on behaviour are well known from honey bees (Amdam *et al.* 2006; Amdam *et al.* 2004) and has also been shown to affect how quickly *Platythyrea punctata* ponerine ants switch from in-nest work to foraging (Walter 2012), so such age-independent effects are possible. Most probably a combination of mechanisms are used, with the simple criterion provided by age being complemented by more precise information provided by physiological factors such as fertility.

High-ranking individuals spent significantly less time foraging and were less likely to engage in nest defence, in keeping with the hypothesis that high-ranking individuals avoid performing tasks that are associated with high mortality risk. Foraging has previously been shown to be one of the most dangerous colony tasks (Schmid-Hempel and Schmid-Hempel 1984; Visscher and Dukas 1997) and nest defence will also carry significant risks. Previous investigations into nest defence behaviour in cooperative vertebrates and primitively eusocial insects have produced extremely mixed results. In Damaraland mole rats it is the dominant individual who defends the nest against conspecific intruders (Cooney 2002), a pattern similar to hover wasps (Cronin and Field 2006), paper wasps (Fishwild and Gamboa 1992) and halictine bees (Bell *et al.* 1974). In contrast to this, in naked mole rats, subordinates defend the nest (Lacey and Sherman 1991; O'Riain and Jarvis 1997), as I have also shown to be the case in *D. quadriceps*. These differences in colony defence strategy may be due to differing evolutionary histories. Nest defence by subordinates in queenless ponerine ants could be a characteristic left over from their highly eusocial ancestor, whereas colony defence by dominant individuals in species descended from a solitary ancestor may be a remnant of natural maternal defence of offspring. An alternative explanation may relate to differing colony sizes, as both naked mole rats and queenless ants have comparatively larger colony sizes. The opportunity for future reproduction in

subordinates varies in relation to colony size (Bourke 1999; Monnin *et al.* 2003), and thus in species with very small colonies, subordinates may be unwilling to defend the nest.

Focal observations of individual behaviour revealed a strong relationship between rank and time spent idle. Several studies have found that workload negatively influences longevity (Schmid-Hempel and Wolf 1988; Tsuji *et al.* 1996) and thus high-rankers should be expected to minimise energy expenditure (Cant and Field 2001), increasing their likelihood of surviving to obtain the reproductive role. I also predicted that high-rankers might spend more time performing self-grooming, a self-directed behaviour which is likely to improve longevity by reducing pathogen load (Fernández-Marín *et al.* 2006; Hughes *et al.* 2002). In contrast to this, I found that medium-rankers performed the greatest amount of self-grooming inside the nest, and low-rankers performed the most outside the nest. However, this is consistent with self-grooming as a mechanism to reduce pathogen load, as medium- and low-ranking foragers are likely to be exposed to pathogens more frequently than non-foragers. Individuals are often observed self-grooming immediately after returning to the nest from a foraging trip, which further supports a key role for self-grooming in disease resistance. I found that high-ranking workers perform the most allogrooming, an unexpected result since allogrooming is expected to improve the health of the recipient. This may possibly indicate that allogrooming plays a social role in *D. quadriceps* (e.g. in maintaining hierarchies), similar to in many primates and other animals (Lazaro-Perea *et al.* 2004; Ren *et al.* 1991; Vervaecke *et al.* 2000).

Brood care was primarily performed by high-ranking individuals, consistent with my hypothesis that this enables them to remain in close proximity to eggs, improving their ability to detect and prevent illicit laying by subordinate workers. Furthermore, egg care behaviours are mostly performed by high-rankers, a relationship that is not true for all larva and pupa care behaviours. Whilst all brood care behaviours are performed in the brood chamber, only the egg stage offers a significant opportunity for cheating. That high-rankers dominate this behaviour but not other brood care activities supports a role for egg care in preventing illicit egg laying. Previous investigations of egg-policing

behaviour in this species have indicated that destruction of worker-laid eggs is performed primarily by the alpha, however other high-rankers are also occasionally involved (Monnin and Peeters 1997). One surprising result is that the beta performs the greatest amount of egg antennation, as I might expect the alpha to have greatest incentive to inspect eggs in order to confirm that they were laid by her. However, it is possible that antennation also enables the beta to assess the fertility of the alpha, and thus judge whether it would be beneficial to attempt to overthrow her. It has previously been shown that ponerine ants are able to distinguish between alpha- and worker-laid eggs, which differ in their in the cuticular hydrocarbon profile (Monnin and Peeters 1997; Tannure-Nascimento *et al.* 2009) and that alpha fertility is also signalled through cuticular hydrocarbons (Cuvillier-Hot *et al.* 2004; Monnin and Peeters 1998). It is therefore plausible that by regularly antennating alpha-laid eggs, the beta may be able to assess her fertility. Attempts to overthrow the alpha will generally be met by high levels of aggression from low-ranking subordinates (Monnin *et al.* 2002) except when alpha fertility is below 75% (Monnin and Ratnieks 2001). Since the beta rank changes regularly, the ability to detect an opportunity to overthrow the alpha represents a major fitness advantage for the beta (Cuvillier-Hot 2004).

To my knowledge, this study is the first to demonstrate a relationship between future reproductive prospects and nest defence and other risk-taking behaviours in a species in which simple society is secondarily derived. The apparent flexibility in task choice exhibited by *D. quadriceps* is likely to have evolved relatively recently, since their divergence from their highly eusocial ancestor. Understanding the organisation of division of labour in simple eusocial societies can greatly inform explanations of the evolution of sociality itself.

Chapter 4

Allogrooming is Social and Hygienic in Dinosaur Ants

4.1 Abstract

Social behaviour is common in the animal kingdom, although only relatively few species have evolved the extreme levels of altruism observed in eusocial societies. One adaptation to social life has been the development of social immunity, which includes allogrooming, whereby one individual grooms another. Allogrooming has been found to play both a hygienic and social role in some primates and other mammals, however this has not previously been investigated in social insects. In the primitively eusocial dinosaur ant, *Dinoponera quadriceps*, six distinct aggressive interactions have been identified which control and maintain the dominance hierarchy. However, non-aggressive interactions may also play a role in hierarchy maintenance. Here I investigate the role of allogrooming in determining and reinforcing the dominance hierarchy in dinosaur ants. Using behavioural observations, I found that high-ranking nest mates perform the majority (75.6%) of allogrooming. This is contrary to the pattern predicted based upon a purely hygienic role of allogrooming, where low-rankers would be expected to engage most in allogrooming, since this class of worker is exposed to the greatest number of pathogens and are least valuable to the colony. Allogrooming performed by high-rankers is frequently preceded by aggression, suggesting that these behaviours may play a role in hierarchy maintenance in this species. I also identified a second peak in allogrooming for low-ranking actors, primarily in interaction with other low-rankers. These allogrooming events were longer and rarely followed aggressive behaviour. I therefore propose a dual role for allogrooming in *Dinoponera quadriceps*; short-duration allogrooms between high-rankers are important in hierarchy maintenance and social cohesion, whilst long-duration allogrooms between low-rankers may serve a more conventional role in pathogen removal. My data suggest that allogrooming may play an important, under-appreciated role in dominance interactions and that making up may be important to the regulation of simple ant societies, just as in those of some vertebrates.

4.2 Introduction

Social behaviour is common in the animal kingdom, although only relatively few species have evolved the extreme levels of altruism observed in eusocial societies. Eusociality exists along a continuum from facultatively social species (e.g. halictid bees), through primitive eusociality (e.g. paper wasps) to the advanced societies of honeybees and leafcutter ants. Eusocial societies are united by reproductive division of labour, with the worker caste forgoing reproduction in favour of assisting the queen to rear offspring. In advanced societies, castes are generally maintained through chemical signalling, however in simpler societies, behavioural maintenance also occurs, and in many species reproduction is determined by a dominance hierarchy. Although grooming is often a self-directed behaviour, allogrooming, whereby one individual grooms another, is observed in many animal species.

Grooming is a ubiquitous behaviour, which plays a role in hygiene and therefore parasite defence. Grooming is the behaviour whereby an individual cleans its skin, fur or feathers, removing foreign objects such as dirt and parasites. In animals which form social groups or aggregations, allogrooming, whereby one individual grooms another, is sometimes observed, in addition to more frequently observed self-grooming. Allogrooming has primarily been associated with a role in pathogen removal (Hart and Hart 1992; Mooring and Hart 1993; Pérez and Veà 2000; Rosengaus et al. 1998; Walker and Hughes 2009). However, a social role is well supported in primates (Easley et al. 1989; Pérez and Veà 2000; Ren et al. 1991; Schino et al. 1988) and more recently social allogrooming has also been identified in birds (Radford 2012) and ungulates (Mooring *et al.* 2004; Sato *et al.* 1993; Schino 1998). Allogrooming has been shown to relieve tension (Radford 2012; Schino *et al.* 1988) and is thought to reinforce social bonds (Carpenter 1964). In some species, allogrooming relates directly to the social hierarchy, either with low-ranking individuals tending to groom higher-ranked individuals (grooming up the hierarchy; (Adiseshan *et al.* 2011; Vervaecke *et al.* 2000)) or vice versa (grooming down the hierarchy; (Parr *et al.* 1997)). Allogrooming is also commonly observed between colony members in social insects (Evans and Spivak 2010; Reber *et al.* 2011; Rosengaus *et al.* 1998; Walker and Hughes 2009; Wilson-Rich *et al.* 2007). In eusocial species, which live in extremely high densities and are

therefore particularly vulnerable to pathogens (Boomsma *et al.* 2005), many species have developed sophisticated strategies for pathogen defence including innate and adaptive immunity, antifungal and antimicrobial secretions, nest hygiene (e.g. corpse removal), grooming and allogrooming (Bulmer *et al.* 2012; Chapuisat *et al.* 2007; Diez *et al.* 2012; Evans and Spivak 2010; Stow *et al.* 2007; Walker and Hughes 2009). Allogrooming forms a key part of social immunity (Cremer *et al.* 2007), and has been shown to have measurable effects on survival (Rosengaus *et al.* 1998; Walker and Hughes 2009).

In advanced eusocial societies, such as those of honeybees or leaf-cutting ants, reproductive castes are determined prior to birth and are irreversible (Oster and Wilson 1978). Reproductive monopoly of the queen over her workers is often signalled chemically using cuticular hydrocarbons (Izzo *et al.* 2010; Mitra and Gadagkar 2011; Mitra *et al.* 2011; Monnin 2006). However, in simpler societies, such as those of the *Polistes* paper wasps or queenless ponerine ants, reproduction is determined through dominance, which is regulated in part by aggressive interactions (Cronin and Field 2007; Cuvillier-Hot *et al.* 2004; Mitra *et al.* 2011; Monnin and Peeters 1999; Pardi 1948). Cuticular hydrocarbons in these species are also important in signalling dominance and fertility (Izzo *et al.* 2010; Mitra and Gadagkar 2011; Mitra *et al.* 2011; Monnin 2006). In the queenless ponerine dinosaur ant, *Dinoponera quadriceps*, a linear hierarchy is maintained through cuticular hydrocarbon signalling, reinforced by ritualised aggressive interactions (Monnin and Peeters 1999). Allogrooming has been observed in this species (Asher *et al.* 2013), but little is known about its role in the colony.

Here I investigate the relationship between allogrooming, rank and aggressive behaviour in the dinosaur ant, *Dinoponera quadriceps*. I propose that, to effectively perform a hygienic role, allogrooming should be performed primarily within low-ranking individuals, as these individuals have the greatest pathogen exposure and are least valuable to the colony. Alternatively, grooming up the hierarchy (low-rankers grooming higher-rankers) might serve to improve longevity of the reproductive, allowing low-rankers to maximise their inclusive fitness. Gamergate turnover reduces

the relatedness between workers, and thus it is beneficial for subordinates to maximise the lifespan of the current gamergate and allogrooming may assist in this. By contrast, a social role for allogrooming in maintaining the hierarchy or reinforcing social cohesion would most likely be characterised by directional grooming down or up the hierarchy, and would be expected to be primarily performed by high-rankers. Grooming down the hierarchy would be a pattern similar to that of aggressive interactions in primitively eusocial species, which primarily occur between high-ranking individuals. I investigate two possible explanations for the role of allogrooming in *D. quadriceps*; allogrooming may serve a hygienic role (removing pathogens) or a social one (colony cohesion, hierarchy maintenance).

4.3 Methods

Behavioural data for this chapter was collected by BW and CA, video observations were performed by BW, and all statistical analyses were completed by CA.

4.3.1 Housing and Husbandry

Colonies of *Dinoponera quadriceps* were collected from Atlantic forest in Sergipe (S11°01’23,W37°12’9), Brazil in 2009 and 2010, and housed at 26-29°C, 70-90% relative humidity and a 12:12 light: dark cycle. Colonies were housed in plastic containers (38cm x 58cm x 18cm) containing a plastic nest chamber (33cm x 19cm x 11cm), divided into 6 compartments by a cardboard divider. Colonies were fed *Tenebrio* mealworms and banana three times a week, corned beef once a month, and provided with water *ad libitum*. To allow individual identification, all ants were tagged with a small unique number tag (E.H. Thorne Ltd).

4.3.2 Behavioural Observations

Behavioural observations were performed on a total of 14 colonies of *D. quadriceps*, with a mean observation time of 12.95 hours ± 2.76 per colony. Colonies were observed for 30-minute bouts, totalling 77.3 hours (mean per colony = 5.52 hours ± 1.08). Additional behavioural and video observations were completed for eight of the colonies. Two 5-hour observations were performed, with a minimum 5-day break

between observations of the same colony, and 3 hours of video monitoring of the nest box, using a Sony DCR-HC62 camcorder, were also performed.

During all observations, aggressive and allogrooming interactions were recorded. Allogrooming behaviour was defined by the actor licking the legs, thorax, head or abdomen of the recipient with its maxillae and labium. This behaviour is clearly distinct from aggressive interactions, in which the actor may bite the recipient using its mandibles. In total, 393 allogrooming events were observed. For each event, the identity of the actor and recipient was recorded, and the date on which the event occurred. For 122 allogrooming events, duration of the allogrooming event was also recorded. Allogrooming events were considered to have been preceded by aggression if an aggressive interaction between the same individuals occurred up to 5 minutes prior to the allogrooming event. Allogrooming events which occurred during the first five minutes of the observation period were necessarily excluded from analyses investigating aggressive interactions. A total of 325 observations were recorded where the occurrence of aggression prior to the allogroom was known.

Table 4.1 Categories of Allogrooming

Categorisation of allogrooming events according to the rank of the actor and recipient. Allogrooming events were categorised according to the direction (up or down the hierarchy) and more precisely into one of 9 categories relating directly to the rank of the actor and recipient.

Recipient	Actor					
	High-Rank		Medium-Rank		Low-Rank	
	Direction	Type	Direction	Type	Direction	Type
High-Rank	0	HH	+1	MH	+2	LH
Medium-Rank	-1	HM	0	MM	+1	LM
Low-Rank	-2	HL	-1	ML	0	LL

4.3.3 Statistical Analyses

Data analyses were performed using R. Each allogrooming event was categorised according to the direction of the interaction, ranging from +2 for a low-ranking actor grooming a high-ranking recipient, to -2 for a high-ranking actor grooming a low-ranking recipient (*table 4.1*). A direction of 0 therefore represents allogrooming within a rank (e.g. a high-ranker grooming a high-ranker). Allogrooming events were also categorised more finely into one of 9 types representing the combination of actor and

recipient rank (*table 4.1*). For both direction and type, χ^2 tests were performed to determine whether the observed occurrence of each category differed significantly from a both a uniform distribution, and a more realistic frequency distribution based on the relative abundance of each rank (*appendix A2*). The proportion of allogrooms expected for each type and direction was calculated based upon the mean proportion of the colony falling into the rank categories of the actor and recipient.

The duration of allogrooming, and whether it was preceded or not by aggression, were analysed using general linear models. Duration of allogrooming was normalised using a log transformation and the following explanatory variables were included in the full model: type, preceded by aggression, actor rank, recipient rank and colony. An R-squared value was calculated for the minimum adequate model. A binomial model was fitted to whether allogrooming was preceded by aggression, with the following explanatory variables: type, actor rank, recipient rank and colony. Minimum adequate models were created using a model simplification procedure based upon both p-values and AIC.

4.4 Results

The direction of allogrooming events departed significantly from a uniform distribution ($\chi^2_{4,3} = 136.51$, $p < 0.0001$), and was also significant different from the expected distribution based on the relative abundance of each rank ($\chi^2_{4,3} = 98.39$, $p < 0.0001$, *figure 4.1a*). The most common direction was 0 (allogrooming within rank), followed by negative directions -2 and -1 (allogrooming down the hierarchy). Allogrooming events with positive directions (allogrooming up the hierarchy) were extremely rare, representing just 5.6% of all events. The type of allogrooming event also departed significantly from an even distribution ($\chi^2_{8,7} = 171.44$, $p < 0.0001$), and a distribution predicted based upon the abundance of each rank ($\chi^2_{8,7} = 309.2897$, $p < 0.0001$, *figure 4.1b*). The most frequently observed allogrooming events had high-ranking actors (HH, HM, HL), together these types of allogrooming represented 75.6% of all events observed. Allogrooming within the low-rankers was the next most common event, representing a further 12.3% of events.

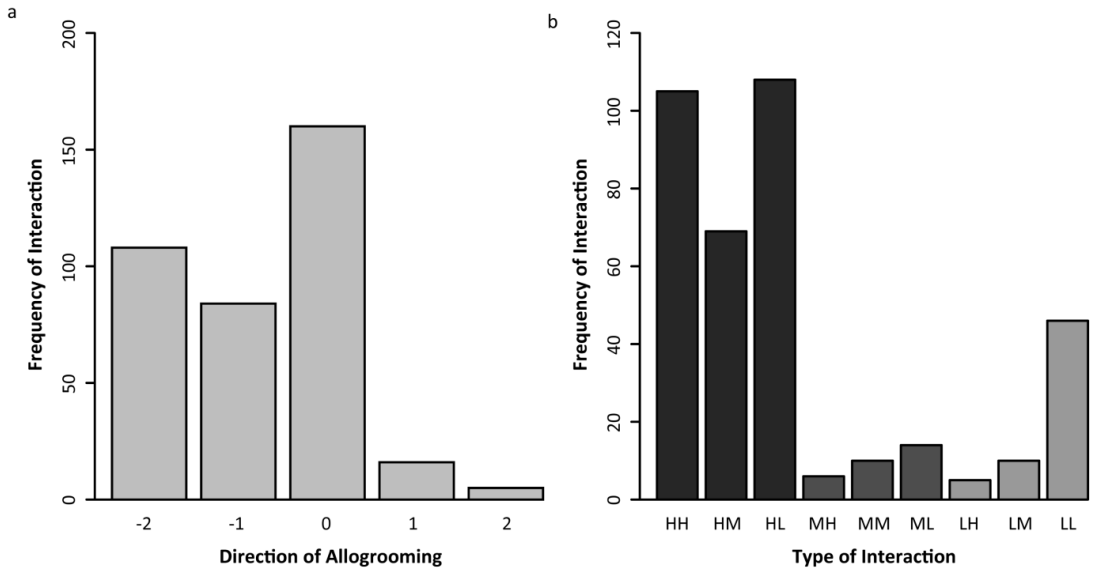


Figure 4.1 Allogrooming Down the Hierarchy

The frequency of different allogrooming events, categorised according to a) direction (+2 e.g low-rank grooming high-rank, +1 e.g medium-rank grooming high-rank, 0 e.g high-rank grooming high-rank, -1 e.g. high-rank grooming medium-rank, -2 e.g high-rank grooming low-rank) of the interaction and b) type, a conglomerate of actor rank and recipient rank (HL = high-rank grooming low-rank, LH = low-rank grooming high-rank). See table 4.1 for full explanation of direction and type categories. Data includes 393 allogrooming events observed in 14 Colonies.

The mean duration of all allogrooming events was 18.46 ± 1.60 seconds. Duration was significantly influenced by the rank of the actor ($F_{33,31}$ = 6.61, p = 0.0041) but not of the recipient ($F_{29,27}$ = 0.91, p = 0.41, *figure 4.2*). Duration was longest for low-ranking actors (36.22 ± 8.43 seconds), and shortest for high-ranking actors (16.63 ± 1.45 seconds). Duration was not significantly different for allogrooming events preceded by aggression ($F_{24,23}$ = 0.047, p = 0.90). Colony also had no significant effect on allogroom duration ($F_{29,27}$ = 0.83, p = 0.45). The minimum adequate model had an R-squared value of 0.647.

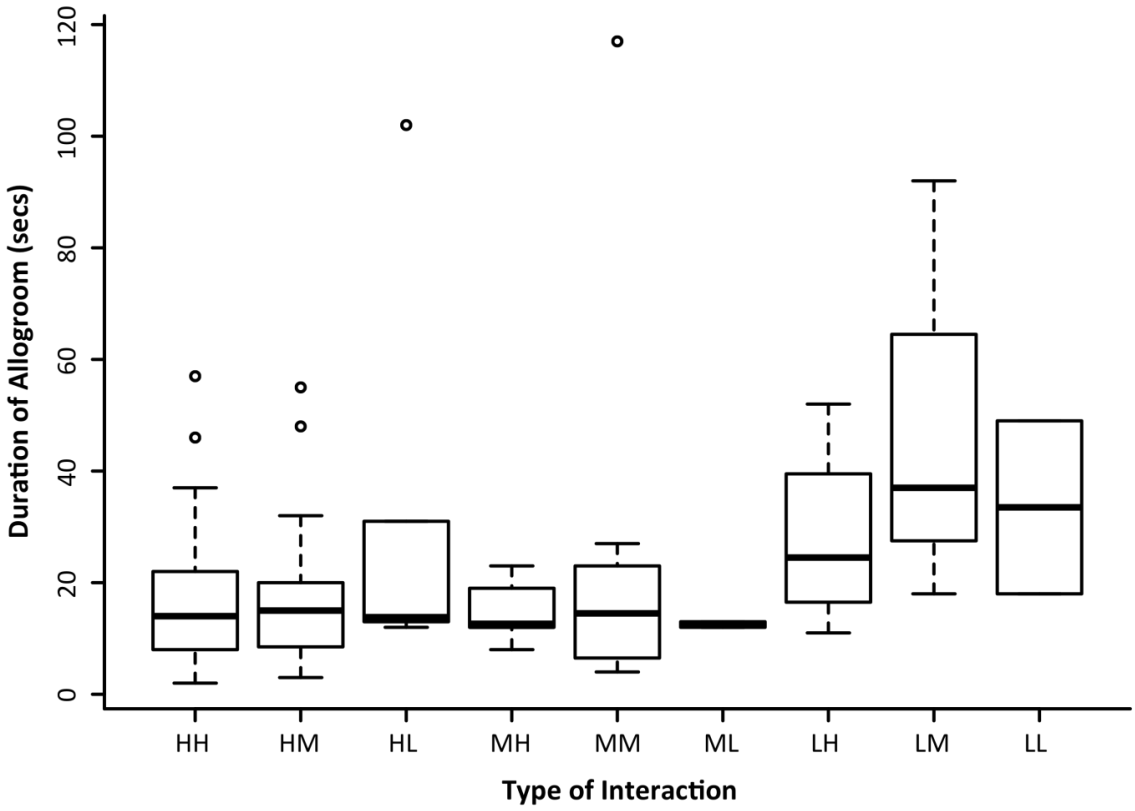


Figure 4.2 Duration of Allogrooming

The duration in seconds of allogrooming events for each type of interaction, where type is a conglomerate of actor rank and recipient rank (e.g. HL = high-rank grooming low-rank, LH = low-rank grooming high-rank). See table 1 for full explanation of type categories. Data for 122 allogrooming events in 9 colonies.

Out of 325 allogrooming events for which it was known whether the event was preceded by an aggressive interaction, 124 allogrooms (38.15%) followed an aggressive interaction between the same pair. There was a significant relationship between whether the allogrooming event was preceded by aggression and both actor rank ($\chi^2_{294,292}$ = -26.31, $p < 0.0001$) and recipient rank ($\chi^2_{294,292}$ = -9.90, $p = 0.0071$, *figure 4.3*). A total of 246 of the 325 allogrooms involved a high-ranking actor, of which 117 (47.56%) were preceded by an aggressive interaction between the same pair. By contrast, only 57 and 18 allogrooms had a low- or medium-ranked actor, with 5.26% and 5.56% of these allogrooms preceded by aggression, respectively. For recipient rank, 86 allogrooms involved a high-ranked recipient, of which 55.81% were preceded by an aggressive interaction. For medium- and low-ranking recipients, there were 56 and 163 allogroom events, with 35.71% and 26.99% of events preceded by an

aggressive interaction, respectively. In addition, the occurrence of aggression prior to allogrooming varied significantly between colonies ($\chi^2_{294,292}$ = -27.71, p = 0.00053).

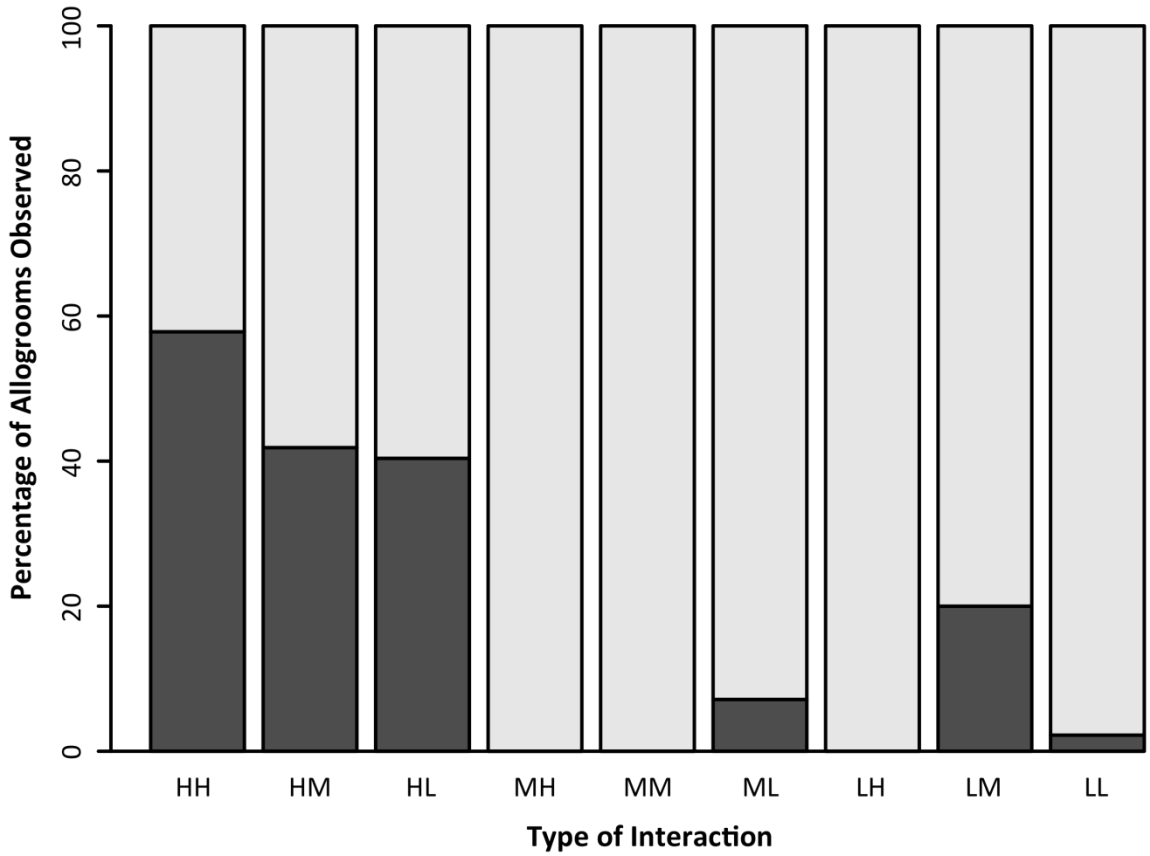


Figure 4.3 Aggression and Allogrooming

Percentage of allogrooms preceded by an aggressive interaction (black) or not (grey) for allogrooms of different type, where type is a conglomerate of actor rank and recipient rank (e.g. HL = high-rank grooming low-rank, LH = low-rank grooming high-rank). See table 1 for full explanation of type categories. Data for 325 allogrooming events in 9 colonies.

4.5 Discussion

Allogrooming events did not occur randomly with respect to the dominance hierarchy. The vast majority of allogrooming events (94.4%) were either within a rank (42.9%), or in a downward direction in the hierarchy. Most allogrooming events involved a high-ranking actor (75.6%), although the next most common type was allogrooming within the low-rank group (*figure 4.1b*). Further, allogrooming events involving high-rankers were significantly more likely to be preceded by an aggressive interaction between the same individuals (*figure 4.3*). These results support a role for allogrooming in reinforcing or maintaining the dominance hierarchy.

4.5.1 A Social Role for Allogrooming

High-ranking individuals in *D. quadriceps* colonies spend the majority of their time caring for the brood and performing social policing (Asher *et al.* 2013; Monnin and Peeters 1999; Monnin and Ratnieks 2001), and are therefore likely to be exposed to fewer pathogens. Despite this, I found that high-rankers perform the majority of allogrooming, and primarily towards other high-rankers. High-rankers also exhibit much higher levels of aggression than low-ranking subordinates, and this too is most frequently directed towards other high-rankers (Monnin and Peeters 1999). Thus, allogrooming may represent a less aggressive dominance interaction, which functions in maintaining and reinforcing the hierarchy. Recipients were often observed to accept allogrooming in a crouched, subordinate pose, consistent with this hypothesis. However, the occurrence of allogrooming between low-rankers indicates that allogrooming is not solely an extension of the aggressive dominance interactions that exist purely for hierarchy maintenance. Further, aggressive dominance interactions are almost never seen between high- and low-rankers in *D. quadriceps* colonies, where as HL allogrooming events are frequent, suggesting that allogrooming may serve as a more general mechanism to reinforce dominance and social cohesion across the colony, while aggressive interactions are more specific to the queue for reproduction amongst the high-rankers.

A social role for allogrooming is well recognised in primates (Easley et al. 1989; Pérez and Veà 2000; Ren et al. 1991; Schino et al. 1988), reducing tension and reinforcing social bonds after aggression in a number of vertebrate species (Radford 2012; Ren *et al.* 1991; Schino *et al.* 1988). Occurrences of social allogrooming in non-primates are less common, but have been demonstrated in birds (Radford 2012) and ungulates (Mooring *et al.* 2004; Sato *et al.* 1993; Schino 1998). Within the social insects, allogrooming has only previously been recorded as a mechanism for pathogen removal (Walker and Hughes 2009; Wilson-Rich *et al.* 2007). In highly eusocial species, allogrooming is unlikely to play a social role, as reproductive castes are morphologically defined and conflict is minimal (Hart and Ratnieks 2005; Sherman *et al.* 1995). However, in more primitively eusocial species, hierarchies are dynamic and

constantly reinforced by aggression and chemical signalling (Cronin and Field 2007; Cuvillier-Hot *et al.* 2004; Monnin 2006; Pardi 1948). In these societies, allogrooming could play an important role both in maintaining the hierarchy and providing access to chemical signals that may provide information about fertility and dominance (Izzo *et al.* 2010; Mitra and Gadagkar 2011; Monnin 2006).

Allogrooming could function as reconciliatory behaviour, reinforcing the hierarchy after aggression. This effect of allogrooming is known in primates, although has rarely been demonstrated in other species. In primates allogrooming has been observed both up the hierarchy (Adiseshan *et al.* 2011; Vervaecke *et al.* 2000) and down the hierarchy (Parr *et al.* 1997). I report allogrooming both within rank and down the hierarchy in *D. quadriceps*. Allogrooming frequently follows aggression and high-high allogrooming events are most likely to be preceded by aggression, consistent with a reconciliatory role for allogrooming in reinforcing the hierarchy and reducing stress after aggressive interactions. The effect of allogrooming, both behavioural and physiological, on actor and recipient in this species remains to be tested.

4.5.2 Keeping the Gamergate Clean

Grooming up the hierarchy, which has previously been reported in primates (Adiseshan *et al.* 2011; Vervaecke *et al.* 2000) and social insects (Winston 1987), is rarely observed in this species, possibly indicating that the high-ranking workers do not need allogrooming in order to reduce their pathogen load. In many advanced eusocial societies, subordinates groom the queen (Holldobler and Wilson 1990; Remolina and Hughes 2008; Trettin *et al.* 2011; Winston 1987), likely extending her longevity by reducing pathogen load. Queens in advanced societies are morphologically and behaviourally adapted to their reproductive role, and thus may have reduced ability to remove pathogens through self-grooming, necessitating allogrooming up the hierarchy. The rarity of allogrooming up the hierarchy in *D. quadriceps* may be explained by reproductives being morphologically identical to subordinates (Peeters 1991), and therefore possess sufficient self-grooming capabilities to negate the requirement for allogrooming up the hierarchy.

4.5.3 Allogrooming as a Source of Information

An alternate explanation for my results could be that allogrooming allows an individual access to a great deal of information about the recipient through cuticular hydrocarbons. A high-ranker grooming an individual immediately below her in the hierarchy could assess the dominance and fertility of the recipient. In a society where illicit egg laying is fairly frequent (Monnin and Ratnieks 2001), assessing the reproductive status of your subordinates may be very beneficial. However, the finding that allogrooming tended to follow aggression suggests that this is not the case; we might expect information gathering allogrooming to occur randomly with respect to aggressive interactions, or tend to precede aggression, should allogrooming reveal a cheating subordinate.

4.5.4 Allogrooming and Primitive Eusociality

Allogrooming is more characteristic of advanced eusocial societies, and in general social immunity is expected to be more prevalent in more complex societies, where colonies sizes tend to be larger (Stow *et al.* 2007). To my knowledge, allogrooming has not been reported for a primitively eusocial species. Allogrooming in *D. quadriceps* may therefore be a behaviour that remains from their advanced eusocial ancestors, having later been secondarily co-opted for a role in social cohesion. Further research into allogrooming in other ponerine ants is needed to investigate this hypothesis more thoroughly. Allogrooming has previously been reported in two queenright ponerine ants; *Pachycondyla apicalis* and *Nothomyrmecia macrops.* In *P. apicalis*, it has been shown to facilitate the transfer of cuticular hydrocarbons between individuals (Soroker *et al.* 1998). It likely contributes to the development of the 'colony odour' (Boulay *et al.* 2004; Soroker *et al.* 1998), therefore, and it may also provide information about identity, dominance and fertility. However, whether allogrooming is utilised for either a social or hygienic role in these or other ponerine ants still needs to be tested.

4.5.5 A Hygienic Role for Allogrooming

The relatively high frequency of low-low allogrooming events compared to other types with a low- or medium-ranked actor suggests that these events may be fulfilling a pathogen-removal function. Very few allogrooms performed by medium- or low-

rankers were preceded by aggressive behaviour, supporting this view. Furthermore, the duration of grooming was significantly longer for allogrooms with a low-ranking actor, again suggesting that these low-rank allogrooms may function primarily in pathogen removal, and can be considered distinct from the short, dominance-related allogrooms observed between high-rankers. Low-rankers are exposed to the greatest number of pathogens as they spend more time outside the nest foraging and come into contact with waste materials and dead ants (Asher *et al.* 2013; Monnin and Peeters 1999). Allogrooming in response to pathogen exposure is common in social insects and has been shown to increase survival (Rosengaus *et al.* 1998; Walker and Hughes 2009; Wilson-Rich *et al.* 2007). Thus, increased allogrooming between low-rankers could serve to remove pathogens from those individuals most at risk.

4.5.6 Concluding Remarks

My data suggests a dual role for allogrooming in colonies of the primitively eusocial ant, *Dinoponera quadriceps*. Allogrooming may have evolved in advanced ponerine ants as a form of social immunity, later being co-opted for a social role after the loss of the queen-caste and reversion to a primitive state. This study therefore represents the first demonstration of a role for allogrooming in social hierarchy maintenance in a social insect. Whether allogrooming may play a more general role in social cohesion in other eusocial species, particularly other queenless ponerine ants, remains to be tested.

Chapter 5

Learning to Police:

The Influence of Experience and Social Environment

5.1 Abstract

The plasticity of behaviour, and the consequent ability of many animals to adjust innate behaviours according to experience and learning, allows animals to better adapt to their environment, and to improve in task performance. Behaviours involving complex cue recognition may particularly benefit from improved task performance due to learning and experience. Subordinate reproduction is an area of conflict in many social insect colonies, and the removal of subordinate eggs requires the recognition of complex cues such as cuticular hydrocarbons. Here, I investigate whether propensity to police is influenced by past experience of illicit eggs, across two temporal scales, in the socially primitive ponerine ant, *Dinoponera quadriceps*. I investigate short-term experience through the introduction of foreign eggs, and long-term experience in the form of naturally occurring illicit-laying by subordinates. I show an increase in time spent inspecting foreign eggs by individuals with increased experience of artificially introduced eggs. Overall policing rates were significantly higher in colonies containing reproductively active subordinates, suggesting that policing behaviour emerges in response to experience of subordinate cheating. Individual past policing behaviour influenced future behaviour, leading to the emergence of a specialised policing workforce in *D. quadriceps*, which may enable colonies to take advantage of the benefits of learning on task performance. Together, these data suggest that learning and past experience play a key role in influencing policing behaviour in the simple societies of *Dinoponera quadriceps*.

5.2 Introduction

Altruism and cooperative behaviour, in spite of appearing contrary to the laws of natural selection, are common across the animal kingdom, and eusocial insects are one of the pinnacles of sociality. Cooperation is maintained in highly altruistic societies

through the resolution of conflicts which arise because individuals within a social group are not genetically identical (Ratnieks *et al.* 2006). Conflict resolution in cooperative interactions can be achieved through a number of mechanisms including physical aggression, but requires cues for identifying non-cooperators who try to cheat the system (Ratnieks *et al.* 2006). Understanding why conflicts arise, and how they are resolved, can bring us closer to understanding how and why social behaviour emerges and is maintained.

5.2.1 Conflict and Conflict Resolution

Social insect colonies represent one of the pinnacles of social evolution, and can provide insight into conflict resolution and the evolutionary maintenance of cooperation. One key area of conflict in eusocial societies arises over reproduction by subordinate group members, and in social insects, workers are commonly able to lay unfertilized male eggs owing to the haplodiploid system of sex determination (Bourke 1988). Laying male eggs offers subordinates an opportunity to 'cheat' the system and gain direct fitness benefits, but carries costs for nest mates and colony productivity (Bourke 1988; Oldroyd 2013; Ratnieks 1988; Teseo *et al.* 2013). Depending upon relatedness between colony members, the queen and/or the workers may benefit from policing reproductively active subordinates (cheaters) (Ratnieks 1988). Worker policing can occur through overt aggression towards the cheater, or destruction of worker-laid eggs (Gobin *et al.* 1999; Halling *et al.* 2001; Kikuta and Tsuji 1999; Liebig *et al.* 1999). In monogynous, monandrous colonies, workers are more closely related to sisters (queen's daughters, r = 0.75), and nephews (r = 0.375) than to brothers (r = 0.25), and thus should not oppose worker-reproduction. However, worker policing occurs frequently in species in which it is not predicted based on relatedness alone (D'Ettorre *et al.* 2004; Foster and Ratnieks 2001a; Hartmann *et al.* 2003; Kikuta and Tsuji 1999; Pirk *et al.* 2003; Ratnieks *et al.* 2001; Teseo *et al.* 2013) and a number of possible hypotheses have been proposed to explain this phenomenon, including costs of worker reproduction to colony efficiency (Ratnieks 1988) and the costs of errors in discriminating the sex of brood (Foster and Ratnieks 2001b). Recent research suggests that subordinate egg-laying may indeed be very costly to colony efficiency (Teseo *et al.* 2013), and this may be sufficient to explain the occurrence of worker policing. An

inability to discriminate sex during early brood development (Foster and Ratnieks 2001a; Nonacs and Carlin 1990), or a tendency for worker-laid eggs to be in competition with queen-laid female eggs, could favour the development of worker policing because average relatedness to the queen's offspring (r = 0.5) is higher than worker offspring. Policing of worker reproduction has been documented in a wide variety of social Hymenoptera including honeybees, wasps and ants, and appears to be a strong deterrent against widespread cheating (D'Ettorre *et al.* 2004; Monnin and Peeters 1997; Pirk *et al.* 2003; Ratnieks and Visscher 1989; Wenseleers and Ratnieks 2006; Wenseleers *et al.* 2005).

5.2.2 Task Specialisation and Division of Labour

Despite the occurrence of worker-reproduction, reproductive skew in most Hymenopteran species is high (Bang and Gadagkar 2012; Hart and Ratnieks 2005; Uddin and Tsuchida 2012), with one or a few individuals dominating reproduction. In addition to reproductive division of labour, social insect colonies are characterized by task specialisation (Robinson 1992). Non-reproductive division of labour may have been key to the success of the social insects, increasing colony efficiency and resource use (Dukas 2008; Dukas and Visscher 1994; Page and Mitchell 1998; Ravary *et al.* 2007; Wilson 1974; Wilson 1985). Specialisation allows colonies to exploit individual differences in task performance (Morse 1978; O'Donnell *et al.* 2000) and the benefits of learning and experience (Dukas and Visscher 1994; Durisko *et al.* 2010; Johnson 1991; O'Donnell and Jeanne 1992). Specialisation has been demonstrated for a variety of tasks including foraging behaviour (Hofstede and Sommeijer 2006; Pinter-Wollman *et al.* 2012; Robinson *et al.* 2009), undertaking (removing dead individuals from the nest) (Julian and Cahan 1999; Trumbo and Robinson 1997), waste management (Waddington and Hughes 2010), collecting building materials and brood transportation during nest relocation (Dornhaus 2008; Dornhaus *et al.* 2009), thermoregulation (Gardner *et al.* 2007), and policing behaviours (Van Zweden *et al.* 2007). Individual specialisation may yield benefits because individuals can become more efficient or effective at their specialized task (Hofstede and Sommeijer 2006; Julian and Cahan 1999), and task specialisation may be improved through learning and experience. Improvements in task performance with experience have been documented for a

range of taxa including apes (Helton 2007), birds (Helton 2007; Yoerg 1994), spiders (Heiling and Herberstein 1999; Morse 2000), cockroaches (Durier and Rivault 2000), and Hymenoptera (Dukas and Visscher 1994; Durisko *et al.* 2010; Johnson 1991; O'Donnell and Jeanne 1992). In this latter group, experience has been shown to improve foraging performance in honeybees (Dukas 2008; Dukas and Visscher 1994), wasps (O'Donnell and Jeanne 1992), bumblebees (Durisko *et al.* 2010; O'Donnell *et al.* 2000) and ants (Johnson 1991), and efficiency in transporting items between nest sites in ants (Langridge *et al.* 2008). Not all behaviours improve through learning and experience, however; honeybee undertakers show no improvements in efficiency during their lifetime (Trumbo and Robinson 1997). The benefits of learning and experience may only be applicable to tasks for which multiple cues can inform behaviour. Improvements with foraging experience in wild honeybees are not replicated when foraging from a feeder, where fewer cues may be necessary to perform the task well (Dukas 2008). Identifying cues associated with worker-laid eggs may be sufficiently complex that it can benefit from learning, and the high-costs associated with mistakes in policing (Keller 1997) would make improvements beneficial. However, to my knowledge the influence of learning and experience on policing behaviour has not been investigated.

5.2.3 Aims and Hypotheses

Here, I provide the first test of the hypothesis that experience influences an individual's propensity, ability and / or speed at policing illicit egg-laying, using the ponerine ant *Dinoponera quadriceps*. Like several other ponerine species, *D. quadriceps* is queenless, meaning that queen caste has been lost, and the reproductive role replaced by workers (Peeters 1991). Colonies are headed by a single, mated female (alpha), followed by a short, linear hierarchy of subordinates, who queue to replace her (Monnin and Peeters 1998, 1999). *D. quadriceps* represents a good model-system for studying worker policing, as subordinate reproduction is likely to be an important factor influencing colony productivity, and policing may be crucial for maintaining group cohesion. It has previously been shown that in about 40% of colonies, high-ranking workers will develop their ovaries and attempt to cheat by laying unfertilized, male eggs (Monnin and Ratnieks 2001), as expected in a

monogynous, monandrous colony. These eggs are detectable by the alpha and other high-ranking workers because of their cuticular hydrocarbon profile (Tannure-Nascimento *et al.* 2009), and most worker-laid eggs are policed (Monnin and Ratnieks 2001). Therefore, individual differences in ability or propensity to police may relate to dominance rank in this species, but it is not clear to what extent past experience influences policing in these ants. I test the hypotheses that (1) certain individuals in *D. quadriceps* specialise in egg policing and that (2) police improve in propensity and / or speed of policing with increasing experience of illicit eggs. To test these hypotheses I investigate the influence of experience on policing behaviour in *Dinoponera quadriceps* at two temporal scales, utilizing both artificial introductions and naturally occurring worker reproduction. Task performance improves with experience for many colony tasks (Dukas and Visscher 1994; Durisko *et al.* 2010; Johnson 1991; O'Donnell and Jeanne 1992), but remains unexplored for egg-policing behaviour.

5.3 Methods

5.3.1 Experimental Set Up

Eleven colonies of *Dinoponera quadriceps* were collected in Sergipe, Brazil in 2009 and 2010, and housed at 26-29°C, 70-90% relative humidity and a 12:12 light: dark cycle. Colonies were housed in plastic containers (38cm x 58cm x 18cm), containing a small plastic nest chamber (33cm x 19cm x 11cm), divided into 6 compartments by a cardboard divider. Colonies were fed *Tenebrio* mealworms and banana three times a week, corned beef once a month, and provided with water *ad libitum*. To allow individual identification, all ants were tagged with a small numbered tag using a resin glue (E.H. Thorne Ltd).

Foreign egg introductions were performed with 10 colonies in 2010 and 2011, using eggs removed from 18 donor colonies. Donor eggs were randomly selected for each trial, and donor colony and egg age were controlled for statistically in later analyses. Non-natal eggs were used in this experiment as a proxy for worker-laid eggs, because worker-laid eggs could not be obtained in sufficient numbers, and previous studies have shown that non-natal eggs are detected by *D. quadriceps* workers, in the same

way as worker-laid eggs, based on their cuticular hydrocarbon (CHC) profile (Monnin and Peeters 1997; Tannure-Nascimento *et al.* 2009). All discriminator colonies had eggs, larvae and pupae present in their colony at the time of the experiment. Eggs were removed from donor colonies, and checked under the microscope for viability. Eggs were assumed viable if they had no visible damage (such as indentation) or dark marks on their surface. After collection, eggs were stored in petri dishes, with a small piece of wet cotton wool to maintain humidity. This appears to be a suitable environment for egg storage since some of the collected eggs developed into larvae during the storage period. Time from egg removal from the donator colony to egg introduction was recorded and included in statistical models.

For seven colonies, 25 egg introductions were performed across 5 consecutive days, and sample size was supplemented with a further four colonies, tested using 15 introductions over 3 days. During each trial, a foreign egg was introduced into the nest area of a discriminator colony. Eggs were selected randomly from donor colonies. The identity of all workers who antennated or picked up the egg was recorded, and the identity of any worker who policed the egg (either by eating it or by removing it from the nest and placing in the foraging area or waste pile) was recorded. The time at which each individual first antennated an egg, as well as the time at which it policed or accepted the egg, was also recorded. Observations began immediately following the introduction and continued until the egg was policed, or for a maximum of 30 min.

5.3.2 Determining Rank and Identifying Subordinate Cheaters

The rank of all colony members was determined using behavioural observations, similar to those described by Monnin and Peeters (1999). Colonies were observed for 30-minute periods, during which the nature of any aggressive interactions was recorded. For each aggressive interaction observed, the type of interaction and the identity of the actor and recipient were recorded. Each colony was observed for a minimum of 150 minutes. Dominance hierarchies were constructed for each colony, based on the method described in Monnin and Peeters (1999). Individuals were assigned to one of three categories, high-, medium- or low-rank. High-rankers are involved in frequent, high intensity dominance interactions, whilst medium-rankers

are involved in aggression less frequently, and low-rankers are rarely involved in any aggression. Following the policing experiment, all individuals were sacrificed and ovary dissections were performed in order to confirm the identity of the alpha and to determine the ovarian development of subordinate workers. Alpha females were identifiable by having 8 – 10, fully developed, yolky oocytes, whereas reproductively active subordinates had fewer (2 – 6) partially developed oocytes.

5.3.3 Statistical Analysis

In one colony, none of the introduced eggs were policed, and this colony was not included in individual-level analyses. Data were analysed using a linear mixed effects model approach in R. Binomial models were created to investigate whether rank, introduction number, proportion of previous eggs policed or time since last foreign egg encounter had an effect on whether or not an individual policed the current egg. Explanatory variables applicable only to an individual's second egg encounter and onwards (e.g. proportion of previous eggs policed) were applied in one model, whilst those applicable to all encounters (e.g. rank) were applied in a second. Colony and individual number were included as random effects, and encounter number included as a repeated measure. Donor colony and egg age were also included in the models.

Additionally, linear mixed effects models with normal distributions were created for time taken to process an egg (seconds from first antennation to policing or acceptance). Explanatory variables were identical to those described above. These explanatory variables were also tested on the type of response to an egg (ignore, accept, eat or waste). Data for colonies undergoing 25 introductions were analysed separately from those undergoing 15 introductions. The effect of rank on the proportion of eggs policed by an individual across all introductions was also investigated using a general mixed effects model with a quasipoisson error structure, including colony as an explanatory variable. Data from colonies undergoing both 25 and 15 introductions was included in this analysis. Additionally, pairwise t-tests for each rank were performed for the proportion of egg encounters policed by each individual. Rank comparisons were performed both considering the gamergate separately from the other ranks, and including her within the high-rankers. P-values

were corrected for multiple comparisons using a bonferroni correction. The relationship between an individuals' choice to police on a given encounter and the time taken to make that decision, was investigated using a normally distributed linear mixed effects model, with colony and individual included as random effects.

Finally, the relationship between the presence of cheaters (reproductively active subordinates) was investigated for three response variables: processing time, response type and, at the colony-level, proportion of introduced eggs policed, using a linear mixed model approach. A Spearman's rank correlation was calculated for the relationship between proportion of eggs policed and proportion of reproductively active subordinates. Data for these analyses was pooled from both the 25-introduction and 15-introduction datasets.

5.4 Results

Mean colony size for the 11 colonies used in this experiment was 39.9 ± 9.18 individuals. A total of 250 individuals encountered a foreign egg at least once, with an average of 3.77 ± 0.228 encounters per ant. Across 210 trials, an average of 39.5% ± 8.69 of introduced eggs were policed, but there was considerable variation across colonies in this (range 0% - 92%).

5.4.1 Specialisation

Only 19.6% of individuals who encountered at least one foreign egg during the experiment ever policed an egg. Further, 56.6% of all policing events were performed by 13 individuals who policed at least 2 foreign eggs, representing just 5.2% of all individuals who ever encountered a foreign egg (*figure 5.1*).

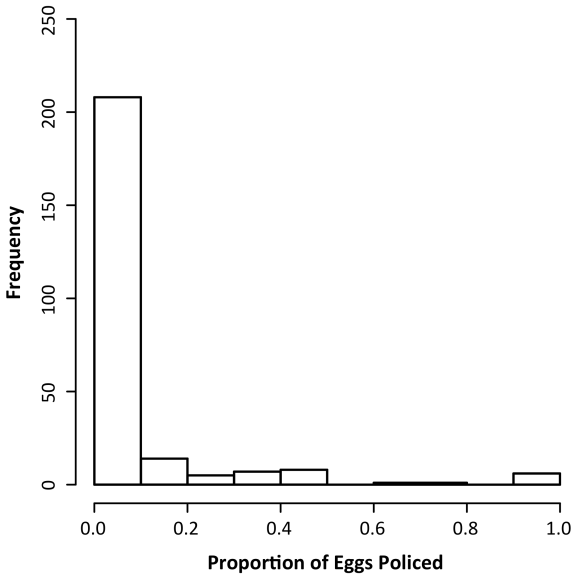


Figure 5.1 Policing in Dinosaur Ants

Histogram showing the number of individuals (frequency) policing different proportions of encountered foreign eggs for 10 colonies, across all egg introductions.

Individuals who had policed a higher proportion of their previous egg encounters were more likely to police their current encounter ($\chi^2_{23,250}$ = 70.7, p < 0.0001, *figure 5.2a*). Speed of policing decisions was also predicted from an individual's past behaviour; the proportion of previous egg encounters policed had a significant effect on the time taken to process a foreign egg for eggs that were policed ($\chi^2_{24,60}$= 95.3, p < 0.0001, *figure 5.2b*) but not for eggs that were not policed ($\chi^2_{24,649}$= 0.68, p = 0.409). The type of response to foreign eggs (eat, removed to waste, accept, ignore) was significantly related to an individual's past behaviour, as measured by the proportion of previous egg encounters that an individual policed ($\chi^2_{24,546}$= 275.1, p < 0.0001, *figure 5.2c*). Mean proportion of past encounters that were policed was highest for individuals who removed eggs to the waste pile (mean = 0.40 ± 0.25), and lowest for individuals who ignored eggs (mean = 0.08 ± 0.001).

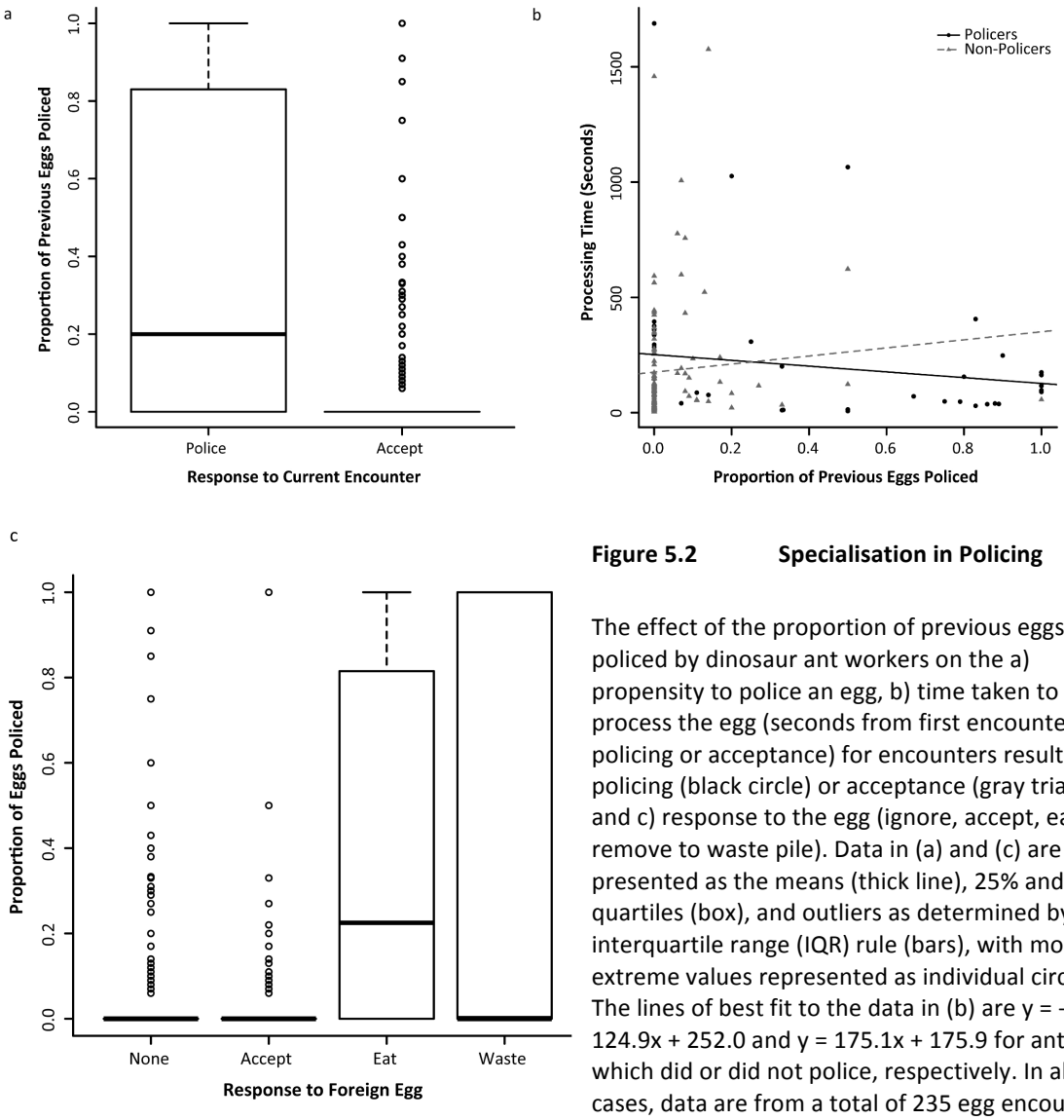


Figure 5.2 Specialisation in Policing

The effect of the proportion of previous eggs policed by dinosaur ant workers on the a) propensity to police an egg, b) time taken to process the egg (seconds from first encounter to policing or acceptance) for encounters resulting in policing (black circle) or acceptance (gray triangle), and c) response to the egg (ignore, accept, eat or remove to waste pile). Data in (a) and (c) are presented as the means (thick line), 25% and 75% quartiles (box), and outliers as determined by the interquartile range (IQR) rule (bars), with more extreme values represented as individual circles. The lines of best fit to the data in (b) are y = -124.9x + 252.0 and y = 175.1x + 175.9 for ants which did or did not police, respectively. In all cases, data are from a total of 235 egg encounters, involving workers from 10 colonies.

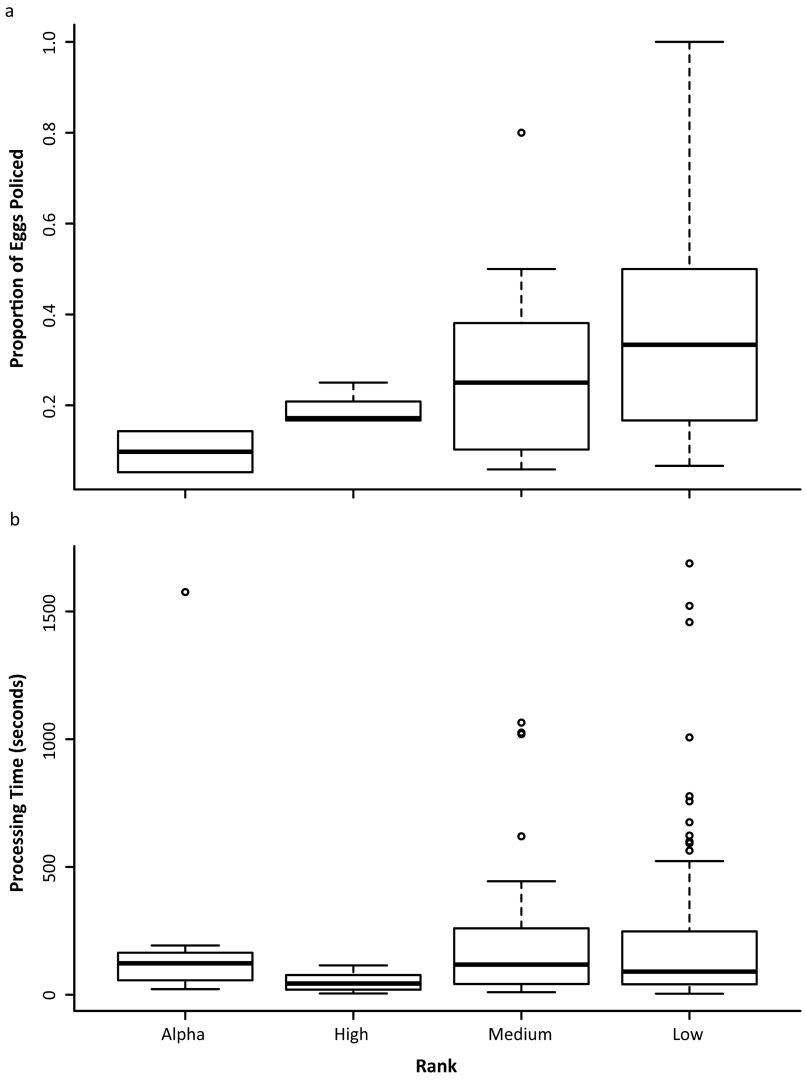


Figure 5.3 Rank and Policing

The effect of dominance rank (alpha, high-, medium- and low) on a) The proportion of foreign eggs policed across 25 foreign egg introductions and b) Time taken to process the foreign egg (seconds from first encounter to policing or acceptance).

I investigated whether individual specialisation in policing related to dominance rank. Rank had no effect on propensity to police the current egg encounter ($\chi^2_{44,709}$ = 5.199, p = 0.158). There was significant effect of an individual's rank on the proportion of foreign eggs policed ($\chi^2_{240,546}$=2.701, p = 0.03), although this was not significant when the gamergate (alpha) was considered separately from other high-rankers ($\chi^2_{240,546}$=2.7275, p = 0.08). However, a pairwise t-test revealed that there was a statistically significant difference in policing rates between high- and low-rankers (t_{188}= 3.0404, p = 0.008 (Bonferroni corrected)), with high-rankers policing 1.9% of introduced eggs compared to 8.3% in low-rankers (*figure 5.3a*). All other pairwise comparisons were non-significant after bonferroni correction. There was also a marginally non-significant effect of rank on processing time for policed eggs ($\chi^2_{37,60}$= 7.06, p = 0.007, *figure 5.3b*), with low-rankers spending 32% longer processing eggs than high-rankers (Mean high-ranker = 137.3 ± 63.5 seconds, mean low-ranker = 201.2 ± 34.15 seconds). Rank

influenced response type ($\chi^2_{44,709}$= 9.77, p = 0.021) through an increased frequency of policing behaviours (egg eating / removal to waste pile) in medium- and low-rankers.

5.4.2 Experience

The decision to police a given foreign egg encounter was not influenced by the number of previous egg introductions, number of previous egg encounters or time since last egg encounter. An effect of introduction number was found, however, for the first 15 introductions ($\chi^2_{12,337}$= 17.08, p < 0.0001) with individuals showing a higher propensity to police in later introductions. Further, the speed of decision-making about whether to police a foreign egg was affected by previous experience; there was a significant effect of number of previous egg encounters ($\chi^2_{48,60}$= 30.47, p = 0.0037, *figure 5.4a*) on the time taken to process a foreign egg, for eggs that were policed. As previous experience of foreign eggs increased, so did processing time. However, this effect was not present when both policed and accepted eggs were included in the model ($\chi^2_{55,709}$= 12.04, p = 0.7409). There was also significant effect of number of previous encounters on the response to a foreign egg ($\chi^2_{44,709}$= 29.55, p = 0.042, *figure 5.4b*) with individuals who had encountered more foreign eggs previously being more likely to accept or eat an egg rather than ignore it or take it to the waste. Introduction number was found to influence processing time, however only for the 15-introduction dataset ($\chi^2_{27,337}$= 14.46, p = 0.0001) with more rapid processing of foreign eggs as introduction number increased (*appendix A3*). There was no significant difference in processing time for eggs that were policed or those that were not ($\chi^2_{49,709}$= 1.15, p = 0.2839). Of the ten colonies for which both policing and ovarian activity information was collected, 5 colonies contained at least one reproductively active subordinate cheater, and 4 of these contained 2 or more cheaters. These cheaters represented between 0.95% and 27% of the total colony population (mean = 13.2% ± 5.2%). The presence of reproductively active subordinates in these colonies appeared to influence policing behaviour. There was a significant difference in the mean proportion of eggs policed for colonies with or without cheaters ($F_{1,9}$= 10.78, p = 0.0111), with cheated colonies policing an average of 54.4 % ± 11.5 of foreign eggs compared to just 12.0% ± 5.9 for uncheated colonies (*figure 5.5a*).

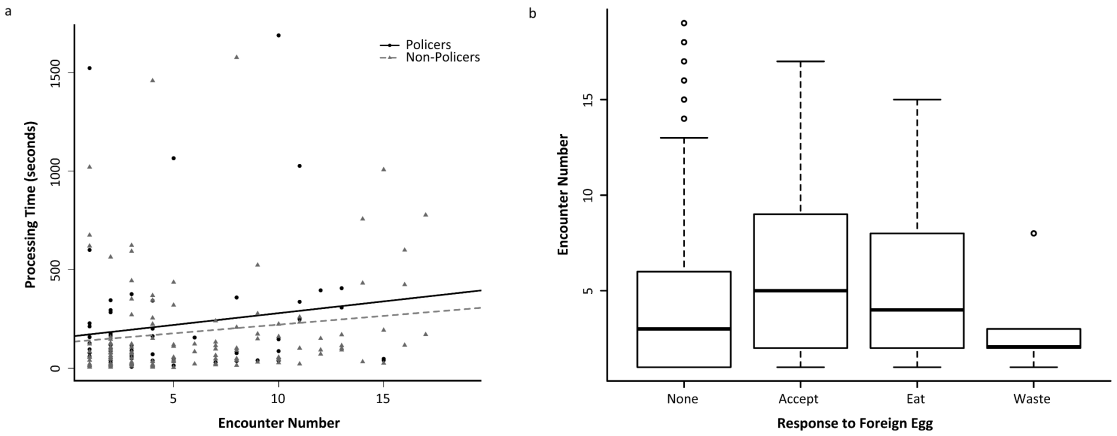


Figure 5.4 Short-Term Experience and Policing

The effect of the number of previous foreign egg encounters experienced during 25 egg introductions on a) The time taken for an individual to process the foreign egg (seconds from first encounter to policing or acceptance) for encounters resulting in policing (black circle) or acceptance (gray triangle) and b) Response to current foreign egg encounter (ignore, accept, eat or remove to waste pile). The lines of best fit to the data in (a) are y = 11.95x + 159.95 and y = 8.84x + 132.91 for ants which did or did not police, respectively.

Furthermore, there was a strong positive correlation between the proportion of eggs policed by a colony and the proportion of high-ranking subordinates attempting to cheat (s = 55.13, rho = 0.666, p = 0.035; *figure 5b*). A significant interaction was found between number of previous encounters and the presence of cheaters on time taken to process foreign eggs ($\chi^2_{20,709}$= 10.33, p = 0.01597), with increasing processing time as encounter number increases for cheated but not uncheated colonies (*figure 5.5c*). Response type (eat, waste, accept, ignore) was significantly influenced by the presence of cheaters ($\chi^2_{49,709}$= 18.047, p < 0.0001), and the proportion of the colony cheating ($\chi^2_{49,709}$= 8.2404, p = 0.0030). This likely relates to increased frequency of policing in general in these colonies, although policing by waste removal was slightly more common in uncheated colonies.

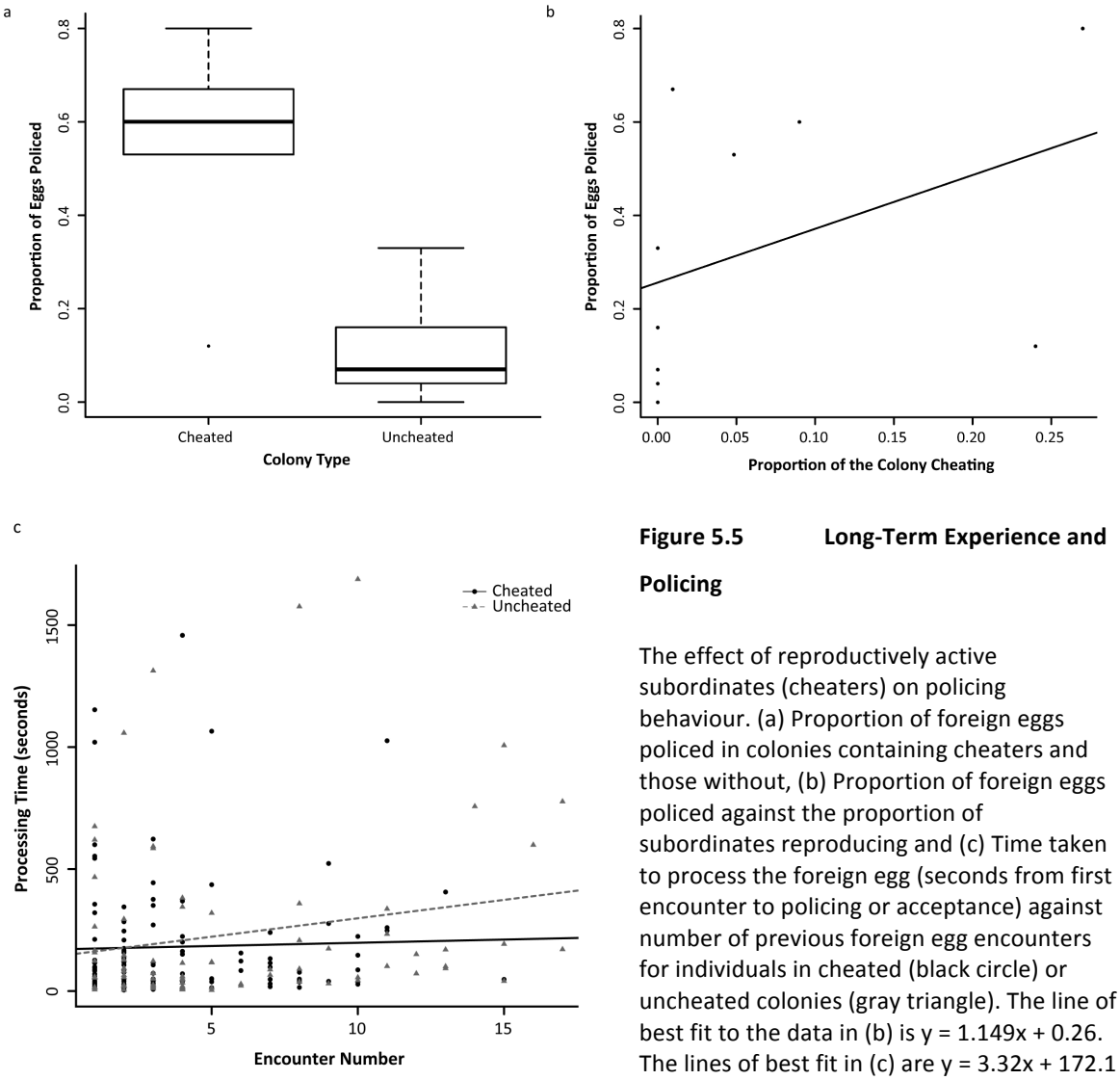


Figure 5.5 Long-Term Experience and Policing

The effect of reproductively active subordinates (cheaters) on policing behaviour. (a) Proportion of foreign eggs policed in colonies containing cheaters and those without, (b) Proportion of foreign eggs policed against the proportion of subordinates reproducing and (c) Time taken to process the foreign egg (seconds from first encounter to policing or acceptance) against number of previous foreign egg encounters for individuals in cheated (black circle) or uncheated colonies (gray triangle). The line of best fit to the data in (b) is y = 1.149x + 0.26. The lines of best fit in (c) are y = 3.32x + 172.1 and y = 21.55x + 88.34 for ants in cheated and uncheated colonies, respectively.

5.5 Discussion

I demonstrate a role of experience in determining policing behaviour in the queenless ponerine ant, *Dinoponera quadriceps*. Individual experience influenced policing behaviour on two temporal scales resulting in individual specialisation and learned increases in cautiousness from past experience. Short-term experience was measured through the effect of successive introductions of foreign eggs; the behaviour of individuals was influenced by their previous encounters with foreign eggs introduced during this experiment. Individuals who had policed more eggs previously were more likely to police again, with a small subset of the colony specializing in policing. Long-term experience was investigated through the impact of resident, naturally occurring

cheaters (reproductively active subordinates) on responses to the introduction of foreign eggs. Cheaters (reproductively active subordinates) occurred in 50% of colonies, consistent with previous reports of the prevalence of worker laying in this species (Monnin and Ratnieks 2001). Workers in colonies with reproductively active subordinates were more likely to police foreign eggs, and past experience of cheating increased individual cautiousness and colony-wide policing response. Together, these data suggest that experience and learning play an important role in egg policing behaviour.

5.5.1 Long-Term Exposure to Illicit Eggs

Policing was significantly more frequent in colonies that were exposed to worker-laid eggs naturally, as indicated by the presence of subordinates with partially developed ovaries. Workers in colonies containing reproductively active subordinates policed 42% more foreign eggs than those without cheaters, and policing rates were low in uncheated colonies. This supports a key role of experience in determining policing behaviour as individuals in cheated colonies would have been exposed to illicit egg laying prior to this experiment, representing greater temporal and numerical exposure to unwanted eggs. Furthermore, a strong positive correlation between the proportion of subordinate cheaters and proportion of introduced eggs policed indicates that the extent of previous experience plays a key role in determining policing behaviour in this species. The speed with which foreign eggs were processed for policing was significantly influenced by past experience as measured by the number of previous encounters with a foreign egg. It might be expected that individuals would improve in the speed of their response to foreign eggs with increased experience, however my results indicate the opposite: processing time increased with number of previous eggs encountered. This relationship was not significant for egg encounters which resulted in egg acceptance, however, despite a similar trend. Both foreign eggs and subordinate-laid eggs are likely to be detected using cuticular hydrocarbons (D'Ettorre *et al.* 2006; Tannure-Nascimento *et al.* 2009). Thus, this relationship may be due to the introduction of a relatively large number of non-natal eggs confusing or diluting the chemical cues used in egg recognitions (D'Ettorre *et al.* 2004; D'Ettorre *et al.* 2006; Endler *et al.* 2004; Monnin and Peeters 1997)

However, a significant interaction between encounter number and the presence of cheaters was found for processing time, suggesting that slower processing might represent an adaptive increase in cautiousness to avoid error. Increases in processing time with increasing experience were only found for colonies without cheaters, suggesting that an initial increase in cautiousness when encountering eggs may occur following the first appearance of foreign eggs in a colony. Colonies where workers frequently encounter non-queen-laid eggs may benefit from extra caution in investigating eggs, as mistakes in policing are likely to be very costly (Keller 1997). Experience has been shown to affect task performance in a range of taxa (Dukas and Visscher 1994; Durisko *et al.* 2010; Helton 2007; Johnson 1991; O'Donnell and Jeanne 1992; Yoerg 1994); usually learning results in increased speed, efficiency, accuracy or success at a particular task. However, specialisation does not always equate to improved task performance (Dornhaus 2008), and in this case experience of illicit egg-laying appears to increase cautiousness in potential policers. Increased cautiousness appears to be a general strategy for all eggs, as there was no significant difference in processing time for eggs that were eventually policed compared to those that were not policed. My results indicate that experience influences policing behaviour over multiple temporal scales.

5.5.2 A Specialist Police Force

The finding that individuals who policed previously were more likely to police again suggests that individual specialisation in policing behaviour occurs in this species. Task specialisation for policing has previously been reported (Van Zweden *et al.* 2007) and increased specialisation is associated with improved task performance in some species (Chittka and Thomson 1997), although this is not always the case (Dornhaus 2008). Specialisation may relate to dominance rank, as my data showed that low-ranking workers policed a larger proportion of eggs than high-rankers. Interestingly, this contrasts with previous work, which indicated an important role for high-ranking workers in egg policing in *D. quadriceps* (Monnin and Peeters 1997; Tannure-Nascimento *et al.* 2009). In natural colonies, worker-laid eggs are generally policed by the alpha (Monnin and Peeters 1997), and in laboratory assays high-ranking workers

make fewer mistakes when differentiating between natal- and non-natal eggs (Tannure-Nascimento *et al.* 2009). However, by placing foreign eggs in the nest area but not directly on the egg pile in this study, may have afforded lower-ranking subordinates the opportunity to encounter and police eggs they may not normally have contact with, and low-rankers in this study spent longer assessing foreign eggs indicating that they may be less adept at discrimination or more cautious in doing so. Importantly, my manipulations have revealed that workers of all rank are capable of detecting and removing foreign eggs when the opportunity arises.

5.5.3 Positive Feedback

Policing behaviour in *D. quadriceps* may represent a positive feedback system, in which individuals who encounter and police a foreign egg become more likely to do so again in the future. This is consistent with previous investigations of specialisation in policing behaviour in another ponerine ant, *Pachycondyla inversa*, where high skew in propensity to police has been found amongst workers, unrelated to an individuals' own ovarian activity (Van Zweden *et al.* 2007). Furthermore, in the ant *Cerapach biroi*, initial foraging success is a strong determinant of future propensity to forage, supporting a role for positive feedback in influencing task performance (Ravary *et al.* 2007). Under natural circumstances, a positive-feedback system would be likely to result in high-rankers performing the most egg policing, because worker-laid eggs would be found in the egg-pile, where high-rankers spend most of their time (Asher *et al.* 2013; Monnin and Peeters 1999). The type of response to a foreign egg (ignore, accept, eat, waste) was influenced by an individual's rank; only low- and medium-rankers policed eggs by waste removal, suggesting that this behaviour may represent an extension of these individuals' normal behavioural repertoire, as medium- and low-rankers are commonly responsible for waste removal (Asher *et al.* 2013). Furthermore, by preferentially eating illicit eggs, high rankers can avoid the risk of encountering pathogens outside the nest. This type of positive feedback mechanism is likely to be mediated through individual reinforcement of response thresholds (Page and Mitchell 1998; Theraulaz *et al.* 1998), as shown by social insects in other contexts (Jeanson *et al.* 2008; Weidenmüller 2004), and past experience determining future behaviour

resulting in task specialisation has previously been demonstrated for foraging behaviour in ants (Chittka and Muller 2009).

5.5.4 Learning and Memory in Insects

Over the short-term, no effect of time between foreign egg encounters was found for any of the variables measured, suggesting that any effects of learning that occur from encountering and / or policing eggs are maintained over a period of several days. The data collected during the 25-introduction trials covered a period of 5 days, and the mean time between encounters was 15.8 ± 0.79 hours. Few studies have investigated the duration of memory in insects, and it appears to be highly variable, with some learned behaviours lasting days and others a lifetime (Huigens *et al.* 2009; Johnson *et al.* 1994; Sheehan and Shelton 1989). In general, memory tends to last longer for species and behaviours in which the environment (social or abiotic) is more predictable (Huigens *et al.* 2009; Johnson *et al.* 1994).

5.5.5 Relatedness, Sex Discrimination and Colony Efficiency

Relatedness may provide an explanation for the occurrence of policing in this species, as colonies of *D. quadriceps* are both monogynous and monandrous, and thus workers are more closely related to the queen's offspring (r = 0.5) than to worker-laid male eggs (r = 0.375)(Mehdiabadi *et al.* 2003). Sex discrimination of brood is unlikely to be possible at the egg-stage (Nonacs and Carlin 1990; Passera and Aron 1996), and thus workers should be expected to police worker-laid eggs despite being more closely related to worker-laid than queen-laid male eggs (r = 0.25)(Mehdiabadi *et al.* 2003). Furthermore, an inability to distinguish male and female brood may provide incentives to police worker-laid male eggs as a means of controlling the sex-ratio of brood whilst reducing the loss of investment caused by removing older brood at a stage when sex determination is possible (Foster and Ratnieks 2001a). Workers can be certain that any worker-laid eggs will be male, since males show no interest in mating with subordinates in this species, who are therefore incapable of laying fertilized (female) eggs (Monnin and Peeters 1998). Worker policing has now been documented in a variety of Hymenopteran species in which workers are more closely related to worker-laid than queen-laid male eggs (Foster and Ratnieks 2001a) including other

monandrous ponerine ants (D'Ettorre *et al.* 2004; Kikuta and Tsuji 1999) and even in some extreme cases where workers are able to clonally produce females (Hartmann *et al.* 2003). However, there are also many examples in which worker policing does not occur (Endler *et al.* 2007; Moritz *et al.* 1999). The mechanisms driving policing likely represent a combination of relatedness, life history and ecological factors. Policing could be favoured regardless of relatedness benefits if worker-reproduction causes a reduction in colony productivity (Ratnieks 1988). The loss of colony productivity caused by worker reproduction in *D. quadriceps* may be minimal since illicit reproduction is generally restricted to high-ranking workers who contribute relatively little to colony tasks anyway (Monnin and Peeters 1999). However, widespread worker reproduction could disrupt colony stability and challenge the dominance of the alpha.

5.5.6 Concluding Remarks

Together, these results suggest that through behavioural plasticity and individual specialisation, dinosaur ant colonies are able to take advantage of the benefits of learning and experience, and maximise colony productivity depending upon the social environment. In colonies without cheating subordinates, the costs of time-consuming egg-checks are avoided, but individuals quickly become more prudent in their assessment of eggs as they encounter interlopers. Whether learning plays a role in social cohesion in other eusocial species remains to be tested.

Chapter 6

Differential Gene Expression relates to Dominance Rank and Division of Labour

6.1 Abstract

Phenotypic plasticity is a key characteristic of most organisms, enabling them to adapt to variation in biotic and abiotic conditions. Phenotypic plasticity is partly mediated through differential expression of shared genes in response to external stimuli, and may be discrete (polyphenism) or continuous (reaction norms). Social insect castes are a classic example of phenotypic plasticity, with different morphological and behavioural castes generated from a shared set of genes. Here I present the first investigation of the transcriptional regulation of division of labour in the queenless ponerine ant, *Dinoponera quadriceps*. Ponerine ants have a secondarily derived primitive social structure and can therefore yield powerful insights into the role of evolutionary history in caste determination and behavioural plasticity. I find that only 5% of genes differ significantly in expression between the brains of dinosaur ants of different rank, reproductive physiology and foraging effort. The greatest differences observed relate to reproductive physiology and differentiate the alpha female from her subordinates. *D. quadriceps* appears to be characterised by a discontinuous social hierarchy, and a great deal of transcriptional diversity may have been lost along with the reversion from a morphologically distinct queen caste to reproductive workers.

6.2 Introduction

Phenotypic plasticity is a fundamental trait of most organisms, better adapting them to variable environments, both biotic and abiotic. Phenotypic plasticity occurs when different phenotypes are generated from the same genotype, through interaction with external stimuli (Evans and Wheeler 2001; Hall 2003; Simpson *et al.* 2011; West-Eberhard 1989; West-Eberhard 2003). Classic examples include sexual dimorphism, cell differentiation in multicellular organisms, seasonal morphs, and castes in social insects (Fusco and Minelli 2010; West-Eberhard 1989; West-Eberhard 2003; Whitman and

Agrawal 2009). Social insects exhibit remarkable phenotypic plasticity; within a colony, a single genotype can produce queens, workers or soldiers, which differ in morphology, physiology and behaviour (Holldobler and Wilson 1990; Wilson 1974). Further, many social insects exhibit behavioural plasticity within castes often generating further division of labour (Holldobler and Wilson 1990; Wilson 1974).

6.2.1 Caste Determination and Behavioural Plasticity

A single genotype can give rise to fixed, discreet polyphenisms (e.g. antlers in red deer), or to more flexible, continuous reaction norms (e.g. temperature and nutritional effects on body size) (Fusco and Minelli 2010; Roff 1996; West-Eberhard 1989; Whitman and Agrawal 2009). Reaction norms describe phenotypic plasticity that varies as a continuous function of the environmental signal (Woltereck 1909). Morphologically distinct castes in social insects (e.g. queens, soldiers) are an example of polyphenism, with caste being nutritionally determined during larval development and fixed for life (Bell *et al.* 1974; Michener 1974; Wilson 1974). In these species, caste is controlled by response thresholds, whereby the 'switch' from one caste to another during development is achieved only when a specific environmental stimulus is exceeded (Bonabeau *et al.* 1996, 1998; Theraulaz *et al.* 1998).

Discreet, morphologically-adapted castes are the hallmark of advanced eusociality (e.g. honeybees), however in eusocial species with simpler societies, reproductive and worker roles are plastic in adulthood, and differentiation between worker behaviour appears to be more continuous (Sherman *et al.* 1995). Therefore, while morphological and behavioural castes in advanced societies can meaningfully be described as polyphenisms, it may be more fruitful to view simple societies as flexible, continuous reaction norms. Despite this, little attention has been paid to whether castes in social insect societies, particularly simple societies, truly represent polyphenisms or continuous reaction norms.

6.2.2 Decoupling of Ancestral Traits

The reproductive ground plan hypothesis suggests that during the evolution of eusociality, the full task repertoire of the solitary ancestor became decoupled into

reproduction (queens) and provisioning (worker) phenotypes (Johnson *et al.* 2010; West-Eberhard 1987). In many solitary species, these behaviours may be temporally decoupled, often existing in cycles of oviposition and foraging, and the mechanisms underlying this cyclic behaviour may have provided the foundation for the evolution of eusociality (West-Eberhard 1987). Evidence from genome-wide expression studies suggests that reproductive physiology explains a large amount of variation in gene expression (Cardoen *et al.* 2011; Chandrasekaran *et al.* 2011; Ometto *et al.* 2010; Zayed and Robinson 2012) and recent work has indicated distinct gene regulatory networks specific to reproduction and provisioning in paper wasps (Ferreira *et al.* 2013).

6.2.3 The Transcriptional Control of Plasticity

The remarkable phenotypic diversity exhibited by social insect societies arises through differential regulation of a shared set of genes (Evans and Wheeler 2001; Gadagkar 1997; Patalano *et al.* 2012). Sociogenomic research has investigated both the genes involved in regulating caste differentiation and the epigenetic mechanisms that regulate those genes (Chittka *et al.* 2012; Gadagkar 1997; Patalano *et al.* 2012), and has revealed a number of genes relating to individual differences in behaviour, physiology and age (Alaux *et al*. 2009; Ben-Shahar *et al*. 2002; Chandrasekaran *et al*. 2011; Grozinger *et al*. 2007; Haisheng *et al*. 2004; Heylen *et al*. 2008; Ingram *et al*. 2005; Lutz *et al*. 2012; Nelson *et al*. 2007; Shorter and Tibbetts 2009; Sullivan *et al*. 2000; Toth *et al*. 2010). These studies, however, have been limited by the requirement to select genes of interest for study. Increasingly, large-scale genomic and transcriptomic data are allowing us to investigate the intricacies of caste and behaviour in eusocial species, without these biases. Several recent studies have investigated transcriptome profiles of different individuals for advanced species (Bonasio *et al.* 2012; Cardoen *et al.* 2011; Colgan *et al.* 2011), and a few studies have investigated caste-biased gene expression in primitively eusocial species, in which caste is plastic (Bonasio *et al.* 2012; Ferreira *et al.* 2013).

6.2.4 Sociogenomics and the Eusociality Continuum

Primitively eusocial species contain subordinates who retain reproductive totipotency into adulthood (Wilson 2000). Primitive eusociality occurs in lower termites, polistine wasps, and halictid and allodapine bees (Hart and Ratnieks 2005), and additionally, a simple social structure and reproductively totipotent work force occurs secondarily in some species of ponerine ant (Monnin and Peeters 2008; Peeters 1991). Ponerine ants cannot be considered primitively eusocial, as they are descended from an advanced ancestor with a morphologically distinct queen caste (Schmidt 2013). However, among the ponerine ants, some species also have reproductively active workers (known as 'gamergates' (Peeters and Crewe 1984), and in some cases have lost the queen caste entirely. These 'queenless' ponerine ants share many aspects of their social structure with truly primitive species such as the paper wasps and halictid bees (Hart and Ratnieks 2005). The ponerine ants are a polyphyletic group, representing multiple independent evolutionary origins of reproductively active (gamergate) workers (Schmidt 2013). Thus dinosaur ants offer the opportunity to investigate secondarily derived simple society, which can be compared with recent genomic and transcriptomic data for ancestrally primitive societies such as the paper wasp *Polistes Canadensis* (Ferreira *et al.* 2013) and the advanced ponerine ant, *Harpegnathos saltator* (Bonasio *et al.* 2010).

6.2.5 Social and Reproductive Dominance

In many primitively eusocial species and ponerine ants societies are defined by a social hierarchy, which is important in determining reproduction (Bridge and Field 2007; Chandrashekara and Gadagkar 1991; Cronin and Field 2007; Monnin and Peeters 1999; Monnin *et al.* 2003; Pardi 1948). Cooperative mammal societies such as those of mongooses and mole rats also display social hierarchies (Clarke and Faulkes 1997; Creel *et al.* 1992; De Luca and Ginsberg 2001; Doolan and Macdonald 1997). Social hierarchies may relate to size, age or aggression, and in social insects are often also reinforced by cuticular hydrocarbon signalling (Cuvillier-Hot *et al.* 2002; D'Ettorre *et al.* 2004; Peeters *et al.* 1999).

In the dinosaur ant, *Dinoponea quadriceps*, colonies are headed by a single reproductively active worker (alpha), followed by a near-linear dominance hierarchy (Monnin and Peeters 1999). Behaviour is strongly related to rank, with low-rankers performing foraging and nest defence behaviours, and high-rankers (alpha, beta, etc) performing brood care and engaging in aggressive social interactions, which are used to maintain the hierarchy (Asher *et al.* 2013; Monnin and Peeters 1999). Reproductively active subordinates are also found in about 40 – 50% of colonies (Monnin and Ratnieks 2001), however only the alpha female ever mates (Monnin and Peeters 1998). Individuals of different rank therefore differ in behaviour, age and reproductive physiology. *Dinoponera* have an unusual evolutionary history, having recently lost the queen caste (Monnin and Peeters 2008; Peeters 1991; Schmidt 2013). The occurrence of gamergates in *Dinoponera* is thought to have arisen less than 20 MYA (Schmidt 2009), and their social organisation now more closely represents that of primitively eusocial species than species with advanced societies (Peeters 1991; Ratnieks *et al.* 2001). However, whether they are transcriptionally more similar to advanced or primitive species has not previously been investigated.

6.2.6 Aims and Hypotheses

This study aims to reveal how gene expression changes with social rank in the primitively eusocial dinosaur ant, *Dinoponera quadriceps*. Specifically, I address two key aspects of eusociality: (1) the continuity of social hierarchies and the (2) genes that underlie them. Using a combination of behavioural observations, radio-frequency tracking and next generation sequencing, I investigate the transcriptional regulation of division of labour and elucidate the gene expression patterns which are specific to dominance, reproduction and provisioning behaviour. To my knowledge, this is the first study to attempt to directly correlate a continuous behavioural variable (foraging effort) with transcriptome-wide gene expression in a social insect.

(1) I test whether the social hierarchy represent a continuum of gene expression, with little difference between adjacent individuals in the queue, or a discontinuous reaction norm with large differences in gene expression associated with the differences

between subordinates (low-rank) and high-ranking reproductives (alpha-rank) and hopeful reproductives (beta-rank).

(2) In addition, I relate differential gene expression to dominance rank (a measure of aggression), ovarian development and mating status (reproduction) and a continuous measure of individual foraging effort (provisioning), enabling us to tease apart the genes underlying specific social traits that are decoupled in sociality.

6.3 Methods

I determined the dominance rank, ovarian activity and behaviour of 18 individuals from 7 colonies of *Dinoponera quadriceps* (*table 6.1*). I generated the first genome and transcriptome sequence data for *D. quadriceps*, using genomic DNA from a whole male body and mRNA from female brains. Brain samples were sequenced individually, and analysed in relation to rank, ovarian development and foraging behaviour.

6.3.1 Contributions

The data presented in this chapter was generated in collaboration with Heinz Himmelbaur's lab at the Centre for Genomic Regulation (CRG), University of Barcelona, and with Afsaneh Maleki at the University of Sheffield. Genome sequencing and assembly was performed by Anna Ferrer Salvador, André Minoche, and Francisco Câmara Ferreira at CRG. The PASA assembly was created by Pedro Ferreira. Transcriptome sequence data was assembled and readcounts normalized by AM. Functional annotation of the transcriptome was performed by AM, Anna Vlasova and CA. Statistical analyses on all gene expression data, as well as Behavioural observations and RFID monitoring, were performed by CA.

6.3.2 Husbandry

Colonies of *Dinoponera quadriceps* were collected from Altantic forest in Sergipe (S11°01'23,W37°12'9) and Campo Formoso (S10°26'972, W40°20'771), Brazil in 2009, '10 and '11. Colonies were housed in plastic containers (38cm x 58cm x 18cm) containing a plastic nest chamber (33cm x 19cm x 11cm) divided into 6 compartments,

at 26-29°C, 70-90% relative humidity and a 12:12 light: dark cycle. Colonies were provided with food (*Tenebrio* mealworms, corned beef, banana) and water. All ants were tagged with a small unique number tag (E.H. Thorne Ltd). For those colonies in which RFID monitoring was performed (*section 6.2.3*), an RFID tag was attached to the thorax of the ant and the number tag affixed to the RFID tag.

Table 6.1 Transcriptome Sequencing Samples

D. quadriceps samples taken for transcriptome sequencing. Whole brains were removed from individual females and transcripts sequenced using Illumina sequencing. Table presents the unique identification code for each ant, the colony, year of collection and colony size at the time of behavioural observations. Further, the rank, mating status and ovarian activity of each ant is listed. Finally, the means of behavioural data collection (observation or RFID) is shown along with the estimated number of trips per day and percentage of time spent outside for each ant.

Ant	Colony	Collection Year	Colony Size	Rank	Mated	Ovarian Activity	Obs. Method	Trips Per Day	% Time outside
2B84	2a	2010	12	Alpha	Yes	Developed	Behaviour	1.488	2.0
2CAL				Beta	No	Undeveloped		NA	NA
2B82				Low	No	Undeveloped		0.844	1.0
12Y30	12a	2010	24	Alpha	Yes	Developed	Behaviour	0.000	0.0
12Y47				Beta	No	Undeveloped		0.000	0.0
12Y51				Low	No	Undeveloped		0.000	0.0
10G48	10b	2011	80	Alpha	Yes	Developed	RFID	0.000	0.0
10G87				Beta	No	Undeveloped		0.000	0.0
10G17				Low	No	Undeveloped		4.302	38.4
23Y79	23b	2011	14	Alpha	Yes	Developed	RFID	0.443	38.0
23Y70				Beta	No	Developed		1.604	9.1
23Y59				Low	No	Undeveloped		14.51	56.3
36W26	36b	2011	19	Alpha	Yes	Developed	RFID	1.558	17.6
36W62				Beta	No	Developed		0.000	0.0
36W35				Low	No	Undeveloped		0.000	0.0
10B70	10a	2010	37	Alpha	Yes	Developed	Behaviour	2.664	8.0
35Y87	35b	2011	68	Alpha	Yes	Developed	None	NA	NA
35Y44				Low	No	Undeveloped		NA	NA

6.3.3 Dominance Hierarchies

The dominance hierarchy in *D. quadriceps* is maintained by frequent ritualised aggressive interactions between high ranking workers (Monnin and Peeters 1999). These 'dominance interactions' have been categorised into 6 types and can be reliably used to determine dominance rank, which is correlated with ovarian activity (Peeters *et al.* 1999). The dominance rank of all individuals was determined during 30-minute behavioural observations totaling 33 hours (mean = 4 hrs 43 mins ± 1 hr 10 mins per colony), recording the type of interaction and the identities of the actor and recipient

for all aggressive interactions. Dominance hierarchies were then constructed for each colony. High-rankers were assigned a precise linear rank, whilst the remaining colony members were assigned to coarse-scale hierarchical categories: medium- and low-rank, based on the method developed by Monnin and Peeters (1999).

6.3.4 Radio Frequency Identification (RFID)

Radio frequency identification tagging was used to monitor the movements of all colony members for three colonies (N = 113, mean colony size = 37.67 ± 21.21). Each ant was tagged using passive RFID, 16 bit programming mode [GiS TS-Q5Bee Tags], 18, 6 x 3 x 2 mm) encoded with unique 4-digit identification numbers, as well as a small unique number tag (E.H. Thorne Ltd). Nests were monitored for a minimum of 5 days continuously (11.33 ± 3.76 days per colony). During RFID monitoring, colonies were housed in small plastic nest boxes, joined to a larger foraging area by a plastic tube. Two RFID antennae were placed around each tube, to monitor movement of individuals from the nest to foraging area. Using two antennae enabled the direction of movement to be determined. The antennae were placed at east 6cm apart, to avoid any overlap in their field of detection. The relative timing of records on each antennae could reveal both the direction of movement (which antennae detected the ant first) as well as discriminating occurrences of ants moving part way through the tunnel and then immediately returning.

RFID data was manipulated using *R* (*appendix A4.1*). The duration of each foraging trip was calculated and the total time spent foraging was calculated for each individual. This was converted into two measures of foraging behaviour for which correlations with gene expression were performed; number of trips per day and the percentage of time spent outside the nest. RFID data for each sequenced individual was additionally checked by hand in order to confirm the accuracy of data included in gene expression analyses.

6.3.5 Behavioural Observations

Behavioural observations were performed on three colonies (N = 73, mean colony size = 24.33 ± 7.22; *Table 6.1*). The task being performed by all colony members was

recorded during 100 samples between 6th July and 7th September 2010. In total, 24 different tasks were recorded. From this, the proportion of time spent outside the nest was calculated. In addition, in order to compare directly with RFID data, an estimate was made of the number of trips per day. This was calculated based upon the number of trips outside the nest per hour during observation periods, however this is not directly comparable since behavioural observations were conducted only between the hours of 8am and 8pm, whereas RFID monitoring was possible 24 hours a day. In colony 2a, a new callow (2CAL) emerged after the observation period but prior to sequencing, and quickly ascended to the beta rank. Transcriptome data was obtained for this individual, however no behavioural data is available.

6.3.6 Genome Sequencing

To facilitate a high-quality assembly of the transcriptome sequences, I additionally generated the first full genome sequence for *Dinoponera quadriceps*. DNA was extracted from the whole body of a single haploid male, thereby minimising variation and improving the quality of the final assembly. DNA extractions were optimised for use in this non-model organism, and several different protocols, tissues and storage mediums were trialled in order to maximise yield (*appendix A4.2*). A single whole body was stored at -80°C prior to the final extraction, following homogenisation, it was split across four phenol-chloroform extractions (*appendix A4.3*). Extracted DNA was stored at -80°C prior to sequencing. DNA sequencing was performed using Illumina sequencing, 540 nt paired-end, with a mate-pair library.

6.3.7 Transcriptome Sequencing

Individual brain transcriptome sequences were obtained for *D. quadriceps* females. A total of 18 individuals of 3 different ranks (alpha, beta and low), from 7 colonies (mean colony size = 36.29 ±10.30) were collected for transcriptome sequencing (*table 6.1*). The sample included 7 alpha females (mated, developed ovaries), 5 beta-ranked workers, (unmated, 3 developed ovaries, 2 undeveloped ovaries), and 6 low-ranking workers (unmated, undeveloped ovaries).

6.3.7.1 Brain mRNA Extraction

Following RFID or behavioural monitoring, individuals were removed from the colony, sacrificed using liquid nitrogen, and stored in RNAlater at -80°C. Brain dissections were performed over ice to reduce RNA degradation, and whole brains were removed and placed immediately in a 1.5ml eppendorf containing 500µl of Trizol reagent, on ice.

Most pre-existing protocols have been optimised for model organisms, and obtaining high quality and quantity of RNA from this non-model organism required the trial and optimisation of several extraction protocols. Initial extractions using a standard Trizol extraction yielded low quantity and quality RNA extractions, and modifications to this protocol failed to improve the result. A Direct-zol RNA miniprep kit was also trialed and optimised, and following inclusion of a DNase treatment phase and extra elution; high quality RNA yields were achieved. RNA extracts were quality checked using Nanodrop and a QIAxcel advanced system, and only samples with at least 1.5µg of RNA and a 260/280 ratio of greater than 2 were sequenced. Quality of RNA is often estimated based upon a ratio between the 18S and 28S bands, however in several non-model organisms the 28S band has been found to dissociate during extraction, resulting in a single band on gel images and making quality more difficult to assess (Gayral *et al.* 2011). RNA extractions were then performed using a Direct-zol RNA miniprep kit according to the manufacturers protocol (*appendix A4.4*).

6.3.7.2 mRNA Library Preparation and Sequencing

19 individual brain RNA samples were sequenced, and libraries were prepared using the TruSeq RNA Sample Prep Kit v2 (RS-122-2001/2, Illumina) according to the manufacturer's protocol (*appendix A4.5*)**.** Final libraries were analyzed using Agilent DNA 1000 chip to estimate the quantity and check size distribution, and were then quantified by qPCR using the KAPA Library Quantification Kit (KK4835, KapaBiosystems) prior to amplification with Illumina's cBot. Libraries were loaded at a concentration of 1.8 pM onto the flowcell, and were sequenced Paired End, 100nts on Illumina's HiSeq 2000.

6.3.8 Sequence Alignment and Annotation

DNA sequence data using evidence modeller (EVM) and Program to Assemble Spliced Alignments (PASA) pipelines (*appendix A4.6*). The completeness of the assembly was estimated using the CEGMA pipeline (Parra *et al.* 2007), which looks for core orthologous proteins (COGS), deemed to be highly conserved and existing in low-copy numbers in the majority of higher eukaryotes. This list consists of 248 COG proteins from 6 species. The most complete sequences are estimated to contain around 90% of COG proteins (Parra *et al.* 2009).

The PASA assemble was created using an input of *D. quadriceps* transcriptome generated using the program cufflinks. Using four brain transcriptome datasets of *D. quadriceps*, RNASeq reads aligned to the *D. quadriceps* genome were assembled into transcripts using cufflinks, resulting in the generation of 27,787 transcripts that were subsequently fed into the PASA pipeline. This pipeline is quite stringent, and enabled 27,141 of the brain-derived transcripts (97.8%) using the mapping tool gmap. This is indicative of a high-quality alignment. To consider alignment valid, PASA required at least 90% of the transcript aligned to the genome. Valid gmap transcript alignments were clustered based on genome mapping location and assembled into gene structures to include the maximal number of compatible transcript alignments. This provided 26,944 PASA assemblies, which generated 18,230 protein-coding transcripts corresponding to 13,688 genes, translating into 16,536 unique proteins. mRNA sequences were aligned using the reference genome sequence using bowtie-0.12.9 (Langmead *et al* 2009), and assembled into transcripts using cufflinks. Reads per kilobase per million mapped reads (RPKM) was calculated for each gene for each of 19 samples, and normalised using BitSeq. BitSeq was then used to convert transcript-level data to gene-level data by averaging RPKM values.

Functional annotation of the transcriptome was performed using a BLASTP of the cufflink-assembled clean transcripts against the EVM consensus annotation. For each transcript, the closest matching annotation sequence was selected. A BLASTP of the EVM consensus annotation was performed against the NR protein database. A sequence was deemed homologous if it shared at least 30% sequence identity and

generated an e-value of less than 10^{-2}. Genes for which no close homologue was available were marked as novel.

6.3.9 Statistical Analysis

Differential expression analysis was primarily performed using BitSeq, a bayesian approach which estimates transcript-level expression while taking into account biological variation (Glaus *et al.* 2012). BitSeq infers relative expression represented by Markov chain Monte Carlo (MCMC) samples from the posterior probability distribution of the read data (Glaus *et al.* 2012). To address aim (1), I investigated expressional differences between individuals of different rank from 7 *D. quadriceps* colonies. I compared 7 individuals of alpha-rank, 5 individuals of beta-rank and 6 individuals of low-rank (*table 6.1*).

In *D. quadriceps*, reproduction is not entirely restricted to the gamergate, although mating is (Monnin and Peeters 1998). Around 40 – 50% of high-ranking individuals develop their ovaries and lay male eggs (Monnin and Peeters 1998). Among the five transcriptome sequences I obtained for beta-ranks, 2 had developed ovaries. Thus, I was able to disentangle the contribution of rank and reproductive physiology to gene expression. In order to address aim 2, I investigated differential gene expression in relation to ovarian development and mating status. These analyses included 9 individuals with developed ovaries and 9 individuals with undeveloped ovaries, 7 mated individuals and 11 unmated individuals (*table 6.1*).

Most studies using BitSeq previously have been interested in the most differentially expressed genes, and so a particular threshold of significance is not well established in the literature, rather most studies merely report the top 50 or 100 most strongly differentially expressed genes. I therefore investigated several probability of positive log-ratio (PPLR) significance thresholds (0.03, 0.04, 0.05), focusing on an intermediate threshold of 0.04, so that so that PPLR values lower than 0.46 (down-regulated) or higher than 0.54 (up-regulated) were considered to show significant differential expression between groups. Further, I investigated using a threshold based upon the

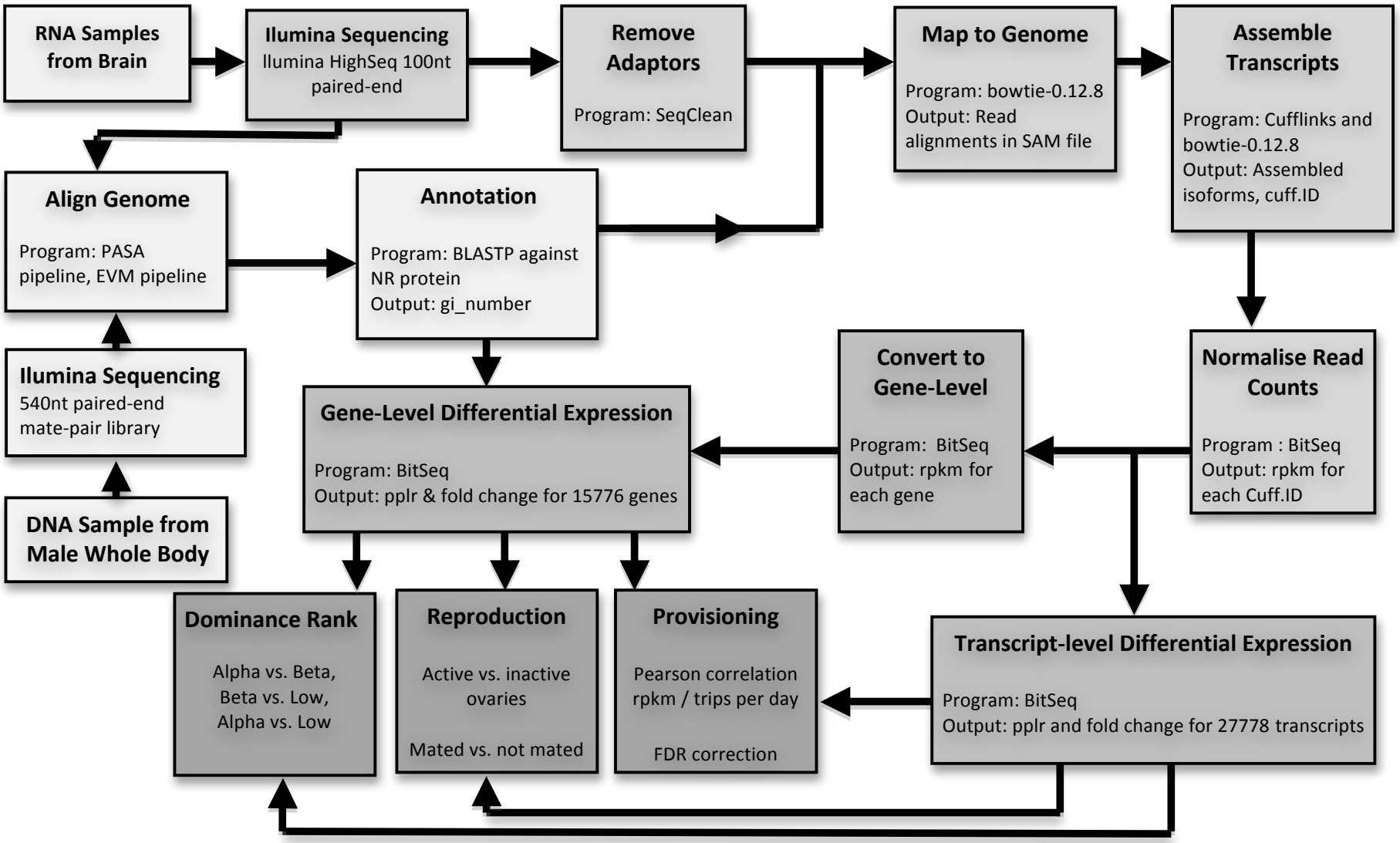
magnitude of expression differences. For this I selected a significance threshold of fold change < 0.9 (down-regulated) or > 1.1 (up-regulated).

Pearson correlations were performed for gene expression and foraging behaviour as measured by the number of foraging trips per day and the proportion of time spent outside, as measured by RFID and behavioural observations. To control for multiple comparisons, the false discovery rate was calculated using the qvalue package in R (Dabney *et al.* 2004).

I investigated functional differences in relation to rank and reproductive status using a GO enrichment analysis performed using the database for annotation, visualisation and integrated discovery (DAVID) version 6.7 (Huang *et al.* 2008; Huang *et al.* 2009). DAVID compares the abundance of different GO functional categories in a gene list of interest against their abundance in the 'background' of all genes in order to identify overrepresented functional categories.

In order to validate the results of BitSeq, rank and ovarian activity analyses were repeated using the EdgeR package. EdgeR uses an overdispersed Poisson model to account for biological variation, and uses an empirical bayes method to moderate overdispersion between transcripts. It is particularly useful for studies with little biological replication (Robinson *et al.* 2010). EdgeR yields a fold change and p-value for each gene for each grouping variable and automatically calculates a false discovery rate corrected 'q-value'. In addition to this basic analysis, comparisons were also investigated using a glm approach within the EdgeR package using the glmLRT function. This again yields a fold change and p-value as well as a corrected q-value for each gene. (*appendix A5.3*).

6.3.10 Transcriptomics Work Flow Chart



6.4 Results

Ovary dissections confirmed the reproductive status of all 7 alpha females and the non-reproductive status of the 6 low-ranking workers. Additionally, partially developed ovaries were detected in 3 of 5 beta females dissected, as is common among high-ranking workers of this species (Monnin and Ratnieks 2001). Measures of foraging behaviour, as recorded by RFID and behavioural observations, indicated a high degree of individual variation in behaviour, particularly between low-ranking workers. Broadly, measures of foraging activity for sequenced individuals indicate that they are representative of their rank (*figure 6.1, table 6.2*).

Table 6.2 Foraging Effort of Sequenced Individuals

Proportion of time spent outside and average number of trips per day ± 1se as recorded by RFID monitoring or behavioural observations. Means for each rank are taken from entire RFID (chapter 2) or behavioural (chapter 3) datasets, number of individuals included in the sample is indicated in brackets.

RFID DATA	**% Time Outside**	**Mean Trips Per Day**	BEHAVIOURAL DATA	**% Time Outside**
Mean Alpha (n = 6)	28.30 ± 7.29	0.69 ± 0.24	**Mean Alpha** (n = 4)	3.25 ± 1.70
10G48	0.00	0.00	2B84	2.00
23Y79	38.00	0.44	12Y30	0.00
36W26	17.60	1.56		
Mean Beta (n = 5)	20.62 ± 11.96	0.72 ± 0.41	**Mean Beta** (n = 4)	0.75 ± 0.48
10G87	0.00	0.00	12Y47	0.00
23Y70	9.06	1.60		
36W62	0.00	0.00		
Mean Low (n = 220)	53.00 ± 21.00	1.17 ± 0.07	**Mean Low** (n = 79)	26.33 ± 3.33
10G17	38.36	4.30	2B82	1.00
23Y59	56.36	14.51	12Y51	0.00
36W35	0.00	0.00		

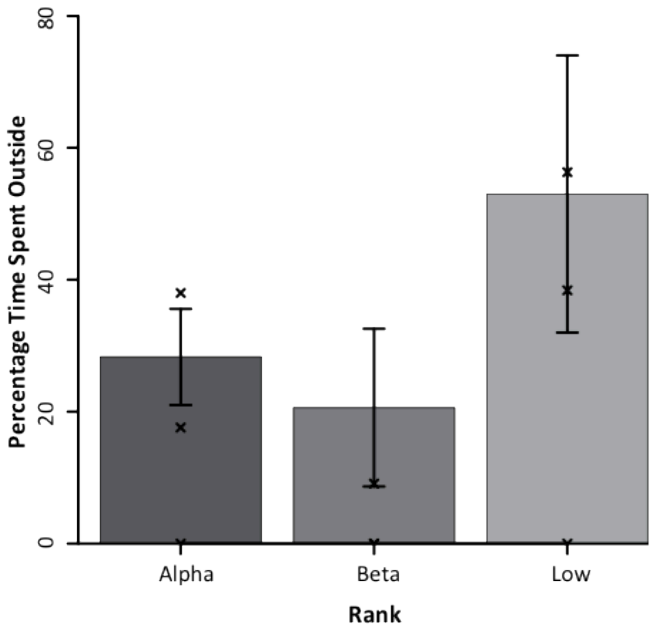


Figure 6.1 Foraging Effort of Sequenced Individuals

Proportion of time spent outside as recorded by RFID monitoring. Means for each rank are taken from entire RFID datasets (chapter 2). Samples used for transcriptome sequences for each rank are indicated as crosses on the graph.

6.4.1 Sequence and Alignment Quality

The genome sequence yielded a high quality assembly, with an N50 scaffold size of 1,359 kb, with a maximum scaffold size of 5,804 kb. Contig N50 was 30.5 kb, and the number of scaffolds and contigs was 14,170. The final assembly size was 261,128,193 bp. Assembly of the transcriptome to the reference genome generated 18,230 protein-coding transcripts corresponding to 13,688 genes, translating into 16,536 unique proteins. Approximately 1,700 transcripts differed only in their untranslated regions and 868 (4.7%) correspond to partial transcripts, lacking a 5' or 3' end sequence. Differential expression analyses were performed 27778 transcripts, mapped to 15776 different proteins. For the *Dinoponera quadriceps* assembly, 97.58% of COG proteins were completely detected, and 99.19% were at least partially detected. Functional annotation of the transcriptome revealed many putative genes for which the same gi_number mapped as the closest homologue, indicating some inaccuracies in the currently available publicly available genomic information. This is not unexpected for a non-model organism, and genome and transcriptome annotations will continue to improve in accuracy, quality and completeness as sequence data is gathered for more species, and as functional studies confirm the roles of candidate genes.

6.4.2 Continuity of Social Hierarchies

In total, I identified 460 genes differentially expressed with regard to rank (*table 6.3*). Overall, the most expression differences were found between the alpha and the beta (173 genes), with most of these differences being up-regulated in the alpha compared to the beta (115 genes; 66.5% of DE genes)(*table 6.3*). I identified 158 genes differentially expressed between beta-ranks and low-rankers, of which most were up-regulated in the low-ranker compared to the beta (101 genes; 63.9% of DE genes). The smallest number of expressional differences were found between the alpha and the low-ranking workers (137 genes), with roughly equal numbers of genes being up-regulated in the alpha (72 genes; 52.6%) and the low-rankers. Using a fold-change threshold of 0.1, very few genes were identified as being differentially expressed in BL and AL comparisons, however most genes differentially expressed between the alpha and beta remained significant. Comparing between lists of up-regulated known homologues (gi_numbers) of genes for each comparison (*appendix A5.1*), the greatest

overlap was between the beta and low-ranked worker (52 shared up-regulated genes), the smallest between the alpha and beta worker
(4 shared up-regulated genes)(*figure 6.2*). Less overlap was found when comparing significantly upregulated genes at the level of putative genes identified from the genome assembly, however. This reflects the fact that during annotation, some putative genes were found to be homologous to the same gene sequence in another species, thereby generating some redundancy in the annotation. In terms of unique genes as identified by the genome assembly, only one was found to be significantly upregulated in both the alpha and beta, two in both the alpha and low- ranked workers, and none were found for the beta and low-ranked workers.

Table 6.3 Differential Expression in Relation to Rank

Numbers of significantly differentially expressed genes in relation to rank at the abs(pplr)>0.4 level, including numbers of genes up-regulated and down-regulated for alpha vs. beta, beta vs. low and alpha vs. low.

Comparison	**Significant** abs(pplr)>0.04	**Significant** fold change > 0.1	Up-regulated	Down-Regulated
Alpha vs. Beta	173	110	115	58
Beta vs. Low	158	2	57	101
Alpha vs. Low	137	51	72	65

I investigated the numbers of differentially expressed genes at three different levels of pplr significance (*appendix A5.2*), however the results broadly agree with each other in terms of patterns of differential expression between ranks. I present here the numbers of differentially expressed genes identified at the abs(pplr)>0.04 significance level, intermediate between the three levels of significance investigated, as this represents a balance between stringency and thorough exploration of the differences between rank. Very few genes were identified as significantly differentially expressed at the abs(pplr)>0.05 level.

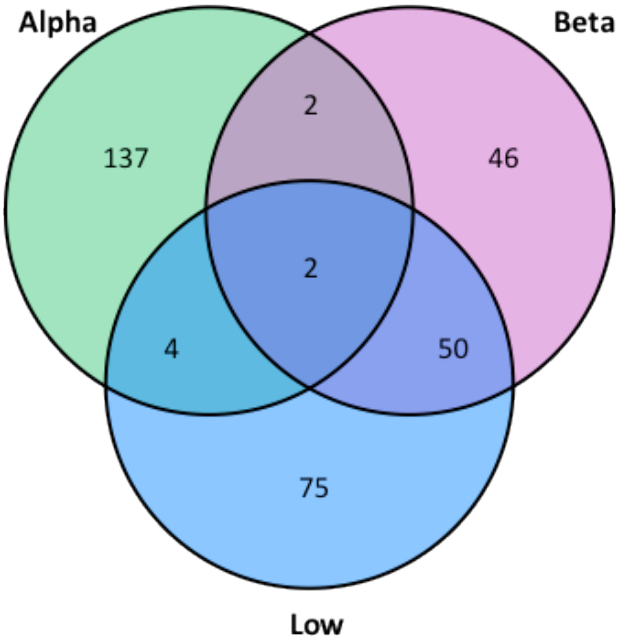


Figure 6.2 Differential Expression in Relation to Rank

Venn diagram of the 460 genes significantly up-regulated in alpha, beta and low-ranked individuals at the pplr threshold of 0.04. Numbers displayed related to the number of unique shared gi_numbers (316) for the closest homologue found during functional annotation.

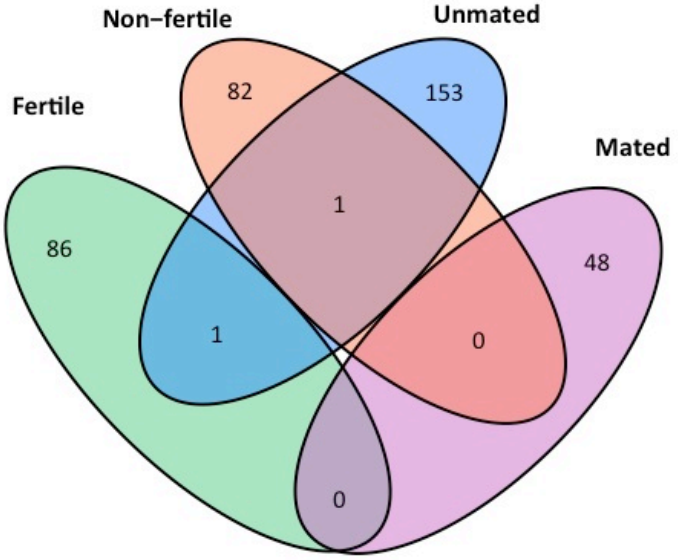


Figure 6.3 Reproductive Physiology and Gene Expression

Venn diagram of the 337 differentially expressed genes (at the pplr cut-off of 0.04) for genes up-regulated in individuals with developed or undeveloped ovaries (fertile vs non-fertile) and for mated and unmated individuals. Numbers displayed related to the number of shared differentially expressed transcripts grouped into genes.

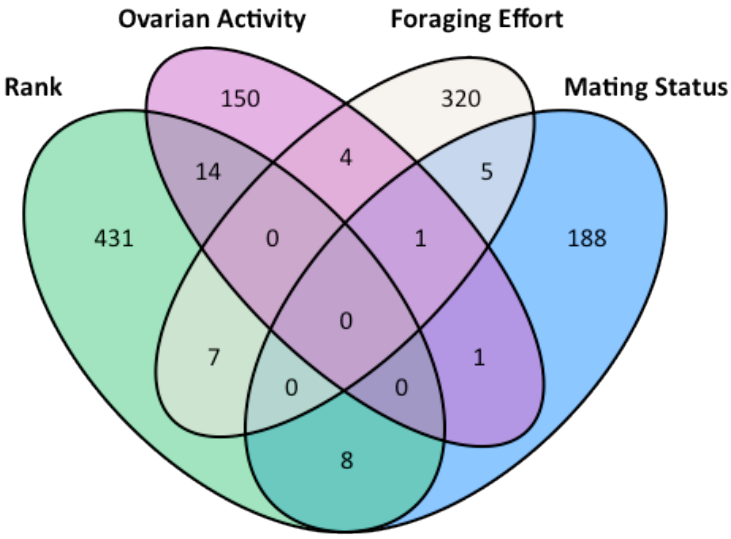


Figure 6.4 Differential Expression in Relation to Rank, Reproduction and Provisioning

Venn diagram of the 1129 unique genes significantly differentially expressed for rank, reproductive physiology (ovarian activity, mating status), and foraging effort (% time spent outside) for the q-value threshold of 0.975. Numbers displayed related to the number of shared differentially expressed transcripts grouped into genes.

6.4.3 Functional Analysis

In order to investigate functional differences between ranks, a GO enrichment analysis was performed for genes identified as up-regulated in relation to rank and ovarian activity, using the Database for Annotation, Visualisation and Integrated Discovery (DAVID) version 6.7 (Huang *et al.* 2008; Huang *et al.* 2009). Very few differentially expressed genes were found in the DAVID database (4 – 16%), making resulting enrichment analyses difficult to interpret. For the alpha rank, GO terms were identified for only 24 out of 195 genes identified as up-regulated in the alpha, and within these 24 genes, no GO categories were found to be enriched. Similarly, only 9 out of 102 genes up-regulated in the beta, and 19 of 131 genes up-regulated in low-rankers were located in the DAVID database. No GO categories were found to be enriched for either rank. Looking at genes up-regulated in reproductively active ants, 3 of 72 genes were identified in the DAVID database, showing no enriched GO categories. Similarly, for reproductively inactive ants, 11 of 71 genes were identified and no GO categories were found to be enriched. I additionally attempted GO enrichment analyses for gene lists of differentially expressed genes for each comparison. For genes differentially expressed between the alpha and beta, only 19 of 177 genes were located in the DAVID database, with one enriched GO category identified: Cytoskeleton. For genes differentially expressed between the beta and low, or alpha and low categories, no enriched GO categories were identified amongst the 21 and 18 genes identified in the DAVID database, respectively.

6.4.4 Underlying Mechanisms of Social Hierarchies

A principal component analysis was performed for the gene expression of all 15776 genes for each individual. Each principal component explained only a small amount of variance in gene expression between individuals; PC1 explained 6.2% of variance between individuals, while PC2 explained a further 6.2% (*table 6.4*). Individuals did not appear to cluster in relation to colony, rank or ovarian activity for principal components 1 and 2 (*figure 6.5)*.

Table 6.4 Importance of PCA components.

Standard deviation and proportion of variance explained for each principal component, and cumulative proportion of variance explained.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9
Standard Deviation	31.34	31.18	31.07	30.91	30.90	30.77	30.58	30.48	30.46
Proportion of Variance	0.062	0.062	0.061	0.061	0.061	0.060	0.059	0.059	0.059
Cumulative Proportion	0.062	0.124	0.185	0.246	0.306	0.366	0.425	0.484	0.543
	PC10	PC11	PC12	PC13	PC14	PC15	PC16	PC17	PC18
Standard Deviation	30.41	30.26	30.15	30.11	29.90	29.88	29.81	29.60	2.808^{e-13}
Proportion of Variance	0.059	0.058	0.058	0.058	0.057	0.057	0.056	0.056	0.000
Cumulative Proportion	0.602	0.660	0.717	0.775	0.832	0.888	0.945	1.000	1.000

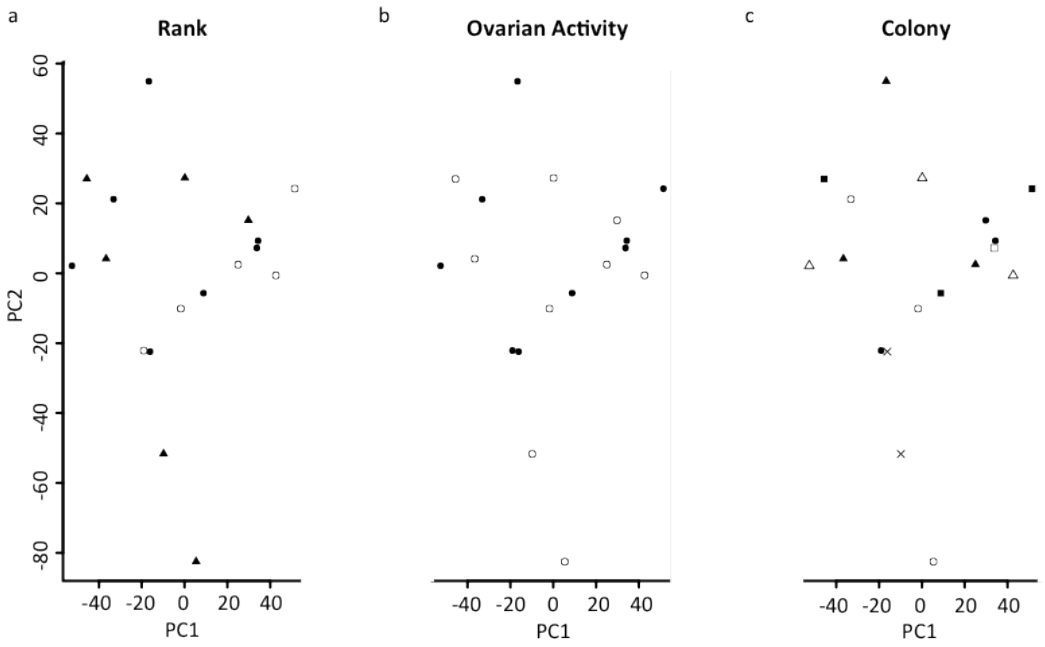


Figure 6.5 Principal Component Analysis of Gene Expression.

PCA of gene expression for 15776 genes in 18 individuals. PC1 against PC2, points coloured according to (a) **rank** – alpha = filled circles, beta = empty circles, low = filled triangles, (b) **ovarian activity** – developed ovaries = filled circles, undeveloped ovaries = empty circles, and (c) **colony** – colony 2a = empty circles, colony 12a = empty triangles, colony 10a = empty squares, colony 36b = filled squares, colony 23b = filled circles, colony 10b = filled circles, colony 35b = crosses.

6.4.4.1 Reproductive Physiology

At the 0.04 significance level, 170 genes were differentially expressed in relation to ovarian activity, 87 of which were up-regulated in reproductive individuals (*table 6.5*). I

found 203 genes that were differentially expressed between mated and unmated individuals; 48 of which were up-regulated in mated individuals. For both ovarian activity and mating status, no genes were significant differentially expressed using the fold change threshold of 0.1.

Table 6.5 Differentially Expressed Genes in Relation to Reproduction and Provisioning

Numbers of significantly differentially expressed genes for reproductive physiology at the abs(pplr)>0.4 level, including numbers of genes up-regulated and down-regulated for each comparison. Numbers of significant pearson correlations (q < 0.97) between foraging behaviour (number of trips per day or percentage of time spent outside, measured by RFID or behavioural observations) and gene expression. The number of significant correlations before FDR correction are shown in brackets.

Comparison	**Significant** abs(pplr)>0.04	**Significant** fold change > 0.1	Up-regulated	Down-Regulated	% Total DE Genes
Ovarian Activity	170	0	87	83	20.73
Mating Status	203	0	48	155	24.76
Foraging Effort (% Time Outside)	11 (755)	NA	7 (391)	4 (364)	1.34
Foraging Effort (Trips Per Day)	3 (818)	NA	1 (436)	2 (382)	0.37

I also compared lists of differentially expressed genes generated in relation to rank and reproductive physiology. Of the 460 genes identified as differentially expressed in relation to rank, 17 were also differentially expressed in relation to ovarian activity, and 10 in relation to mating status (*figure 6.3*). The greatest overlap existed for genes identified as up-regulated in the alpha and individuals with active ovaries (7 genes), and between low-rankers and individuals with inactive ovaries (5 genes). Larger overlaps in differentially expressed gene lists were observed when considering the homologous genes identified in the functional annotation, however the overall trends remained the same. For those genes for which functional annotation was available, the following genes were identified: Masquerade isoform B, Zinc finger protein 13, FAD-dependent oxidoreductase, ATP-binding cassette sub-family G, Sushi, von Willebrand factor type A, distal-less, 1-phosphatidylinositol-4,5-bisphosphate, futsch, pax-1, beta-mannosidase, O-mannosyltransferase 1, M-phase phosphoprotein 1, guanylate cyclase beta, Odorant receptor 2a, USF 2, Cadhern, Palmitoyltransferase, Dopey-1, eIF 3, Spatacsin and SLC12A9 (*appendix A5.1*).

6.4.4.2 Foraging Effort

I investigated the relationship between foraging effort and gene expression, by performing pearson correlations between individual gene expression for each gene against two measures individual extranidal activity (percent of time spent outside, number of trips per day) as measured using either RFID monitoring or behaviour observations. Prior to the false discovery rate correction, I identified 755 and 818 genes that showed significant ($p > 0.05$) correlations between gene expression and time spent outside the nest and trips per day respectively. FDR yielded pi0 values of 0.995 and 0.972 respectively, indicating that for both analyses, approximately 3 – 4 significant p-values represent genuine true positives. Q-values were universally high (smallest q-value, $q = 0.57$). However, the developers of the q-value algorithm recommend that q-values be considered independent of an arbitrary 0.05 significance threshold (Storey and Tibshirani 2003). Considering the histogram of q-values, my data for both measures of foraging effort indicate a bimodal distribution. For example, for time spent outside the nest, I observe a mode at 0.97, followed by a second, larger mode at 0.975, with very few intermediate q-values. Using a q-value threshold of 0.97 yields 11 significant correlations, which is close to the four expected based upon the pi0 value. I have therefore considered correlations with a q-value < 0.97 to be significant, with genes lying in the $q = 0.97 – 0.975$ region to be potential true positives. To maximise the depth of data analysis, I have included genes with correlations $q < 0.975$ for all diagram and for gene list comparisons. Likewise, for my second measure of foraging effort, trips per day, I have used a threshold of $q < 0.89$ for significant, yielding three significant correlations, whilst considering q values between 0.89 and 0.91 as possible true positives (30 genes).

In order to investigate the relationship between gene expression, rank and foraging behaviour, I compared the list of 820 genes differentially expressed for rank and reproductive physiology with the list of true positives and potential true positives for correlations between foraging effort (time spent outside) and gene expression. A total of 14 genes were identified as being differentially expressed in relation to both rank and foraging effort, while a further 9 genes differentially expressed for ovarian activity

and 8 genes differentially expressed for mating status were also correlated with foraging effort (*figure 6.4*). Again, greater overlap was found for gene homologues (gi_numbers) than for assembled genes, but yielding the same trend. Functional annotation was available for the following genes: pax-1, Beta-mannosidase, M-phase phosphoprotein 1, Sphingosine-1-phosphate phosphatase, odorant receptor 2a, USF2 and cadherin (*appendix A5.1*).

6.5 Discussion

Here I present the first investigation of the expressional control of reproductive and behavioural division of labour in the secondarily primitive ponerine ant, *Dinoponera quadriceps*.

6.5.1 Continuity of Social Hierarchies

The first aim of this study was to investigate the continuity of social hierarchies, specifically investigating whether the social hierarchy observed in *D. quadriceps* represents a continuous or discontinuous hierarchy. Overall, I found relatively few genes showed differential expression with respect to rank, with more differences in gene expression between the alpha (mated reproductive) and her subordinates. Consistent with this, the largest number of differentially expressed genes were detected in relation to mating status. Other studies have found gene expression differences between queens, reproductively active and inactive workers (Cardoen *et al.* 2011; Grozinger *et al.* 2007), and several studies have found substantial gene expression differences between mated and unmated female paper wasps (Sumner *et al.* 2006; Toth *et al.* 2010).

Patterns of up-regulation between ranks are also more consistent with a discontinuous social hierarchy; I found that more genes were significantly up regulated in the alpha compared to other ranks. Comparing between the alpha and other ranks, 60% of differentially expressed genes were up-regulated in the alpha. In advanced societies, microarray data from advanced species indicates that the numbers of up-regulated genes in the queen and worker castes is roughly equal (Grozinger *et al.* 2007; Ometto

et al. 2010). However, in primitively eusocial paper wasps 94% of differentially expressed genes are up-regulated in the worker caste (Ferreira *et al.* 2013). Further, a pooled-sample transcriptome analysis for another ponerine ant, in which the queen caste is still present, showed that 62% of differentially expressed genes between gamergates and workers were up-regulated in the gamergate (Bonasio *et al.* 2010). This is strongly consistent with my result that around 60 – 70% of differentially expressed genes are up-regulated in the gamergate compared to other ranks, and is also consistent with higher levels of up-regulation in reproductive active honeybee workers (Grozinger *et al.* 2007). In general I found relatively small magnitude differences in expression (gene expression fold change), with fewer genes meeting the foldchange > 0.1 threshold than the pplr < 0.04 threshold. Larger magnitude expressional differences were observed between the alpha female and other ranks. This provides more convincing support for a discontinuous social hierarchy in which the alpha is most distinct from other colony members.

6.5.2 Underlying Mechanisms of Social Hierarchies

6.5.2.1 Reproductive Physiology

In total, 460 genes were differentially expressed in relation to rank, with a further 170 in relation to ovarian activity and 203 in relation to mating status. Rank comprises a complex set of characteristics, including age differences, cuticular hydrocarbon differences, mating status and ovarian activity, and thus the greater number of differentially expressed genes in relation to rank likely reflects the complexity of the variable. Despite this, together ovarian activity and mating status accounted for 45.49% of all differentially expressed genes, indicating a key role for reproductive physiology in determining gene expression. Differences in brain gene expression patterns in relation to reproductive physiology, however, tended to be small in magnitude, with no genes meeting the foldchange > 0.1 threshold. That very few high magnitude differences were observed in relation to ovarian activity likely reflects the use of exclusively brain tissue in this study.

6.5.2.2 Foraging Effort

I performed pearson correlations between the expression level of each gene and two measures of foraging effort. Correlational analyses in gene expression studies have rarely been performed, and to my knowledge this is the first attempt at directly correlating a continuous behavioural variable (foraging effort) with transcriptome-wide gene expression patterns in a social insect. I detected 11 genes that showed significant correlations between time spent outside and gene expression, and a further 326 genes were identified as possible true positives by the FDR correction. Correlation coefficients for these correlations were also frequently high, with foraging explaining up to 82.6% of the variance in gene expression. The highest correlation coefficients obtained were negative, again indicating that gene expression tended to be lower for foraging individuals and suggesting that foragers tend to be a more specialised and committed caste. Other studies have found a key role for foraging behaviour in determining gene expression patterns in *Apis mellifera* (Whitfield 2003) and *Polistes metricus* (Toth *et al.* 2010).

Radio frequency identification tagging (RFID) tended to over estimate the percentage of time spent outside the nest compared to behavioural observations (*table 6.2, figure 6.1*); errors in the RFID estimates could be caused due to limitations in processing complex data from multiple antennae, tag reading errors or interference from multiple ants passing the same antenna simultaneously. However, trends in the data remained the same; the greatest amount of extranidal activity was performed by low-rankers, and the alpha female spent slightly more time outside than the beta.

6.5.2.3 Age

One unexpected result is that the number of differentially expressed genes between the alpha and beta is higher than the number between the alpha and low. This apparently paradoxical result indicates some shared traits between the alpha and low-ranking workers that have an influence on gene expression. One possible candidate for this is age. Based on the known age structure of hierarchies in this species (Monnin and Peeters 1999), I can expect that the beta rank should always be the youngest

colony member, low-rankers will be amongst the oldest, and the alpha rank could represent a range of ages. Thus it is possible that those genes for which expression is shared between alphas and low-rankers relate to older age, shared between low-rankers and older alphas. Developmental stage has been shown to have a large influence on gene expression differences (Colgan *et al.* 2011; Hoffman and Goodisman 2007; Ometto *et al.* 2010), however relatively few studies have investigated the influence of adult age. Further, the influence of age cannot easily be distinguished from experience. However, age has been shown to influence gene expression in honeybees (Alaux *et al.* 2009) and paper wasps (Ferreira *et al.* 2013), although relatively few methylation changes have been found with age when compared to behavioural differences (Lockett *et al.* 2011).

6.5.3 The Loss of the Queen Caste

Out of a total of 15776 genes, only 479 (3%) were identified as significantly differentially expressed with respect to rank or reproductive status. This is in contrast to microarray studies of caste-biased gene expression in highly eusocial species, where between 25% and 34% of genes show differentially expression in relation to caste in the honeybee (Evans and Wheeler 1999; Grozinger *et al.* 2007) and the fire ant (Ometto *et al.* 2010). However, these comparisons are between workers and true queens, whereas *Dinoponera quadriceps* has replaced the queen caste with reproductively active workers. The relatively low numbers of differentially expressed genes identified in this study are more consistent with the numbers of differentially expressed genes identified within the worker caste in *Apis mellifera*, where between 3% and 12% of genes studied differ in expression between reproductively active and inactive workers (Cardoen *et al.* 2011; Grozinger *et al.* 2007). My results are also broadly consistent with findings for primitively eusocial species. In the primitively eusocial paper wasp, *P. Canadensis*, similarly low levels of differential expression were also observed between caste, with 1909 (7%) differentially expressed between queens and workers. Therefore the results of this study suggest that in the loss of the queen caste, *D. quadriceps* eliminated much of the differential gene expression between individuals, and that alphas in these colonies truly are reproductively active workers as opposed to queens in terms of their gene expression profiles.

The validity of this idea could be informed through comparison with other ponerine species in which the queen caste remains in conjunction with gamergates. In *Harpegnathos saltator*, colonies are founded by morphologically adapted queens, whose daughters take over reproduction and become gamergates following her death. *Harpegnathos saltator* societies are thought to represent an intermediate point between advanced and secondarily primitive (Monnin and Peeters 2008), and pooled transcriptome data is available for comparison between the gamergate and non-reproductive worker. In *H. saltator*, 742 genes (3.8%) were differentially expressed between the gamergate and subordinates. However, this study did not include samples from the morphological queen caste.

6.5.4 Caste Commitment

There are many parallels between the process of cell commitment during development and the process of caste determination; cells (or individuals) are initially totipotent, slowly becoming increasingly committed to a particular role (Chittka *et al.* 2012; Patalano *et al.* 2012). Differences in the numbers of genes up-regulated in different castes and reproductive groups may inform us about caste commitment. Overall the beta had the greatest amount of down-regulation. In the paper wasp, *Polistes canadensis*, newly emerged wasps (callows) were found to down-regulate many genes compared to queens, workers and foundresses (Ferreira *et al.* 2013). Similarly, I find that beta-ranked workers, the youngest in the colony, tend to down-regulate genes relative to the other ranks. This down-regulation in younger individuals may represent a relatively undifferentiated state, neither 'worker' nor 'queen' (Ferreira *et al.* 2013), and this is certainly the case for the beta-ranked worker in a dinosaur ant society.

In *D. quadriceps*, I found that roughly half of all differentially expressed genes were up-regulated in individuals with developed ovaries compared to reproductively inactive workers, similar to queen-worker comparisons in advanced societies (Grozinger *et al.* 2007; Ometto *et al.* 2010), and suggesting roughly equal caste commitment. By contrast, in the primitively eusocial species *Polistes canadensis*, more genes were up-

regulated in workers (78%) compared to queens, consistent with continued reproductive totipotency of the worker caste into adulthood (Ferreira *et al.* 2013). Thus, dinosaur ants appear to show a greater degree of caste commitment in terms of gene expression patterns than primitively eusocial species, which is consistent with their evolutionary history. However, more data on a wider range of taxa is needed to thoroughly investigate this hypothesis.

6.5.5 Functional Redundancy in Genome Annotation

As with all studies utilising large-scale genomic or transcriptomic data, my results are limited by the quality of assembly and annotation. Quality assessment indicates that the genome assembly presented is of high-quality, covering 97% of core orthologous proteins. However, the fields of genome and transcriptome assembly and annotation remain in their infancy, and a major challenge for molecular biologists and bioinformaticians over the coming decades will be to refine and improve these methods within the constraints of computing power available (Frishman, 2007; Rust *et al* 2002) . As next-generation sequencing technology becomes increasingly affordable, a greater availability of genomic data from a range of model organisms, along with focused functional analyses on a wider range of genes, will greatly improve our understanding of genomic and transcriptomic data (Frishman, 2007; Rust *et al* 2002). Within the genome annotation presented here, a number of putative genes share their closest homologue with the same gene sequence in another species, generating some functional redundancy within my data. In particular it has affected the determination of the number of shared up-regulated genes between different groups, with analyses at the level of putative genes and transcripts yielding fewer shared 'genes' than those performed at the level of id. Overall, these annotation inaccuracies should have only a relatively small influence on my analyses and the overall interpretation of the data since the majority of my analysis were performed on genes as predicted by the genome assembly.

6.5.6 Conclusions

Here I present the first investigation of the transcriptional control of a social hierarchy in a secondarily derived primitively eusocial ant, *Dinoponera quadriceps*. Using

transcriptome data from 18 individuals of three different ranks, known reproductive physiology and foraging effort, I find relatively few expressional differences between individuals. The largest expressional differences were identified in relation to rank, and my results indicate a discontinuous social hierarchy in which the gamergate is the most distinct, with many expressional differences relating to reproductive physiology. The beta rank may represent a relatively undifferentiated state, with lower levels of gene expression prior to committing to either a gamergate or low-rank phenotype. I propose that the comparatively small differences between gamergates and non-reproductive workers in *Dinoponera quadriceps* is a result of the recent loss of the queen caste in which much differentiation between colony members was lost. The remaining differences are more comparable with those differences observed between reproductively active and inactive workers of highly eusocial species. The transcriptional profile of *D. quadriceps* highlights their unusual evolutionary history and will provide a powerful comparison to other simple societies.

Chapter 7

Testing the Toolkit: Conservation versus Novelty in the Evolution of Eusociality

7.1 Abstract

During the evolution of eusociality, ancestral traits relating to reproduction and provisioning behaviour are believed to have become decoupled into distinct 'queen' and 'worker' phenotypes. The toolkit hypothesis suggests that across multiple independent origins of eusociality, the same ancestral genes may have been co-opted for a role in social behaviour, and several studies have now identified key toolkit genes involved in caste determination and division of labour across a range of eusocial species. The ponerine ants exhibit a simple social structure, but are descended from a recent advanced ancestor, thereby offering an unusual opportunity to investigate the role of evolutionary history in shaping the transcriptional control of caste and division of labour. Here, I investigate the relative importance of conserved toolkit genes and novel, taxa-specific genes, in the reproductive division of labour in the queenless ponerine ant, *Dinoponera quadriceps*. I find a greater number of novel up-regulated genes associated with the reproductive phenotype. In general levels of novelty are lower in *D. quadriceps* than in other simple societies. I also identify 17 toolkit genes are differentially expressed in relation to rank or ovarian activity in *D. quadriceps*. These results suggest that both conserved and novel genes have played a role in the social evolution of dinosaur ants, but that novel genes may have been more important in the emergence of the reproductively active worker phenotype, which represents the key evolutionary innovation that characterises queenless ponerine ants.

7.2 Introduction

The evolution of eusociality was a major transition in evolution (Maynard Smith and Szathmary 1995). Eusociality is characterised by three key traits: reproductive division of labour, cooperative care of young, and an overlap of at least two generations, so that offspring assist their parents (Maynard Smith and Szathmary 1995; Wilson 1974,

2000). Like other major evolutionary transitions, eusociality involves the advent of higher-order organisation and cooperation between previously separate entities (Maynard Smith and Szathmary 1995; Queller 2000), which then lead to subsequent specialisation of the previously separate units to perform specific jobs (Bourke 2011). Sharing the same genome, individual units achieved specialisation through differences in gene expression. Multicellularity and eusociality are two examples of this type of 'fraternal' major transition (Queller 2000). Research in the field of 'evodevo' has yielded many insights into the evolution of multicellularity, in particular the identification of homeobox genes, which are fundamental in multicellular organisation across taxa (Hall 2003; Pearson *et al.* 2005). An emerging hypothesis for the evolution of polyphenism and polyethism in social insects is that specific ancestral genes have been involved in the evolution of eusociality across different lineages. Just as the homeobox genes have provided a toolkit for the evolution of cell differentiation during development, some scientists believe that a similar toolkit of 'sociality genes' may play a conserved role in caste differentiation and division of labour in different species (Johnson *et al.* 2010; Toth and Robinson 2007).

Just as cells begin totipotent, slowly becoming increasingly specialised for a particular task and expressing only a subset of the genes and behaviours of a single-celled organism, social insect castes each display only a small number of the behaviours performed by solitary insects (Chittka *et al.* 2012). Solitary insects must reproduce and provide food for the offspring, whereas in eusociality these two tasks are 'decoupled' into distinct castes; the queen caste performs reproduction while the worker caste provisions (Johnson *et al.* 2010; West-Eberhard 1987). In many solitary species, these behaviours may be temporally decoupled, with seasonal cycles of oviposition and foraging (West-Eberhard 1987). The genes regulating this cyclic behaviour may have provided the foundation for the evolution of eusociality (Ament *et al.* 2010; Ihle *et al.* 2010; Tibbetts *et al.* 2011; Toth *et al.* 2007; West-Eberhard 1987). The reproductive ground plan hypothesis has lead to the concept of toolkit genes, which have been evolutionarily predisposed to becoming decoupled, in generating phenotypic plasticity in social insects (Johnson *et al.* 2010; Toth and Robinson 2007).

7.2.1 Toolkit Genes in the Evolution of Sociality

There is some evidence for candidate 'toolkit' genes underlying queen and worker phenotypes (Ament *et al.* 2011; Bonasio *et al.* 2012; Ferreira *et al.* 2013; Robinson *et al.* 2005; Shorter and Tibbetts 2009; Smith *et al.* 2008; Toth *et al.* 2010; Woodard *et al.* 2011). A number of genes have now been identified which appear to play a crucial role in caste differentiation and behavioural plasticity across lineages representing independent origins of eusociality, and may together form a toolkit for sociality. In particular, five genes have been investigated as potential toolkit genes including Juvenile hormone (Giray *et al.* 2005; Whitfield 2003), vitellogenin (Graff *et al.* 2007; Sumner *et al.* 2006; Weil *et al.* 2007; Wurm *et al.* 2011), insulin-signalling genes (Daugherty *et al.* 2011), members of the major royal jelly protein family (*MRJP*)(Drapeau *et al.* 2006; Sumner *et al.* 2006) and *foraging*, a gene encoding a cGMP-dependent protein kinase (Ben-Shahar *et al.* 2002; Ingram *et al.* 2005; Osborne *et al.* 1997). However, as next-generation sequencing technologies permit genome- and transcriptome-wide investigations of caste-bias, more genes are being identified as potential toolkit genes. In particular, cytochrome p450 (Cardoen *et al.* 2011; Colgan *et al.* 2011; Weil *et al.* 2007), hexamerin (Colgan *et al.* 2011; Sumner *et al.* 2006), histone 2A (Graff *et al.* 2007; Weil *et al.* 2007), oxidoreductase (Daugherty *et al.* 2011; Whitfield *et al.* 2006) and yellow (Cardoen *et al.* 2011; Graff *et al.* 2007) have been identified as caste-biased in a number of species.

Juvenile hormone (*JH*) has been shown to influence both reproductive division of labour and temporal polyethism in honeybees (Watson 1985; Whitfield *et al.* 2006) and also regulates foraging behaviour in paper wasps (*Polistes dominulus*, Shorter and Tibbetts 2009; Tibbetts *et al.* 2011; *Polistes canadensis,* Giray *et al.* 2005) and termites (*Cryptotermes secundus*, Weil *et al.* 2007), and major / minor differentiation in carpenter ants (*Camponotus floridanus*, Simola et al. 2013). Changes in *JH* levels during behavioural maturation are regulated by a mutually inhibitory relationship with vitellogenin (Guidugli *et al.* 2005; Sullivan *et al.* 2000). *Vitellogenin* is a yolk precursor protein (Tian *et al.* 2004), which also plays a role in foraging behaviour in honeybees (Amdam et al. 2004; Guidugli et al. 2005; Nelson et al. 2007a), as well as queen-worker

caste differentiation in termites (Weil *et al.* 2009; Weil *et al.* 2007) and fire ants (Wurm *et al.* 2011), and worker caste differentiation in the carpenter ant (Simola et al. 2013). The *major royal jelly proteins* (MRJPs) are a group of nine glycoproteins essential for nutritional provisioning of queen-destined larvae in honeybees, which also show differential expression in relation to caste in adults (Drapeau *et al.* 2006; Thompson *et al.* 2006). Furthermore, major royal jelly proteins have been identified in several other eusocial species such as red imported fire ants (Tian *et al.* 2004) and paper wasps (Sumner *et al.* 2006), where they appear to play a role in reproductive division of labour. Expression of the *foraging* (*for*) gene influences division of labour in honeybees (Ben-Shahar *et al.* 2002), two species of ants (Ingram *et al.* 2005; Lucas and Sokolowski 2009) and in bumblebees (Kodaira *et al.* 2009; Tobback *et al.* 2010). Another pathway that influences division of labour in honeybees is the insulin / insulin-like growth factor signalling pathway (Ament *et al.* 2008; Hattori *et al.* 2013). IIS levels are significantly higher in the brain and abdomen of foraging workers than nurses, and inhibition of this pathway delays behavioural maturation in young honeybees (Ament *et al.* 2008). RNAi of the insulin receptor substrate (IRS) prevented development of the queen phenotype in honeybee larvae (Wolschin *et al.* 2011) and IIS genes have also been found to be important in soldier caste development in termites (Hattori *et al.* 2013).

7.2.2 The Importance of Novelty

A toolkit for sociality may have mediated some of the evolutionary changes that generate the polyphenisms we observe in social insects. However, caste differentiation cannot be mediated purely by the action of such a small number of genes, and an emerging picture is that novel genes also play an important role in insect phenotypes (Ferreira *et al.* 2013; Johnson and Tsutsui 2011; Weil *et al.* 2009). The importance of novel genes in the evolution of phenotypic innovation is emerging in a range of taxa (Dai *et al.* 2008; Ferreira *et al.* 2013; Fry *et al.* 2010; Khalturin *et al.* 2008; Simola *et al.* 2013; Woodard *et al.* 2011), and novel genes may in fact be more important in generating adaptive variation than duplication events (Carvunis *et al.* 2012; Ding *et al.* 2012; Zhang 2003). Genome-wide studies are increasingly revealing a large contribution of novel genes to polyphenism in social insects. Across the honeybee genome, 696 genes (6%) are found only in insects, 182 of which are found exclusively

in the honeybee, and these genes tended to be more highly expressed in workers compared to queens, which is consistent with the fact that workers exhibit more novel behaviours than queens (Johnson and Tsutsui 2011). Furthermore, across 9 bee species spanning 3 independent origins of eusociality, 10% of genes showing recent rapid evolution are unique to either advanced or primitive species and a greater number of these novel genes were found to be associated with primitive eusociality than advanced eusociality (Woodard *et al.* 2011). In two closely related species of termite, only 3 genes with caste-biased expression patterns were conserved between species, and novel genes appear to have contributed to eusocial evolution even over small evolutionary timescales (Weil *et al.* 2009).

Novel genes appear to have been of greater importance in the emergence of the worker phenotype than for differences in behaviour within the worker caste, such as differences between nurses and foragers (Johnson and Tsutsui 2011; Toth *et al.* 2010). Genes involved in provisioning behaviour are conserved between honeybees and paper wasps, a relationship that does not exist for reproduction (Toth *et al.* 2010). Thus, it appears that while conserved 'toolkit' genes may have played a role in the evolution of eusociality, novel genes have also been of great importance, with conserved and novel genes contributing differently to different polyphenisms.

7.2.3 Epigenetics and Caste Differentiation

Changes in gene expression patterns are mediated epigenetically (Patalano *et al.* 2012). DNA methylation is thought to be a key epigenetic modification (Cedar and Bergman 2009). DNA methylation changes dynamically and shows strong associations with gene expression (Elango *et al.* 2009; Glastad *et al.* 2012; Suzuki and Bird 2008). MicroRNAs and methyltransferases are important in epigenetic regulation and display caste-biased expression in honeybees (Behura and Whitfield 2010; Greenberg *et al.* 2012) and ants (Bonasio *et al.* 2010). Furthermore, whole methylome studies have revealed methylation biases between castes (Bonasio *et al.* 2012; Lockett *et al.* 2011), and chromatin maps indicate a strong relationship between histone methylation and caste biased gene expression (Simola et al. 2013). Epigenetic modification in social insects is largely implemented through alternative splicing (Bonasio 2012; Lyko *et al.*

2010), which is also correlated with methylation patterns (Li-Byarlay *et al.* 2013). Caste-biased gene expression appears to be mediated by a relatively small number of highly conserved transcription factors (Ament *et al.* 2012; Chandrasekaran *et al.* 2011; Zayed and Robinson 2012), indicating the possibility of a transcriptional toolkit for sociality.

7.2.4 Conservation and Novelty across the Spectrum of Eusociality

Most investigations of the transcriptional control of caste and division of labour in social insects have focused on advanced societies such as those of the honeybee, bumblebee, fire ant and harvester ant (Fischman *et al.* 2011; Robinson *et al.* 2005; Whitfield *et al.* 2006). More recently, several studies have investigated these phenomenon in primitively eusocial species, which has revealed many of the same toolkit genes involved in sociality through independent evolutionary origins (Bonasio *et al.* 2010; Ferreira *et al.* 2013; Toth *et al.* 2010; Toth *et al.* 2007). However, novel genes also appear to have played a key role in social evolution. High levels of novelty have been found in primitively eusocial paper wasps (Ferreira *et al.* 2013), however here the definition of novelty is based upon an inherently biased selection of available genomic data. A greater level of novelty in *Polistes* may therefore be more reflective of the paucity of sequence data available for the wasps rather than their primitive social structure. A comparison of the genomes of the ponerine ant *H. saltator* and the advanced ant *C. floridanus* revealed a greater number of species-specific (novel) genes in *C. floridanus*, enriched for functions relating to sensory and particularly odorant-binding processes, and detoxification (Bonasio *et al.* 2010). However, numbers of novel genes were low compared to results for *P. canadensis* (Ferreira *et al.* 2013). Thus, this suggests reduced levels of novelty in secondarily primitive species, although more data is needed to investigate this initial trend further.

The ponerine ants offer a unique opportunity to study the dynamics of social evolution. Ponerine ants include species that have either partly or completely replaced the queen caste with reproductively active workers, secondarily reverting to a state of primitive eusociality (Monnin and Peeters 2008; Peeters 1991; Schmidt 2013). *H. saltator* represents an intermediate step between advanced society (morphological

queen, worker sterility) and secondary primitive behaviour (no queen caste, reproductive workers)(Monnin and Peeters 2008; Peeters 1991). Their societies include queens, who are replaced by gamergates following her death, thereby extending colony longevity (Monnin and Peeters 2008; Peeters 1991). Other species such as the dinosaur ant, *Dinoponera quadriceps*, have completely lost the queen caste. Colonies are headed by a singly-mated reproductive female known as the gamergate (alpha female), followed by a hierarchy of potential reproductives who queue to replace the current gamergate when she dies (Monnin and Peeters 1999). Dinosaur ants therefore offer the opportunity to investigate how division of labour, both reproductive- and non-reproductive, is achieved in a species with a primitive social structure, but advanced ancestry. Here, I investigate the relative contributions of conserved and novel genes to polyphenism in the dinosaur ant, *Dinoponera quadriceps*.

7.2.5 Aims and Hypotheses

Using next generation sequencing, I investigate the role of novel and conserved genes in creating division of labour within a secondarily primitive ant, *Dinoponera quadriceps*. Specifically, I investigate the relative contribution of conserved and novel genes to rank phenotypes, and to reproductive phenotype. In addition, I look for genes identified as caste-biased in other eusocial species in the transcriptome of *Dinoponera quadriceps*, and compare their expression pattern with other species.

7.3 Methods

7.3.1 Genome and Transcriptome Sequencing, Alignment and Annotation

In order to investigate the contribution of conserved and novel genes to caste evolution in *D. quadriceps*, I first obtained a full genome sequence from a single haploid male, together with brain transcriptome sequences for 18 adult females of three ranks (‘alpha’ – gamergate / reproductive worker, ’beta’ – highest ranked subordinate, ‘low’ – low-ranked subordinate)(see chapter 6 for full methods). Briefly, DNA was extracted using a phenol-chloroform extraction, sequenced using Illumina sequencing 540nt paired-end. RNA extractions were performed on 18 females of known rank and ovarian activity using a Direct-Zol RNA miniprep kit. Rank of each

female was determined using behavioural observations of ritualised aggressive interactions (chapter 2) and ovarian activity was determined with ovary dissections. RNA sequencing was performed using Illumina HiSeq 2000, 100nts paired-end reads. DNA sequence data was aligned using the EVM and PASA pipelines. The genome sequence provided a reference genome to improve alignment of the transcriptome sequences. mRNA sequences were aligned to the reference genome and assembled into transcripts using bowtie-0.12.9 and cufflinks. Read counts were calculated, normalised and converted to gene-level using BitSeq. Differential expression analyses were performed for pairwise rank comparisons (alpha vs. beta, beta vs. low and alpha vs. low), and for ovarian activity (developed vs. undeveloped) using BitSeq. Lists of differentially expressed genes were generated for each comparison, as well as lists of up-regulated genes for each phenotype. Genes were considered to be significantly differentially expressed if they had a probability of positive log ratio (pplr) of less than 0.46 (down-regulated) or greater than 0.54 (up-regulated).

Functional annotation of the transcriptome was performed using a BLASTP of the consensus protein set against all available sequence data on NCBI (Sayers *et al.* 2009). Hits with a greater than 50% sequence homology and $e10^{-2}$ were considered to be homologous, and the gi_number of the closest match (gene with the highest sequence identity) was associated with that transcript. In each case, the unique, species- and gene-specific identifier generated by NCBI, 'gi_number', of the closest homologue was assigned to each gene. Genes for which no close homologue was available were marked as novel. In addition, CA performed a BLASTP of the consensus protein set against the *Apis mellifera* genome, in order to determine the closest honeybee homologue for each transcript. This enabled a more accurate investigation of novelty in *D. quadriceps*, as the availability of several ant genome sequences (Bonasio *et al.* 2010; Nygaard *et al.* 2011; Smith *et al.* 2011; Suen *et al.* 2011; Wurm *et al.* 2011) might lead to an underestimate of novelty in comparison with other taxa such as wasps in which sequence data is available for only very few species (Werren *et al.* 2010).

7.3.2 Comparing Gene Lists

In order to investigate the contribution of previously identified toolkit genes to the differentially expressed gene lists generated for *Dinoponera quadriceps*, I performed a literature search and generated a list of 180 genes which have previously been identified as differentially expressed in other eusocial hymenopteran species (*table 7.3*). This list included genes from 14 species, in 26 academic papers (*appendix A6*). I then attempted to locate these genes in the functional annotation of the *D.quadriceps* transcriptome, and obtained a gi_number for the closest homologues found (gene with the highest sequence identity). This generated a final list of 373 unique gi_numbers that map to putative genes within the transcriptome of *D. quadriceps*, which was compared to the lists of differentially expressed genes generated for *D. quadriceps* in relation to rank or ovarian activity.

7.4 Results

7.4.1 Novel and Conserved Genes

The number of unannotated genes across the transcriptome as a whole was 2851 (18.1%). A slightly, but non-significantly, greater than average percentage of those genes identified as differentially expressed between the alpha and low-ranked individuals were unannotated (24.6%; $\chi^2 = 0.946$, p = 0.369; *table 7.1*).

Table 7.1 Novel and Known Differentially Expressed Genes

The total number of differentially expressed genes at the pplr > 0.04 level for rank and ovarian activity. Number of those genes which matched known genes in other species for which sequence data is available, and the number of those that were up-regulated in each comparison. Number of genes for which no match was found ('novel genes'), and the number of those up-regulated.

Comparison	Significant	Known Genes	Known Genes Up-Regulated	Novel Genes	Novel Genes Up-Regulated
Alpha vs. Beta	173	141	118	**32** (18.5%)	26
Beta vs. Low	158	128	56	**31** (19.6%)	11
Alpha vs. Low	137	104	79	**34** (24.6%)	28
Ovarian Activity	170	134	70	**37** (21.6%)	26

More novel genes were up-regulated in the Alpha compared to the other two ranks, around 80% of novel differentially expressed genes being up-regulated in the alpha compared to the beta and low-ranker (*figure 7.1*). In contrast, only 35% of novel

differentially expressed genes were up-regulated in the beta compared to the low-ranker. This mirrors a general pattern of down-regulation of genes in the beta compared to the other two ranks. Ovarian activity also showed a large number of differentially expressed novel genes, with around 70% of genes differentially expressed between reproductively active and inactive workers being novel.

In terms of the total number of up-regulated genes, for both alpha-beta and beta-low comparisons, the percentage of up-regulated genes that were novel was similar to the transcriptome-wide mean of 18%. However, for alpha-low and ovarian activity comparisons, there was a significantly higher percentage of novel genes present in the list of differentially expressed genes (χ^2 = 0.998, p = 0.042; *table 7.2*).

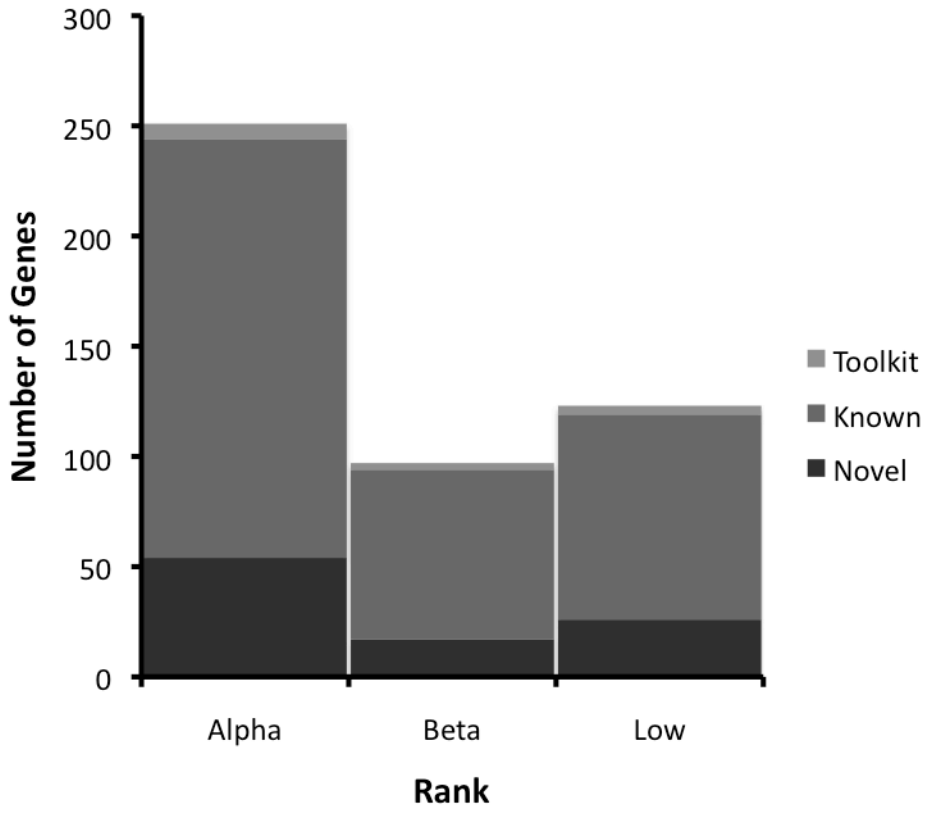


Figure 7.1 Up-Regulation of Novel, Conserved and Toolkit Genes

Stacked bar chart indicating numbers of up-regulated genes in each rank in terms of (1) novel genes, (2) known genes and (3) toolkit genes.

Table 7.2 Up-Regulation of Novel Genes

The contribution of novel up-regulated genes to the total number of novel genes and to the total number of up-regulated genes.

Comparison	Novel Up-Regulated Genes as % of Novel Genes	Novel Up-regulated Genes as % of Up-regulated Genes
Alpha vs. Beta	81.25%	18.06%
Beta vs. Low	35.48%	16.42%
Alpha vs. Low	82.35%	26.17%
Ovarian Activity	70.27%	27.08%

Most genes in *D. quadriceps* shared their closest homologue with another ant species (10464, 66.33%), particularly *C. floridanus* (6836, 43.33%), *H. saltator* (3177 genes, 20.14%) and *S. Invicta* (314, 1.99%). However, many of these 'genes' were duplicates, where one gene sequence from another species mapped as the closest homologue to more than one putative gene in *D. quadriceps*. Amongst the 7443 unique gi_numbers that mapped as the closest homologue to a *D. quadriceps* gene, 79.45% were from another ant species. Of these, 3852 (51.76%) were from *C. floridanus*, 1780 (23.92%) from *H. saltator*, and 192 (2.58%) in *S. invicta*. A direct comparison of *D. quadriceps* transcribed genes with data for *Apis mellifera* found that 65.25% of all *D.quadriceps* genes found a significant homologue in *Apis mellifera*.

7.4.2 Toolkit Genes

Most differentially expressed genes were known genes. Known genes were defined as those for which a homologue was found in another species with greater than 50% sequence homology to *D. quadriceps*. Within these 'known genes' I looked for putative toolkit genes, identified as differentially expressed among provisioners and reproductives in other social insects. I compared differentially expressed gene lists from each comparison with a list of toolkit genes.

My list included 81 genes for Queen-Worker differences, 63 for Worker-Worker differences and 46 for differences between fertile and infertile workers (*table 7.3*). These genes were collected from lists for 14 species. Starting with 180 putative toolkit genes, I identified 112 genes in *D.quadriceps* represented by 373 unique gi_numbers (*figure 7.2*). A total of 78 (41%) putative toolkit genes had no homologue in the *D.*

quadriceps transcriptome. This could be due to variation in the naming and description of genes, because these genes have undergone substantial sequence evolution since their divergence with species for which sequence data is available, or because these genes were not expressed in sufficiently high quantities in the brains of dinosaur ants to be detected in this study. These genes may be expressed in other tissue types.

Table 7.3 Toolkit Gene List

List of toolkit genes compiled from the literature and compared with differentially expressed genes identified in relation to rank and reproductive status in *Dinoponera quadriceps*.

Gene Name	Species	Up-regulated	Reference	Homologue(s)
Queen-Worker				
Actin	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307166491
Alpha-glucosidase	*Bombus terrestris*	Worker	(Colgan *et al.* 2011)	gi_307207957
Alpha-2-macroglobulin	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307191156, gi_307181438
Alpha-mannosidase	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	gi_307176273, gi_340729800
Anarchy-1	*Polistes metricus*	Queen	(Toth *et al.* 2010)	
Argenine Kinase	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307202259, gi_148189777
Arrestin	*Polistes canadensis*	Worker	(Sumner *et al.* 2006)	gi_307176567, gi_110748994,
	Polistes metricus	Queen	(Toth *et al.* 2010)	gi_110748994, gi_307178159
ASP 1	*Lasius Niger*	Queen	(Graff *et al.* 2007)	
ATP-synthase beta chain	*Bombus terrestris*	Worker	(Pereboom *et al.* 2005)	gi_268607737, gi_307174076,
	Polistes metricus	Worker	(Toth *et al.* 2010)	gi_307181472
Beta-tubulin	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307184740, gi_12585365
Black	*Apis mellifera*	Worker	(Grozinger *et al.* 2007)	
Bomboilitin	*Bombus terrestris*	Worker	(Colgan *et al.* 2011)	
Carboxylesterase	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_345497204, gi_340722695
Chymotrypsin-2	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307184098
Cr-PII allergen	*Vespula squamata*	Worker	(Hoffman and Goodisman 2007)	
Cytochrome oxidase I	*Bombus terrestris*	Worker	(Pereboom *et al.* 2005)	
Cytochrome P450	*Bombus terrestris*	Worker	(Colgan *et al.* 2011)	
	Melipona quadrifasciata	Worker	(Judice *et al.* 2004)	> 5 gi_numbers
	Polistes metricus	Worker	(Toth *et al.* 2010)	
Cytochrome P450 reductase	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307211203
Ribosomal protein S29	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_110756649

DAP3				
Dmrt93	*Apis mellifera*	Queen	(Grozinger *et al.* 2007)	
Epidermal Growth Factor EGF-R	*Apis mellifera*	Queen	(Grozinger *et al.* 2007)	gi_307167404
Egg-derived tyrosine-like	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	
Eye-specific diacylglycerol kinase	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307168937
Fatty acid binding protein	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_ 307169705
Foraging	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_239946290, gi_307195802, gi_307180736, gi_307195803
Glutamate Transporter Am-EAAT	*Polistes metricus*	Worker	(Toth *et al.* 2010)	
Glycerol-3-phosphate dehydrogenase	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307174031, gi_156540304
Gram-negative binding protein Gnbp3	*Apis mellifera*	Queen	(Grozinger *et al.* 2007)	gi_254548011
Guanylate cyclase soluble subunit beta-1	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_340715029
Heat Shock Protein HSP83	*Polistes metricus*	Worker	(Toth *et al.* 2010)	> 5 gi_numbers
Hexamerin	*Bombus Terrestris*	Gyne	(Colgan *et al.* 2011)	gi_149939403, gi_307203246,
Hexamerin 2	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	gi_307181851, gi_307174516
Histone 2A	*Lasius Niger*	Queen	(Graff *et al.* 2007)	gi_110749621,
	Polistes metricus	Worker	(Toth *et al.* 2010)	gi_307178968, gi_291242773,
Histone 2B	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307168955
Immune reactive putative protease inhibitor Prlnh6	*Polistes metricus*	Worker	(Toth *et al.* 2010)	
Insulin-like peptide 1	*Apis mellifera*	Worker	(Corona *et al.* 2007)	
Insulin-like peptide receptor	*Apis mellifera*	Worker	(Corona *et al.* 2007)	gi_307188412
kazal-type proteinase inhibitor	*Vespula squamata*	Worker	(Hoffman and Goodisman 2007)	gi_307181886
Kettin	*Melipona quadricfasciata*	Queen	(Judice *et al.* 2004)	
Kinesin family member 21A	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307203097
Major Royal Jelly Protein	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	gi_307175856
Methionine suloxide reductase	*Polistes metricus*	Worker	(Toth *et al.* 2010)	
Mitochondrial malate dehydrogenase	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307180366, gi_115651961, gi_156555485
Large Conductance Calcium Activated Potassium	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_328787228
Long-wave Opsin	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_118150512, gi_157109734

Long-wavelength Rhodopsin	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_2499367, gi_307171284
Myosin	*Melipona quadricfasciata*	Worker	(Judice *et al.* 2004)	< 5 gi_numbers
Odorant-binding protein OBP-1 precursor	*Vespula squamata*	Worker	(Hoffman and Goodisman 2007)	
Period	*Apis mellifera*	Queen	(Grozinger *et al.* 2007)	gi_340723130, gi_307176609
Perioredoxin	*Bombus terrestris*	Queen	(Pereboom *et al.* 2005)	gi_307175821,
	Polistes canadensis	Worker	(Sumner *et al.* 2006)	gi_307207876, gi_283436152
Phosphoenolpyruvate carboxykinase	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307171353
Phospholipase C	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	
1-phosphatidylinositol-4,5-bisphosphate	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307167498, gi_307207893, gi_307189725, gi_307171492, gi_307204257
Poly-ADP-ribose polymerase	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	
Porin Q8T4K0	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	
Prophenoloxidase activating factor	*Polistes metricus*	Queen	(Toth *et al.* 2010)	
Projectin	*Melipona quadricfasciata*	Worker	(Judice *et al.* 2004)	
Prolyl Endopeptidase	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	gi_307177097
Pyruvate Kinase	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307177342
Rab11 G protein	*Melipona quadricfasciata*	Worker	(Judice *et al.* 2004)	gi_307200531
Rab geranylgeranyltransferase beta subunit	*Polistes metricus*	Queen	(Hoffman and Goodisman 2007)	> 5 gi_numbers
Ribonuclease T2 family	*Polistes metricus*	Worker	(Toth *et al.* 2010)	
Ribonucleoprotein F	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_156550801, gi_110760095
Ribosomal protein L9	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307208757, gi_307179102, gI_170055556
Ribsomonal protein S8	*Polistes metricus*	Queen	(Toth *et al.* 2010)	
Sallimus	*Bombus terrestris*	Worker	(Colgan *et al.* 2011)	
Sas10 (UTP3)	*Melipona quadricfasciata*	Worker	(Judice *et al.* 2004)	
Small Heatshock Protein	*Polistes metricus*	Worker	(Toth *et al.* 2010)	> 5 gi_numbers
Sorbitol dehydrogenase SodH1	*Apis mellifera*	Queen	(Grozinger *et al.* 2007)	gi_307181502, gi_307204829
SPARC	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307178967
Schwannomin interacting protein 1	*Polistes metricus*	Queen	(Toth *et al.* 2010)	
ss-alanyl conjugating enzyme	*Polistes metricus*	Queen	(Toth *et al.* 2010)	
Thrombin inhibitor protein	*Lasius Niger*	Queen	(Graff *et al.* 2007)	

Transferrin	*Apis mellifera*	Queen	(Grozinger *et al.* 2007)	gi_307206988, gi_307173763, gi_307175377
	Polistes canadensis	Queen	(Sumner *et al.* 2006)	
Troponin C type I	*Polistes metricus*	Queen	(Toth *et al.* 2010)	gi_307169927, gi_121543993
Tubulin alpha-1 chain	*Polistes canadensis*	Queen	(Sumner *et al.* 2006)	gi_307178877, gi_156548149, gi_110755732
Ubiquinol-cytochrome c oxidoreductase subunit	*Polistes metricus*	Queen	(Toth *et al.* 2010)	
Ubiquitin conjugating enzyme E2	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_114051115
Ubiquitin-specific protease 47	*Polistes metricus*	Worker	(Toth *et al.* 2010)	
Vitellogenin	*Lasius niger*	Queen	(Graff *et al.* 2007)	
	Polistes Canadensis	Queen	(Sumner *et al.* 2006)	
	Polistes metricus	Queen	(Toth *et al.* 2010)	gi_307208884, gi_307182721
Vitellogenin Vg 1, Vg4	*Solenopsis invicta*	Worker	(Wurm *et al.* 2011)	
Vitellogenin Vg2, Vg3	*Solenopsis invicta*	Queen	(Wurm *et al.* 2011)	
Yellow g2	*Lasius niger*	Queen	(Graff *et al.* 2007)	gi_110762773
Zinc binding FYVE Finger Protein	*Polistes metricus*	Worker	(Toth *et al.* 2010)	gi_307173674, gi_307179417, gi_307172323, gi_307192904, gi_156551702
Small zinc finger-like protein	*Polistes metricus*	Queen	(Toth *et al.* 2010)	< 5 gi_numbers
Worker - Worker				
Acetylecholinesterase	*Apis mellifera*	Forager	(Shapira *et al.* 2001)	
Alpha-glucosidase	*Apis mellifera*	Forager	(Kucharski and Maleszka 2002)	gi_307188051, gi_307185295, gi_307207957
Alpha-glucosidase 2	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	
Amino-peptidase	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	gi_307210584, gi_307175765, gi_307168388
Amino-peptidase 2	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	
Amino transferase	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	> 5 gi_numbers
ATP synthase beta chain	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	gi_268607737, gi_307174076, gi_307181472
Carbonate dehydrase CAH1	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
Cox1	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	gi_307186856, gi_307206206, gi_110763486
Cox10	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	
Cu / Zn Superoxide dismutase	*Polistes metricus*	Forager	(Daugherty *et al.* 2011)	gi_307183176, gi_296232048, gi_307165952, gi_295849286
Cysteine protease inhibitor CG12163	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	

Cytochrome P450	*Reticulitermes flavipes*	Soldier	(Tarver *et al.* 2012)	> 5 gi_numbers
Cytoskeletal protein binding Mlc-c	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
Elongation Factor Ef2b	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	> 5 gi_numbers
Eukaryotic initiation factor elF-4a	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	gi_307169387, gi_307189936
Endopeptidase inhibitor CG32354	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
Fax	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
Foraging	*Apis mellifera*	Forager	(Ben-Shahar *et al.* 2002)	gi_239946290, gi_307195802, gi_307180736, gi_307195803
	Pogonomyrmex barbatus	Nurse	(Ingram *et al.* 2005)	
Gld	*Apis mellifera*	Forager	(Kucharski and Maleszka 2002)	gi_307207584
Glutamate 5-kinase	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
Glutamate Synthase Gs2	*Apis mellifera*	Forager	(Kucharski and Maleszka 2002)	gi_307171087
Heat shock protein 20	*Apis mellifera*	Forager	(Kucharski and Maleszka 2002)	> 5 gi_numbers
Heat shock protein 83	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	
Hexamerin	*Camponotus floridanus*	Minor	(Simola *et al.* 2013)	gi_149939403, gi_307203246, gi_307181851, gi_307174516
Hymenoptaecin	*Camponotus floridanus*	Minor	(Simola *et al.* 2013)	gi_307199121
	Apis mellifera	Non-Forager	(Kucharski and Maleszka 2002)	
Inositol 1,4,5-triphosphate 3 kinase	*Apis mellifera*	Forager	(Kucharski and Maleszka 2002)	
Inositol-3-phosphate (Inos)	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
Insulin-like peptide	*Polistes metricus*	Non-Forager	(Daugherty *et al.* 2011)	
	Apis mellifera	Forager	(Ament *et al.* 2010)	
Insulin-like receptor	*Homotermopsis sjostedti*	Soldier	(Hattori *et al.* 2013)	gi_307188412
	Apis mellifera	Forager	(Ament *et al.* 2010)	
Juvenile hormone	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
	Polistes dominulus	Forager	(Shorter and Tibbetts 2009)	
Juvenile Hormone Esterase	*Camponotus floridanus*	Minor	(Simola *et al.* 2013)	
Kuzbanian	*Polistes metricus*	Forager	(Daugherty *et al.* 2011)	
Lectin	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	
Malvolio	*Apis mellifera*	Forager	(Ben-Shahar *et al.* 2004)	gi_307174133
Major Royal Jelly Protein 2	*Apis mellifera*	Forager	(Kucharski and Maleszka 2002)	gi_307175856

MAP Kinase (ERK7)	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	gi_112982906
Mesoderm development CG11314	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
metabotropic glutamate receptor mGluR2	*Camponotus floridanus*	Minor	(Simola *et al.* 2013)	
Monocarboxylate porter CG8271	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	> 5 gi_numbers
Myosin regulatory light chain	*Polistes canadensis*	Newly Emerged	(Sumner *et al.* 2006)	gi_307212512, gi_307171954
Nucleic acid and Zn bindin	*Polistes metricus*	Forager	(Daugherty *et al.* 2011)	
Origin recognition complex Orc 1	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
Organic cation porter CG7442	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	> 5 gi_numbers
Oxidoreductase CG6910	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	gi_307189198, gi_328789575
	Polistes metricus	Forager	(Daugherty *et al.* 2011)	
Pebill	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
Penelope transposable element	*Polistes canadensis*	Newly Emerged	(Sumner *et al.* 2006)	
Period	*Apis mellifera*	Forager	(Bloch 2010)	gi_340723130
Peritrophin	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	gi_307192229, gi_307212772
Prolyl isomerase	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	gi_307185712
Protein Kinase B	*Homotermopsis sjostedti*	Soldier	(Hattori *et al.* 2013)	gi_307183121, gi_307183119
Receptor signalling protein CG30387	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
Retinoid / fatty acid binding protein	*Polistes metricus*	Forager	(Daugherty *et al.* 2011)	
RNA methyltransferase CG30387	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	gi_307183163, gi_307182612, gi_307197937
RNA splicing factor U2af50	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	
Ribosomal protein RpS19	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	gi_307196693
Scp1	*Apis mellifera*	Non-Forager	(Kucharski and Maleszka 2002)	
Sh38	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
SPARC	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	gi_307178967, gi_328793504
	Apis mellifera	Non-Forager	(Kucharski and Maleszka 2002)	
Small ribonucleoprotein SmD3	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	gi_307185014, gi_156550773
Translationally controlled tumour protein Tctp	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
Tungus	*Polistes metricus*	Non-Forager	(Daugherty *et al.* 2011)	
Target of Rapamycin (TOR)	*Polistes metricus*	Forager	(Daugherty *et al.* 2011)	

Translation initiation factor CG11334	*Apis mellifera*	Forager	(Whitfield *et al.* 2006)	> 5 gi_numbers
Ultraspiracle	*Polistes metricus*	Non-Forager	(Daugherty *et al.* 2011)	
Vitellogenin	*Camponotus floridanus*	Major	(Simola *et al.* 2013)	gi_307208884, gi_307182721
Zormin	*Apis mellifera*	Nurse	(Whitfield *et al.* 2006)	
Fertile Worker – Infertile Worker				
Beta-glucosidase	*Cryptotermes secundus*	Fertile Worker	(Weil *et al.* 2007)	gi_307175771, gi_189234578
Cabut	*Apis mellifera*	Infertile Worker	(Cardoen *et al.* 2011)	
Chymotrypsin	*Bombus terrestris*	Infertile Worker	(Pereboom *et al.* 2005)	gi_307212124, gi_307180993, gi_307184098
Cytochrome oxidase I	*Bombus terrestris*	Infertile Worker	(Pereboom *et al.* 2005)	
Cytochrome P450	*Apis mellifera*	Infertile Worker	(Cardoen *et al.* 2011)	52 gi_numbers
E3 ubiquitin-protein ligase MARCH3	*Cryptotermes secundus*	Fertile Worker	(Weil *et al.* 2007)	
	Apis mellifera	Infertile Worker	(Cardoen *et al.* 2011)	> 5 gi_numbers
Ecdysteroid-regulated gene E93/mblk-1	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	gi_307181445, gi_110764750
Epac isoform C	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	
FAM47C	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	
Farnesyl pyrophosphate synthase	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	gi_307166112
Fep3C	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	
G2/mitotic-specific cyclin A	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	gi_156549324
Histone H4	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	gi_221090895
Glucocerebrosidase	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	
Guanylate cyclase	*Cryptotermes secundus*	Fertile Worker	(Weil *et al.* 2007)	gi_307166642, gi_307210703, gi_307182432, gi_307212420
Histone 2A	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	gi_110749621, gi_307178968, gi_291242773,
Hoepel1	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	
Huckebein	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	
Integral membrane protein DUF6	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	
Juvenile hormone esterase (Neofem1)	*Cryptotermes secundus*	Fertile Worker	(Weil *et al.* 2007)	
Kinesin 8	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	> 5 gi_numbers
Ligand-gated chloride	*Apis mellifera*	Infertile	(Cardoen *et al.*	gi_118150482

channel homolog 3		Worker	2011)	
Maelstrom	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	
Major Royal Jelly Proteins (2, 3, 4, 5, 7)	*Apis melligera*	Infertile Worker	(Thompson *et al.* 2006)	gi_307175856
Mapmodulin	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	
Myosin	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	> 5 gi_numbers
Netrin receptor unc5	*Apis mellifera*	Infertile Worker	(Cardoen *et al.* 2011)	gi_328776954, gi_307173827
NPC2	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	gi_307171456, gi_156552270
Obstractor d	*Apis mellifera*	Infertile Worker	(Cardoen *et al.* 2011)	
Odorant binding protein	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	
Ribosomal protein RpL6	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	gi_307178359
Ribosomal protein RpS19e	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	gi_307196693
Serpin (Serine protease inhibitor)	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	gi_307182573
Spc24 subunit of Ndc80	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	gi_307181208
Synapsin	*Apis mellifera*	Infertile Worker	(Thompson *et al.* 2006)	gi_307176394, gi_307176392
Targeting protein for xklp2	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	gi_307181326
Triglyceride lipase	*Apis mellifera*	Infertile Worker	(Cardoen *et al.* 2011)	
Ubiquitin	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	
Vitellogenin	*Cryptotermes secundus*	Fertile Worker	(Weil *et al.* 2007)	gi_307208884, gi_307182721
	Apis mellifera	Nurse	(Corona *et al.* 2007)	
Voltage-gated potassium channel	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	> 5 gi_numbers
Wech	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	
Yellow-g	*Apis mellifera*	Fertile Worker	(Cardoen *et al.* 2011)	gi_110762773
Z band alt. Spliced PDX-Motif protein 66	*Apis mellifera*	Infertile Wo	(Cardoen *et al.* 2011)	gi_289629210
Zinc finger protein	*Cryptotermes cynocephalus*	Fertile Worker	(Weil *et al.* 2009)	> 5 gi_numbers

I was able to identify 17 putative toolkit genes within my list of differentially expressed genes (*table 7.4, figure 7.2*). Seven toolkit genes were found to be differentially expressed between the alpha and beta in this study, four were differentially expressed between the beta and low, and a further three between alpha and low (*figure* 7.3). Additionally, three toolkit genes were identified as being differentially expressed in

relation to ovarian activity. Of particular interest, histone 4A, cytochrome P450, guanylate cyclase, ribosomal protein L9, hexamerin and a zinc finger protein were among genes identified as differentially expressed in *D. quadriceps*.

Five putative toolkit genes were identified as being up-regulated in the alpha (histone H4 replacement, cytochrome P450, hexamerin, ribosomal protein L9 and 1-phosphatidylinositol-4,5-bisphosphate). Four genes were up-regulated in the beta-rank (tubulin beta-1, guanylate cyclase, aspartate aminotransferase, cytochrome P450), and five genes were up-regulated in low-ranking workers (monocarboxylate transporters 9 and 12, cytochrome P450, zinc finger protein 800 and gram-negative bacterial binding protein). Cytochrome P450 and aminopeptidase N were up-reguated in reproductive individuals (all alphas and some betas), while 1-phosphatidylinositol-4,5-bisphosphate was up-regulated in non-reproductive individuals (all low-ranks and some betas).

Table 7.4 Differentially Expressed Toolkit Genes

Toolkit genes identified as differentially expressed between individuals of different rank in *Dinoponera quadriceps*. The name and gi_number of homologues identified in *D.quadriecps* are listed, along with details of previous data for the gene in other species (comparison type, caste in which it is up-regulated) and the pplr and fold change results for the particular comparison in dinosaur ants. Arrows indicate whether the fold change represents an up-regulation (fold change > 1) or down-regulation (fold change < 1) in the primary caste. Finally, the number of *D. quadriceps* genes which map to that particular gi_number is listed, along with the number of those for which a significant difference was identified at the pplr threshold of 0.04. Agreement between the result for *D.quadriceps* and other species is indicated as follows (darker grey – high agreement, lighter grey – agreement, white – no agreement).

Gene	Gi Number	Social Insects		Dinosaur Ant		
		Comparison	Up-regulated	Abs(pplr)	Fold Change	No. Genes in *D. quadriceps* (significant)
Alpha vs. Beta						
Similar to Histone H4 Replacement [*Harpegnathos saltator*]	221090895	FWIFW	Fertile Worker (*Apis mellifera*)	0.054	↑ 1.082	3 (1)
Cytochrome P450 4g15 [*Camponotus floridanus*]	307178521	FWIFW	Fertile Worker (*Cryptotermes secundus*)	0.052	↑ 1.045	10 (1)
		FWIFW	Infertile Worker (*Apis mellifera*)			
		QW	Worker (*Bombus terrestris*)			
Hexamerin [*Harpegnathos saltator*]	307203246	QW	Gyne (*Bombus terrestris*)	0.042	↑ 1.025	2 (1)
		QW	Queen (*Polistes*			

			Canadensis)			
Tubulin beta-1 chain [*Manduca sexta*]	12585365	QW	Queen (*Polistes metricus*)	0.046	↓ 0.7083	2 (1)
Guanylate cyclase soluble subunit beta-1-like [Bombus terrestris]	340715029	QW	Queen (*Polistes metricus*)	0.041	↓ 0.9568	1 (1)
39S Ribosomal Protein L9, mitochondrial [*Camponotus floridanus*]	307179102	QW	Queen (*Polistes metricus*)	0.042	↑ 1.0392	1 (1)
Aspartate aminotransferase [*Camponotus floridanus*]	307180800	WW	Non-Forager (*Apis mellifera*)	0.041	↓ 0.9639	2 (1)
Beta vs. Low						
Cytochrome P450 4C1 [*Camponotus floridanus*]	307183577	FWIFW FWIFW QW	Fertile Worker (*Cryptotermes secundus*) Infertile Worker (*Apis mellifera*) Worker (*Bombus terrestris*)	0.057	↑ 1.079	3 (1)
Monocarboxylate transporter 12 [*Camponotus floridanus*]	307189012	WW	Forager (*Apis mellifera*)	0.043	↓ 0.9281	4 (1)
Monocarboxylate transporter 9 [*Camponotus floridanus*]	307178517	WW	Forager (*Apis mellifera*)	0.047	↓ 0.9752	1 (1)
Alpha vs. Low						
Probable Cytochrome P450 6a13 [*Harpegnathos saltator*]	307181693	FWIFW FWIFW QW	Fertile Worker (*Cryptotermes secundus*) Infertile Worker (*Apis mellifera*) Worker (*Bombus terrestris*)	0.041	↓ 0.9448	3 (1)
Zinc Finger Protein 800 [*Camponotus floridanus*]	307182790	FWIFW	Fertile Worker (*Cryptotermes cyanocephalus*)	0.041	↓ 0.9448	1 (1)
Gram-negative bacteria binding protein [*Myrmica ruginodis*]	254548011	QW	Queen (*Apis mellifera*)	0.041	↓ 0.9775	1 (1)
1-phosphatidylinositol-4,5-	307171492	QW	Queen (*Polistes metricus*)	0.041	↑ 1.061	6 (1)

bisphosphate [*Camponotus floridanus*]						
Ovarian Activity						
Cytochrome P450 9e2 [*Harpegnathos saltator*]	307194839	FWIFW	Fertile Worker (*Cryptotermes secundus*)	0.044	↑ 1.0320	12 (1)
		FWIFW	Infertile Worker (*Apis mellifera*)			
		QW	Worker (*Bombus terrestris*)			
1-phosphatidylinositol-4,5-bisphosphate [*Camponotus floridanus*]	307171492	QW	Queen (*Polistes metricus*)	0.042	↓ 0.9608 33861	6 (1)
Aminopeptidase N [*Campontus floridanus*]	307168388	WW	Non-Forager (*Apis mellifera*)	0.048	↑ 1.0734 72327	12 (1)

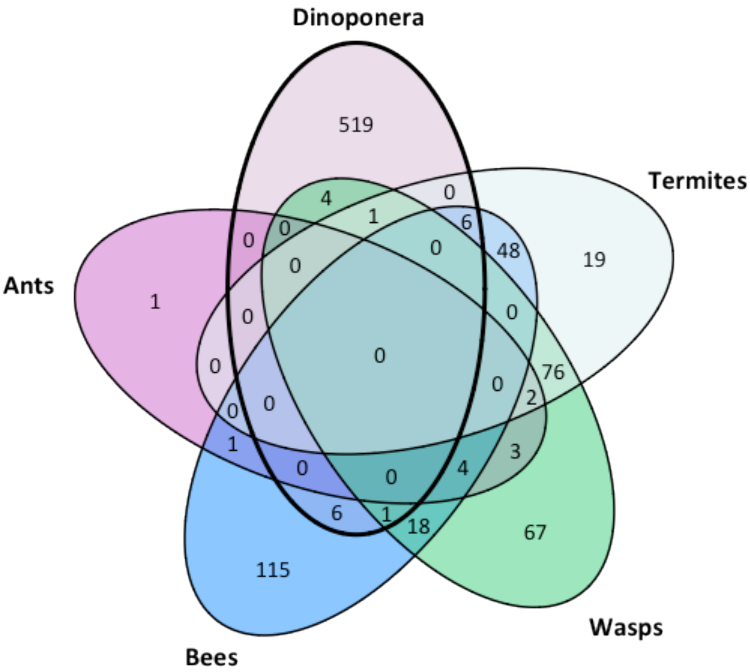


Figure 7.3 Toolkit Genes in Eusocial Insects

Venn diagram of toolkit genes for (1) *Dinoponera quadriceps*, (2) Ants, (3) Bees, (4) Wasps and (5) termites, showing overlap between known genes identified as differentially expressed in *D. quadriceps* in relation to rank or ovarian activity, and known genes in *D. quadriceps* that were identified as being differentially expressed in other social insects.

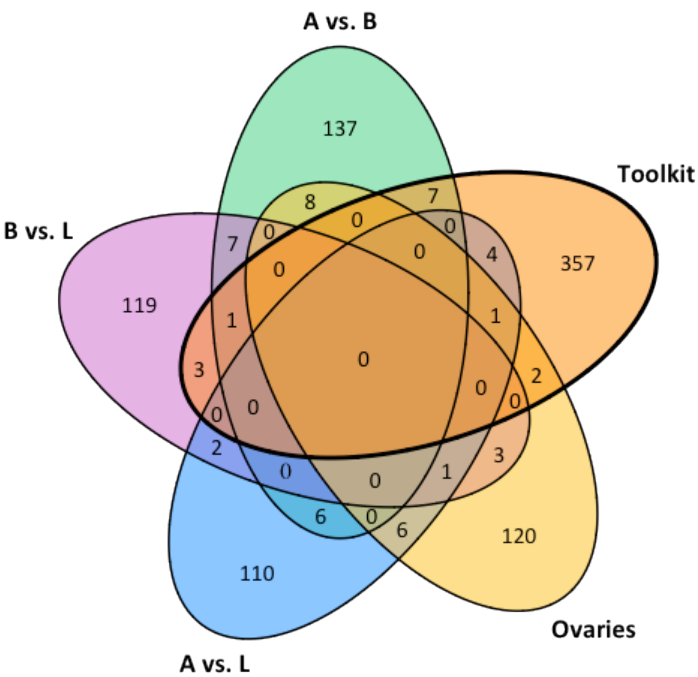


Figure 7.2 Toolkit Genes in Rank and Reproduction

The numbers of genes identified as differentially expressed at the abs(pplr) significance level of 0.04 for pairwise comparisons of rank (alpha vs. beta, beta vs. low, alpha vs. low) and for ovarian activity compared with the list of toolkit genes.

7.5 Discussion

7.5.1 Taxonomically Restricted Genes

Across the entire transcriptome, I found 2851 genes for which functional annotation was not possible, indicating that these genes shared no close, known homologues in other species for which sequence data is available. This represented around 18% of all expressed genes. These genes are therefore likely to be taxonomically restricted, appearing only in *D. quadriceps*, or higher taxonomic groupings such as *Dinoponera*. The closest relative of *D. quadriceps* for which sequence data is available is *Harpegnathos saltator*, which shares a common ancestor with dinosaur ants approximately 70 MYA ago (Schmidt 2013). Thus, 18% of the *D.quadriceps* transcriptome consists of genes which have appeared since their divergence from a common ancestor with *H.saltator*, or which have diverged sufficiently in this time to share less than 50% sequence homology with this species. Taxonomically restricted genes have previously shown to be widespread in other eusocial species including honeybees (Johnson and Tsutsui 2011; Woodard *et al.* 2011) and paper wasps (Ferreira *et al.* 2013), and have been found to contribute disproportionally to the worker caste (Ferreira *et al.* 2013; Johnson and Tsutsui 2011).

The number of novel genes in *D. quadriceps* is much lower than was recently observed for another socially simple species, *P. canadensis*, where 75% of differentially expressed genes were novel (Ferreira *et al.* 2013). One explanation for the overall higher proportion of novel genes in *P. canadensis* reflects the limited availability of sequence data for wasps. 10464 of the 15776 genes found their nearest homologue in another ant; 20.14% in *Harpegnathos saltator*, 43.33% in *Camponotus floridanus*, 1.99% in *Solenopsis invicta*. However, when I compare *D. quadriceps* with the honeybee, I find that 65% of genes in *D. quadriceps* have a homologue with over 30% sequence homology in *Apis mellifera*. This is in contrast to just 31% of genes in *P. canadensis* (e value 10^{-6})(Ferreira *et al.* 2013). Thus this suggests that the relatively small number of novel genes identified in *D. quadriceps* when compared to another socially simple insect seems to reflect a genuine difference rather than a bias in the available sequence data.

Similar levels of unannotated genes were found in my differentially expressed gene lists and the transcriptome as a whole, and no significant departure from the transcriptome-wide mean was found for these lists. However, I did find a significant overrepresentation of novel genes amongst genes up-regulated in the alpha compared to a low-ranker, and between reproductively active and inactive workers. Around 80% of novel genes are up-regulated in the gamergate compared to other ranks. This is in contrast to recent data on *Polistes canadensis*, where 90% of novel genes were up-regulated in the worker caste (Ferreira *et al.* 2013). Unlike the primitively eusocial wasp, where novel genes have contributed more to the worker phenotype, my data suggest that in the secondarily primitive dinosaur ant, *D. quadriceps*, novel genes have contributed to a greater extent to the gamergate phenotype. This may reflect their differing evolutionary histories. *Polistes* have evolved from a solitary ancestor; one of their most recent evolutionary advances, therefore, has been the generation of a reproductively-inactive (albeit temporarily) worker caste (Bell and Sumner 2013). By contrast, *Dinoponera* have evolved from a recent, highly-eusocial ancestor; their most recent evolutionary innovation has been the gamergate phenotype of reproductively active workers where previously workers were universally sterile. Paper wasp societies are comprised of one or a few reproductive females ('queens'), and subordinate workers who retain reproductive totipotency into adulthood, and can take over a dominant role if the opportunity arises (Bell and Sumner 2013).

7.5.2 Toolkit Genes

Of the 180 toolkit genes identified in the literature, 78 (43%) were not found within the transcriptome of *Dinoponera quadriceps*. The remaining 112 yielded a total of 373 similar genes in the *D. quadriceps* transcriptome. Seventeen of these genes were also identified as being differentially expressed in *D. quadriceps*. In many cases, the direction of up-regulation agreed with other species, however this was not always the case, and some genes showed opposite regulatory patterns. This is not unusual, however, in fact several well-characterised toolkit genes such as the foraging gene and vitellogenin have been found to show caste-biased differential expression in opposing directions, or between different castes across species (Ben-Shahar *et al.* 2002; Ingram

et al. 2005; Kodaira *et al.* 2009). This is to be expected, since these toolkit genes must have been independently co-opted for sociality through multiple separate evolutionary origins of eusociality (Johnson *et al.* 2010; West-Eberhard 1987). Below I discuss the relevance of these 17 putatively conserved genes.

7.5.2.1 Cytochrome P450s

In *Dinoponera quadriceps*, I find Cytochrome P450 4g15 to be significantly up-regulated in the alpha compared to the beta, Cytochrome P450 4C1 to be up-regulated in the beta compared to low-rankers, and Cytochrome P450 9e2 to be up-regulated in reproductive active vs inactive workers. These results are most strongly consistent with the previous findings for the damp wood termite, *Cryptotermes secundus,* in which Cytochrome P450 is up-regulated in neonates (reproductive replacements of the queen)(Weil *et al.* 2007). I similarly find up-regulation in the alpha and beta-ranks, representing current and hopefully reproductives, respectively. The alpha female of a dinosaur ant colony is a reproductively active worker rather than a true queen. I also find Cytochrome P450 to be differentially expressed in relation to ovarian activity, further supporting it's involvement in reproduction physiology. A fourth homologue to cytochrome P450 – 'probable cytochrome p450 6a13' – is up-regulated in low-ranked subordinates compared to the alpha. This is more consistent with results for several species of bee, in which Cytochrome P450 is up-regulated in sterile workers (Cardoen *et al.* 2011; Colgan *et al.* 2011; Judice *et al.* 2004).

Cytochrome P450s are a diverse group of heme-containing endoplasmic reticulum-bound enzymes, which catalyse a variety of different oxidative reactions (Feyereisen 1999; Sigel *et al.* 2007). A plethora of P450s have been found, thought to have been generated through repeated duplications of an ancestral gene (Scott and Wen 2001). They are involved in key metabolic processes including lipid metabolism and detoxification (Sigel *et al.* 2007), and also play a role in the oxidation of many hormones and pheromones, including the toolkit hormone JH (Feyereisen 1999; Scott and Wen 2001). In honeybees, Cytochrome P450 is overexpressed during the final larval stages during which caste determination occurs (Corona *et al.* 2007; Evans and Wheeler 2000). It has also been shown to be involved in soldier caste differentiation in

several species of termite, and it is in part regulated by the well-characterised toolkit gene juvenile hormone (Cornette *et al.* 2006; Tarver *et al.* 2012; Zhou *et al.* 2006). Cytochrome P450 metabolises juvenile hormone in house flies (Andersen *et al.* 1997) and cockroaches (Sutherland *et al.* 1998), and may therefore be involved in a regulatory feedback loop with JH.

7.5.2.2 Guanylate Cyclases

In *D. quadriceps,* guanylate cyclase soluble subunit beta-1 is up-regulated in beta-ranked subordinates compared to the alpha. Guanylate cyclases have previously been found to be caste-biased in both paper wasps and termites, in which they are up-regulated in queens and fertile workers respectively (Toth *et al.* 2010; Weil *et al.* 2007). Guanylate cyclase small subunit beta-1 is sensitive to nitric oxide signalling (Hobbs 1997). Some nitric oxide-sensitive guanylate cyclases have been implicated in neuronal maturation in insects (Truman *et al.* 1996). Beta-ranked workers are likely to be the youngest individuals in the colony (Monnin and Peeters 1999), so the overexpression of a NO-sensitive guanylate cyclise in the brains of beta-ranked workers may be involved in age-related neuronal maturation.

Guanylate cyclases are a family of enzymes that catalyse the conversion of GTP into cGMP (Lucas *et al.* 2000). They are therefore important in a number of signalling cascades including regulation of cGMP-regulated phosphodiesterases, cGMP-dependent protein kinases and cyclic nucleotide-gates ion channels (Lucas *et al.* 2000). The role of cGMP-dependent protein kinases in phenotypic plasticity in social insects is well documented in the form of the foraging gene, which has been linked to caste differentiation in a variety of species of both bees and ants (Ben-Shahar *et al.* 2002; Ingram *et al.* 2005; Kodaira *et al.* 2009). Although the foraging gene was not identified as being differentially expressed in relation to rank in *D. quadriceps*, guanylate cyclase is, which may be involved in regulating downstream signaling cascades involving cGMP-dependent protein kinases like the foraging gene.

7.5.2.3 Tubulin and Cytoskeletal Components

Here, I find that tubulin beta-1 chain is up-regulated in the beta compared to the alpha of *D. quadriceps*. The alpha and beta-ranked workers differ in mating status, rank and ovarian activity, however beta-tubulin was not identified as differentially expressed in relation to ovarian activity, suggesting it's differential expression between ranks may represent either a direct effect of rank, or of mating status. Tubulins are cytoskeletal proteins, and together with alpha-tubulin, beta-tubulin is integral in the formation of microtubules (Nielsen *et al.* 2010). Beta-tubulin has previously been shown to be up-regulated in queens compared to workers in the paper wasp *P. metricus* (Toth *et al.* 2010), while alpha-tubulin is up-regulated in queens of *P. Canadensis* (Sumner *et al.* 2006). Cytoskeletal proteins in general may be important in alpha-beta differentiation; I also found the GO functional category 'cytoskeleton' to be overrepresented in genes differentially expressed between the alpha and beta rank (*chapter 6*).

7.5.2.4 Hexamerins

Here, I demonstrate differential expression of hexamerin between the mated reproductive (gamergate) and unmated high-ranking workers of the secondarily primitive ant *Dinoponera quadriceps*, with expression higher in the alpha (gamergate) than the beta-ranked worker. Hexamerin has previously been shown to be up-regulated in queens compared to workers of *Polistes canadensis* (Sumner *et al.* 2006) and up-regulated in gynes of *Bombus terrestris* (Colgan *et al.* 2011), and was found to be up-regulated in the alpha compared to the beta of *D.quadriceps* in this study. Recently, they have also been identified in relation to worker-worker caste differentiation in the carpenter ant, with higher expression of two hexamerin proteins in the brains of major compared to minor workers (Simola et al. 2013). Hexamerins are storage proteins originally derived from hemocyanin proteins. In honeybees, they have been shown to contain a possible ultraspiracle binding site, which is in turn a target of juvenile hormone (Martins *et al.* 2010), and hexamerins have been found to modulate JH availability in termites (Zhou *et al.* 2007). Hex110 and hex70a are both differentially expressed in the fat bodies of adult honeybees, with higher expression in workers than queens (Martins *et al.* 2010). Hexamerins have also been implicated downstream of

the insulin / insulin-like growth factor (IIS) pathway; in honeybees, RNAi of the insulin receptor substrate (IRS) altered hexamerin 110 levels, and was able to override diet and prevent larvae developing as queens (Wolschin *et al.* 2011). In *Polistes metricus*, RNAi of hexamerin 2 failed to produce significant differences in larval or adult traits, but there was a trend towards longer development time and reduced ovary development in treated wasps (Hunt et al. 2011). In *Vespula squamata*, four different ESTs of hexamerin showed different caste-biased expression patterns; two ESTs were up-regulated in queen-destined larvae, the other two ESTs, most similar to hex70a, were up-regulated in worker-destined larvae (Hoffman and Goodisman 2007).

7.5.2.5 Intracellular Signalling

In *D. quadriceps*, 1-phosphatidylinositol-4,5-bisphosphate, I find conflicting patterns in different comparisons. I found up-regulation in the alpha relative to the low-ranked workers, however when comparing between reproductive active and inactive workers, I found up-regulation in non-reproductive ants. 1-phosphatidylinositol-4,5-bisphosphate mediates second messenger molecule production of diacylglycerol and inositol 1,4,5-trishosphate and is involved in the regulation of intracellular signalling cascades. The gene has previously been reported to show caste-biased expression in *P. metricus*, in which is it up-regulated in the queen caste (Toth *et al.* 2010), thus this pattern is consistent with my findings for the alpha vs. beta comparison but not the ovarian activity comparison. A homologue of this gene has been found to be exclusively expressed in the mushroom bodies of honeybee foragers, and may be involved in neuronal transmission (Kamikouchi *et al.* 1998).

Further, two monocarboxylate transporters (9 and 12) appeared on my toolkit gene list and were also differentially expressed in *D. quadriceps*. Monocarboxylate porter CG8271 was found to be up-regulated in foragers compared to workers of *Apis mellifera*, and consistent with this, both monocarboxylate transporters 9 and 12 were found to be up-regulated in low-ranking workers compared to the beta-rank, with log fold changes of -0.035 (pplr = 0.453) and - 0.11 (pplr = 0.457) respectively. In *D.quadriceps*, the beta- and low-ranking workers differ primarily in behaviour, with low-rankers predominantly performing foraging and waste removal tasks, and high-

rankers remaining inside the nest to perform nurse tasks and engage in aggressive interactions which reinforce the dominance hierarchy (Asher *et al.* 2013; Monnin and Peeters 1999). These two genes were not identified as being differentially expressed between fertile and infertile individuals, however, and only between the beta- and low-ranked workers, which may represent their differing behaviour in terms of foraging and brood care.

Monocarboxylate transporters are a family of transporter molecules that mediate the proton-linked transport of monocarboxylates such as lactate and pyruvate across the plasma membrane (Halestrap and Price 1999). Monocarboxylates are important in cellular metabolism and metabolic signalling. Although the family now contains 14 related genes (Halestrap and Meredith 2004), monocarboxylate transporter activity has so far only been confirmed for MCT-1 to MCT-4, thus the role of MCTs 9 and 12 is currently unknown (Halestrap and Price 1999; Halestrap and Meredith 2004). Monocarboxylate porters may therefore be a good target for a toolkit gene, if by gene duplication these genes became available for mediating social evolution across multiple independent origins.

7.5.2.6 Immunity

I find that the immune gene, gram-negative bacterial binding protein (Gnbp) is up-regulated in low-ranking workers compared to the gamergate. Low-ranking workers are exposed to a greater number of pathogens during foraging and waste removal activities (Asher *et al.* 2013; Monnin and Peeters 1999), so we might expect them to up-regulate genes involved in immunity. Gnbp is part of the insect innate immune response, functioning as a pattern recognition enzyme which binds to gram-negative bacteria stimulating an immune response via the toll pathway (Hoffmann *et al.* 1996; Warr *et al.* 2008). Gnbp3 has previously been show to be up-regulated in queens compared to workers in *Apis mellifera* (Grozinger *et al.* 2007), the opposite trend to the one I report for *D. quadriceps*.

7.5.2.7 Amino Acid Synthesis and Catalysis

I find differential expression of aminopeptidase N between reproductively active and inactive dinosaur ants, and aspartate aminotransferase between the alpha and beta-ranked ants. Aminotransferases catalyse reactions between amino acids and keto acids, and are important in the synthesis of some amino acids, while aminopeptidases catalyse the cleavage of amino acids from peptides, and so are essential for many cellular functions and may play a role in protein degradation (Taylor 1993). Aminopeptidase N is up-regulated in reproductively active individuals (alphas and some betas), while aspartate aminotransferase is up-regulated in the beta-ranked worker. Aminopeptidase N is a membrane glycoprotein, thought to be involved in the metabolism of regulatory peptides (Sjöström *et al.* 2002). Two aminopeptidases (1 and 2) and an aminotransferase are overexpressed in non-foraging workers of the honeybee (Kucharski and Maleszka 2002).

7.5.2.8 Ribosomal Proteins

In the dinosaur ant, ribosomal protein L9 is differentially expressed between the alpha and beta-ranks, with higher expression in the gamergate than high-ranking subordinates. Ribosomal proteins are involved in ribosome assembly and function, and some ribosomal proteins also perform extra-ribosomal roles including DNA repair and transcriptional regulation (Marygold *et al.* 2007). A number of ribosomal proteins have been implicated as part of the sociality toolkit, including both large (L) and small (S) subunits. Ribosomal proteins S29 and L9 are involved in queen-worker differentiation in *P. metricus*, while ribosomal proteins S19 and L6 have been found to differ between infertile and fertile honeybees, and in differentiation between foragers and non-foraging bees (Kucharski and Maleszka 2002; Thompson *et al.* 2006).

7.5.2.9 Gene Regulation and Epigenetic Modification

I report the up-regulation of a putative histone H4 replacement gene in the brains of gamergates compared to beta-ranked subordinates in the dinosaur ant, *Dinoponera quadriceps*. Germinal histone 4A has previously been identified as being up-regulated in fertile workers compared to infertile workers of the honeybee, and the closest

homologue in *D. quadriceps* was 'similar to histone H4 replacement', which I find to be up-regulated in the Alpha compared to the Beta rank in *D. quadriceps*. Histones are important epigenetic regulators (Cedar and Bergman 2009), and histone methylation patterns are strongly correlated with DNA methylation patterns, and appear to be strongly conserved across taxa (Hunt *et al.* 2013; Meissner *et al.* 2008). Histone modifications regulate alternative splicing in humans, via their influence on splicing regulators which control splice switching (Luco *et al.* 2010). Several novel histones have been identified in honey bees, which are differentially methylated between castes, and are thought to be involved in several epigenetic functions including transcription initiation and termination (Lyko *et al.* 2010). Furthermore, acetylation of histones H3 and H4 appears to be particularly common in honeybees (Dickman *et al.* 2013). Recently, a whole genome chromatin map of the carpenter ant *Camponous floridanus* revealed specific histone modifications which showed strong caste-bias between major and minor workers (Simola et al. 2013). Histone modifications, particularly acetylation of H3K27, was strongly correlated with caste-biased gene expression (Simola et al. 2013).

Histones identified as being differentially expressed between castes in the honey bee were exclusively histone variants containing introns, differentiating them from non-caste-biased canonical histones which lack introns (Lyko *et al.* 2010). The histone H4 replacement gene in *Drosophila melanogaster*, for which I find a differentially expressed homologue in *D. quadriceps*, also contains introns (Akhmanova *et al.* 1996), consistent with the results for honeybees. These intron-containing histones are thought to have diverged from canonical histones early in eukaryotic evolution, and play a different cellular role, being not directly regulated by the cell cycle as are their canonical counterparts (Akhmanova *et al.* 1996).

I also identify a zinc finger protein (ZFP 800) to be up-regulated in low-rankers compared to the alpha in *D. quadriceps* This is in contrast to the pattern seen in termites, where a zinc finger protein was up-regulated in reproductive compared to infertile workers (Weil *et al.* 2009). Proteins containing zinc finger domains frequently code for transcription regulatory proteins, and include the kruppel family of

transcription factors. Kruppel has been shown to be important in neuronal plasticity in honeybees and fruit flies (Hewes 2008). In particular, I find that a homologue of zing finger protein 800 is differentially expressed between the alpha female (gamergate) and low-ranking workers. Zinc finger protein 800 is a transcription factor and is a member of the krueppel C2H2-type zinc-finger protein (Sayers *et al.* 2009).

7.5.3 Tissue-Specific Expression of Toolkit Genes in Social Insects

This study measured brain mRNA expression in the dinosaur ant*, D. quadriceps*. Thus, differential expression identified in this study and in studies utilising other tissue types are not directly comparable. Many studies of caste-biased gene expression utilise whole bodies, and the size of insect brains may preclude sufficiently large quantities of RNA being extracted for sequencing. The large size of the dinosaur ant allowed me to obtain sufficiently large quantities of mRNA from individual ant brains, thereby capturing individual as well as between-caste variation in gene expression.

The use of brain-tissue may prevent the identification of differential expression for genes which are not directly involved in functions in the brain, for instance genes relating to ovarian activity or immunity. However, by reducing noise caused by tissue-specific expression, the use of brain tissue in this study may enable the identification of brain-specific caste-biased differential expression which might not be detected in whole-body samples (Chittka *et al.* 2012; Simola *et al.* 2013). A few studies have compared the results of differential expression analyses on different tissue types, and found the results to be broadly correlated (Simola *et al.* 2013; Thompson *et al.* 2006), with some tissue-specific genes identified as differentially expressed only in single-tissue samples (Simola et al. 2013).

7.5.4 Conclusions

This represents the first investigation of the importance of novel and conserved genes to caste evolution in the secondarily primitively eusocial ant *Dinoponera quadriceps*. I find around 18% of genes expressed in the brains of dinosaur ants are novel, a number intermediate between advanced and primitively eusocial species (Ferreira *et al.* 2013; Johnson *et al.* 2010). In *D. quadriceps,* greater novelty is observed in genes up-

regulated in the alpha, possibly reflecting the evolutionary history of ponerine ants, in which the reproductively active worker phenotype represents the most recent evolutionary innovation. Although I was unable to identify differential expression for many well supported toolkit genes such as vitellogenin, major royal jelly protein or *for*, I did identify seventeen genes which are differentially expressed in dinosaur ants as well as other eusocial insects. Several of these genes may be involved in the regulation of, or be stimulated by key toolkit genes such as JH and *for*. Several of the 'toolkit' genes I identify as being differentially expressed in *D. quadriceps* are involved in epigenetic modification, and therefore the regulation of other genes. It is possible, therefore, that genes involved in epigenetic modifications are more readily detected as differentially expressed between castes if their differential expression modulates smaller scale changes in a number of down-stream genes which together generate a caste-specific phenotypic effect. I find some evidence for the importance of toolkit genes in *D. quadriceps*, and in particular my results highlight the possibility of a transcriptional toolkit for sociality. Novelty also appears to play a key role, however the majority (78.7%) of transcriptional differences between ranks are in known genes that have not been identified as caste-biased in any other eusocial species to date. Some of these genes may later emerge as important regulators in other social insects, however this remains to be seen.

Chapter 8

General Discussion

8.1 Dinosaur Ant Colonies Are Dynamic

The data I present demonstrates the plasticity of behaviour exhibited by workers in *D. quadriceps* colonies. *Dinoponera* are thought to have evolved their queenless condition in the last 70MY (Schmidt 2009), but despite their recent divergence from a highly eusocial ancestor, behavioural castes are extremely plastic. Rank is strongly predictive of behaviour, but within rank there is still substantial individual variation. Previous work has shown that dinosaur ant workers are able to completely reverse their behaviour in response to changing colony circumstances; attempts at gamergate overthrow are met with extreme aggression when her fertility is high, but workers will support the coup if her fertility has dropped below 75% (Monnin *et al.* 2002). Here, I report further evidence that subordinate behaviour is highly plastic in relation to social circumstance. Individuals will alter their behaviour in relation to their position in the dominance hierarchy, to past experiences of illicit subordinate egg-laying and to the population demography of their environment.

8.1.1 Behavioural Plasticity in Task Choice

I show marked differences in behaviour between ranks, with low-ranking workers dominating foraging and nest defence, and high-rankers specialising in brood care and other intranidal tasks such as nest maintenance. The attention an individual pays to processing new eggs is also a highly plastic behaviour, increasing in response to past experience of foul play. Furthermore, provisioning also appears to be dynamic in *D. quadriceps*, with some individuals choosing to provision more than one neighbouring nest.

8.1.2 Individual Selfishness or Shared Optima?

My data suggest several ways in which individuals may be acting to maximise their indirect fitness. Where the optima varies between individuals and the colony, these

behaviours may be selfishly motivated, however in other cases the individual and colony optima agree and behavioural plasticity may act to maximise colony productivity. The rank-dependent pattern of task choice exhibited by *D. quadriceps*' may represent an attempt by high-rankers to ensure their survival to attain the reproductive role (Cant and Field 2001; Chandrashekara and Gadagkar 1991; Field and Cant 2006; Field *et al.* 2006; O'Donnell 1998). However, low-rankers have little hope of direct reproduction and should therefore be selected to behave so as to maximise indirect fitness. In *D. quadriceps* the low-rankers are older and thus their survival may be of less value to the colony, and their fertility may be reduced, explaining why they preferentially perform dangerous tasks and do not attempt to take over the reproductive role. The rank-based division of labour observed in *D. quadriceps* may therefore represent the optimum for all individuals and may not be the source of significant conflict in the colony. Minimal aggression is directed towards low-ranking workers (Monnin and Peeters 1999), indicating that coercion to perform risky tasks is not necessary in this species. This is in contrast to primitively eusocial wasps in which queens have been observed to physically coerce subordinates into foraging (Bruyndonckx *et al.* 2006; Reeve and Gamboa 1987; Souza and Prezoto 2012). In many social wasps, dominance is regulated by a positive age-based hierarchy (Bridge and Field 2007; Cronin and Field 2007; Pardi 1948), which means that the subordinates are younger, and may explain the need for queen coercion in these species.

While division of labour may not be a source of conflict in *D. quadriceps*, illicit subordinate egg-laying certainly is, and in chapter five I present data demonstrating plasticity in worker policing informed by past experience. Furthermore, the preference of high-rankers for brood care tasks, particularly egg-care, may increase their ability to detect and prevent illicit egg laying by fellow subordinates. I find that individuals increase their cautiousness in processing eggs as their experience of foreign and illicitly laid eggs increases. This suggests that individuals are able to change their behaviour, possibly through alterations to their response thresholds to egg-identity cues, based on past exposure. By doing so, they avoid time-consuming egg checks in colonies where no subordinates are trying to lay eggs. Here, individual experience generates a

plastic colony-level response, allowing colonies to maximise efficiency whilst responding to changing social conditions.

I find further evidence for plasticity in *D. quadriceps* behaviour in chapter two, where I present preliminary evidence for nest drifting over distances of up to 31 meters. This is unlikely to represent errors in nest recognition, but may be explained as a deliberate strategy to maximise inclusive fitness benefits either by provisioning multiple, related nests or through social parasitism (Birmingham *et al.* 2004; Nanork *et al.* 2007; Sumner *et al.* 2007). The latter case is a highly selfish behaviour, however the former may represent the optima for both colonies. Social parasitism, if it occurs in *D. quadriceps,* would be the source of further selection favouring egg policing in colonies where parasitism occurs. Reproduction by fission may mean that neighbouring nests represent 'parent' and 'daughter' nests, in which case nest drifting may be essential to ensure the survival of small, newly-fissioned nests. Further investigations of nest drifting in *D. quadriceps* are needed to determine the individual- and colony-level fitness benefits of this behaviour.

8.1.3 Behavioural Plasticity in Dinosaur Ants

Here, I show considerable behavioural plasticity in the dinosaur ant, *D. quadriceps*. Individuals are able to modify their behaviour in relation to social and ecological conditions, and likely act to maximise their indirect fitness. High-rankers avoid risks, increasing their prospects of gaining direct fitness, and lower-rankers may be increasing their indirect fitness by drifting to other nests. Indirect fitness benefits are maximised through facultative egg-policing and by restricting risky tasks to low-ranking workers, which together maximise colony productivity. In many cases, plasticity may act to maximise the colony-wide fitness, however in other cases individuals may be acting more selfishly.

8.2 Gamergates are Mated Workers

8.2.1 Physiological Differences

The term gamergate literally translates to ‘married worker’ (Peeters and Crewe 1984), and dinosaur ant queens are definitely workers. They show relatively low fertility and live quite short lives (Monnin and Peeters 2008). Similarly, I find relatively few expressional differences between the brains of gamergates and subordinates in relation to rank, foraging behaviour and reproductive physiology (mating status, ovarian activity), despite marked behavioural differences. Dinosaur ant gamergates display a very similar pattern of gene expression to beta-ranked workers, and most differentially expressed genes were up-regulated in the gamergate compared to other workers. The hierarchy of *D. quadriceps* appears to be discontinuous, with the gamergate representing the most distinct phenotype. By contrast, gamergates are behaviourally similar to other high-rankers, engaging in brood care and aggressive interactions. Ovarian activity and mating status are responsible for large expressional differences between individuals, and a greater number than for foraging behaviour, indicating that reproductive physiology may be a key determinant of gene expression.

Overall, relatively few genes mediate the behavioural and physiological differences in the brains of dinosaur ants. The small number and magnitude of differences between behaviourally distinct groups are reminiscent of the pattern observed within the worker caste in advanced eusocial species such as the honeybee (Cardoen *et al.* 2011; Grozinger *et al.* 2007). This is logical, since in evolutionary terms, all dinosaur ants are workers, and the behavioural differences we term ‘caste’ are really accentuated differences within a previously committed worker caste.

8.2.2 Evolutionary Innovations

In many eusocial societies, more genes are up-regulated in workers than in other castes, and novel genes tend to contribute more to the worker phenotype (Ferreira *et*

al. 2013; Johnson and Tsutsui 2011; Toth *et al.* 2010). This may be explained by the fact that the non-reproductive worker caste is the key evolutionary innovation associated with the evolution of eusociality. By contrast, I report a greater number of up-regulated genes and a greater contribution of novel genes to the reproductive phenotype in *D. quadriceps.* This may reflect their unusual evolutionary history, meaning that the emergence of a reproductive active worker (gamergate) phenotype is the most recent evolutionary innovation in this species (Peeters 1991; Schmidt 2013). This view is supported by the finding that reproductive physiology is more important than foraging behaviour in determining gene expression differences between individuals; variation between individuals in foraging behaviour is likely to have been present within the worker caste in their advanced ancestor, whereas differences in reproductive physiology are likely to be a more recent evolutionary innovation.

8.2.3 Transcriptional Control of Behaviour and Physiology

Here, I report a relatively small number of expressional differences in the brains of *D. quadriceps* in relation to dominance rank, provisioning and reproduction, despite substantial differences in behaviour. This may be a result of their evolutionary history, with the differences observed between phenotypes representing a small part of the ancestral variation between castes. The patterns of expressional differences observed in *D. quadriceps* mirrors their evolutionary history and recent loss of the queen caste.

8.3 Dinosaur Ants Retain Remnants of Advanced Society

8.3.1 Allogrooming

Although *D. quadriceps* shows remarkable behavioural plasticity in a variety of circumstances, a number of behaviours are reminiscent of their advanced ancestors. In chapters three and four, I present the first data pertaining to allogrooming in *D. quadriceps*. Eusocial insects, living in large groups of highly related individuals, are particularly vulnerable to parasites and pathogens (Boomsma *et al.* 2005), and several forms of social immunity have evolved in adaptation to this. Allogrooming has not previously been reported in eusocial species with simple society, and in general is

more characteristic of advanced societies where colony sizes tend to be larger (Stow *et al.* 2007). Thus, allogrooming in *D. quadriceps* may be a behaviour conserved from its advanced ancestor. The use of allogrooming in maintaining and stabilising the hierarchy is likely to have evolved since the loss of the queen caste, however, since in advanced societies caste-commitment eliminates the need for a hierarchy.

8.3.2 A Sociality Toolkit

Finally, I present evidence that some genes that form a putative sociality 'toolkit' have been co-opted during the evolution of simple society in *D. quadriceps*. Of 180 toolkit genes identified in other eusocial species, I find 17 are also differentially expressed in dinosaur ants. In particular, I find that several genes associated with epigenetic regulation, such as histones and zinc finger proteins, are differentially expressed between castes. This supports an emerging picture that sociality may be regulated by a transcriptional toolkit, more so than a genetic one (Ament *et al.* 2012; Chandrasekaran *et al.* 2011; Zayed and Robinson 2012).

8.3.3 Ancestral Traits in Dinoponera

Dinoponera quadriceps recently diverged from an advanced ancestor (Peeters 1991; Schmidt 2013), and this is evident in their behaviour and physiology. Allogrooming is a behaviour usually observed in advanced societies, but I also report its occurrence in *D. quadriceps*. As well as playing a continued role in social immunity, allogrooming has also been co-opted for a social role in *D. quadriceps,* most likely after the loss of the queen caste. Several toolkit genes, identified as underpinning phenotypic differentiation in other eusocial species, also play a role in division of labour in *D. quadriceps*, although many key toolkit genes are not involved in generating caste phenotypes. During the reversion to simple sociality, ancestral behaviours and genes have been utilised to generate differences within the previously sterile worker caste, and to stabilise the social hierarchy after the loss of the queen caste.

8.4 Concluding Remarks

In this thesis, I have demonstrated high-levels of behavioural plasticity at both the individual and colony levels, in response to social and environmental conditions. Ponerine ants have secondarily lost the queen caste, and re-evolved a new caste system within the ancestral worker caste. This may explain why we see such high levels of behaviour plasticity, and also explains the occurrence of behaviours that are more characteristic of advanced societies. Higher levels of novelty and a general up-regulation of genes in the reproductive caste is also likely a result of this unusual evolutionary history; in *Dinoponera quadriceps*, the gamergate is a recent evolutionary innovation. However, I show that this plasticity is underlain by relatively few transcriptional differences. The evolutionary history of the ponerine ants has left a clear mark in both the behaviour and physiology of castes. Comparisons between *Dinoponera*, primitively eusocial insects and cooperatively breeding mammals such as meerkats will continue to yield insights into the dynamics of social evolution and social behaviour across the animal kingdom.

Bibliography

Akhmanova, A., K. Miedema and W. Hennig (1996). Identification and Characterization of the *Drosophila* Histone H4 Replacement Gene. FEBS Lett **388**: 219-222.

Alaux, C., S. Sinha, L. Hasadsri, G. J. Hunt, E. Guzmán-Novoa, G. DeGrandi-Hoffman, J. L. Uribe-Rubio, B. R. Southey, S. Rodriguez-Zas and G. E. Robinson (2009). Honey Bee Aggression Supports a Link between Gene Regulation and Behavioral Evolution. Proc Natl Acad Sci USA **106**(36): 15400-15405.

Ament, S., Y. Wang and G. Robinson (2010). Nutritional Regulation of Division of Labor in Honey Bees: Toward a Systems Biology Perspective. Wiley Interdisciplinary Reviews: Systems Biology and Medicine **2**: 566-576.

Andersen, J., J. Walding, P. Evans, W. Bowers and R. Feyereisen (1997). Substrate Specificity for the Epoxidation of Terpenoids and Active Site Topology of House Fly Cytochrome P450 6a1. Chem Res Toxicol **10**(2): 156-164.

Antweb. (2012). "Ant Web." Retrieved 26 January, 2012, from www.antweb.org.

Araujo, A. and Z. Rodrigues (2006). Foraging Behavior of the Queenless Ant *Dinoponera Quadriceps* Santschi (Hymenoptera : Formicidae). Neotrop Entomol **35**(2): 159-164.

Asher, C., S. Sumner, F. Nascimento and W. Hughes (2013). Division of Labour and Risk Taking in the Dinosaur Ant, *Dinoponera Quadriceps*. Myrmecol News **18**: 121-129.

Bell, E. and S. Sumner (2013). Ecology and Social Organisation of Wasps, eLS. John Wiley & Sons, Ltd: Chichester.

Bell, W. J., M. D. Breed, K. W. Richards and C. D. Michener (1974). Social, Stimulatory and Motivational Factors Involved in Intraspecific Nest Defense of a Primitively Eusocial Halictine Bee. J Comp Physiol A **93**: 173-181.

Ben-Shahar, Y., A. Robichon, M. B. Sokolowski and G. E. Robinson (2002). Influence of Gene Action across Different Time Scales on Behavior. Science **296**(5568): 741-744.

Ben-Shahar, Y., N. Dudek and G. Robinson (2004). Phenotypic Deconstruction Reveals Involvement of Manganese Transporter Malvolio in Honey Bee Division of Labor. J Exp Biol **207**(19): 3281-3288.

Beshers, S. N. and J. H. Fewell (2001). Models of Division of Labor in Social Insects. Annu Rev Entomol **46**: 413-440.

Bloch, G. (2010). The Social Clock of the Honeybee. J Biol Rhythms **25**: 307.

Bonabeau, E., G. Theraulaz and J. L. Deneubourg (1996). Quantitative Study of the Fixed Threshold Model for the Regulation of Division of Labour in Insect Societies. P Roy Soc B-Biol Sci **263**: 1565-1569.

Bonasio, R., G. Zhang, C. Ye, N. S. Mutti, X. Fang, N. Qin, G. Donahue, P. Yang, Q. Li, C. Li, P. Zhang, Z. Huang, S. L. Berger, D. Reinberg, J. Wang and J. Liebig (2010). Genomic Comparison of the Ants *Camponotus Floridanus* and *Harpegnathos Saltator*. Science **329**(5995): 1068-1071.

Bonasio, R., Q. Li, J. Lian, N. S. Mutti, L. Jin, H. Zhao, P. Zhang, P. Wen, H. Xiang, Y. Ding, Z. Jin, S. S. Shen, Z. Wang, W. Wang, J. Wang, S. L. Berger, J. Liebig, G. Zhang and D. Reinberg (2012). Genome-Wide and Caste-Specific DNA Methylomes of the Ants *Camponotus Floridanus* and *Harpegnathos Saltator*. Curr Biol **22**(19): 1755-1764.

Boomsma, J. J. (2009). Lifetime Monogamy and the Evolution of Eusociality. Philos T R Soc B **364**(1533): 3191-3207.

Boomsma, J. J. (1989). Sex-Investment Ratios in Ants: Has Female Bias Been Systematically Overestimated? American Naturalist **133**(4): 517-532.

Boomsma, J. J., P. Schmid-Hempel and W. Hughes (2005). Life Histories and Parasite Pressure across the Major Groups of Social Insects. Insect Evol Ecol. M. D. E. Fellowes, G. J. Holloway and J. Rolff. Wallingford, UK, CABI Publishing**:** 139-175.

Bourke, A. F. G. (1988). Worker Reproduction in the Higher Eusocial Hymenoptera. Q Rev Biol **63**(3): 291-311.

Bourke, A. F. G. (1999). Colony Size, Social Complexity and Reproductive Conflict in Social Insects. J Evol Biol **12**(2): 245-257.

Bourke, A. F. G. (2011). Principles of Social Evolution. Oxford, Oxford University Press.

Brady, S., T. Schultz, B. Fisher and P. Ward (2006). Evaluating Alternative Hypotheses for the Early Evolution and Diversification of Ants. Proc Natl Acad Sci USA **103**(48): 18172.

Cant, A. M. and J. Field (2001). Helping Effort and Future Fitness in Cooperative Animal Societies. P Roy Soc Lond B-Biol Sci **268**: 1959-1964.
Cant, A. M. and J. Field (2005). Helping Effort in a Dominance Hierarchy. Behav Ecol **16**(4): 708-715.
Cardoen, D., T. Wenseleers, U. Ernst, E. Danneels, D. Laget, D. De Graaf, L. Schoofs and P. Verleyen (2011). Genome-Wide Analysis of Alternative Reproductive Phenotypes in Honeybee Workers. Mol Ecol **20**(19): 4070-4084.
Carpenter, C. (1964). Naturalistic Behaviour in Nonhuman Primates, Pennsylvania State University Press.
Carroll, C. and D. H. Janzen (1973). Ecology of Foraging by Ants. Annu Rev Ecol Syst: 231-257.
Cedar, H. and Y. Bergman (2009). Linking DNA Methylation and Histone Modification: Patterns and Paradigms. Nat Rev Genet **10**(5): 295-304.
Chittka, A., Y. Wurm and L. Chittka (2012). Epigenetics: The Making of Ant Castes. Curr Biol **22**(19): R835-R838.
Chittka, L. and J. D. Thomson (1997). Sensori-Motor Learning and Its Relevance for Task Specialization in Bumble Bees. Behav Ecol Sociobiol **41**(6): 385-398.
Chittka, L. and H. Muller (2009). Learning, Specialization, Efficiency and Task Allocation in Social Insects. Commun Integr Biol **2**(2): 151-154.
Colgan, T. J., J. C. Carolan, S. J. Bridgett, S. Sumner, M. L. Blaxter and M. J. Brown (2011). Polyphenism in Social Insects: Insights from a Transcriptome-Wide Analysis of Gene Expression in the Life Stages of the Key Pollinator, *Bombus Terrestris*. BMC Genomics **12**(1): 623-643.
Cooney, R. (2002). Colony Defense in Damaraland Mole-Rats, *Cryptomys Damarensis*. Behav Ecol **13**: 160-162.
Corona, M., R. Velarde, S. Remolina, A. Moran-Lauter, Y. Wang, K. Hughes and G. Robinson (2007). Vitellogenin, Juvenile Hormone, Insulin Signaling, and Queen Honey Bee Longevity. Proc Natl Acad Sci USA **104**(17): 7128-7133.
Cremer, S., S. Armitage and P. Schmid-Hempel (2007). Social Immunity. Curr Biol **17**(16): R693-R702.
Crespi, B. and D. Yanega (1995). The Definition of Eusociality. Behav Ecol **6**(1): 109 - 115.
Cronin, A. L. and J. Field (2007). Rank and Colony Defense against Conspecifics in a Facultatively Eusocial Hover Wasp. Behav Ecol **12**: 331-336.
Cuvillier-Hot, V. (2004). Reproductive Monopoly Enforced by Sterile Police Workers in a Queenless Ant. Behav Ecol **15**(6): 970-975.
D'Ettorre, P., A. Tofilski, J. Heinze and F. L. W. Ratnieks (2006). Non-Transferable Signals on Ant Queen Eggs. Naturwissenschaften **93**(3): 136-140.
Dabney, A., J. D. Storey and w. a. f. G. R. Warnes (2004). Qvalue: Q-Value Estimation for False Discovery Rate Control. R Package Version 1.34.0.
Danforth, B. N. (2002). Evolution of Sociality in a Primitively Eusocial Lineage of Bees. Proc Natl Acad Sci USA **99**: 286-290.
Darwin, C. (1859). On the Origin of Species, John Murray, London.
Daugherty, T., A. Toth and G. Robinson (2011). Nutrition and Division of Labor: Effects on Foraging and Brain Gene Expression in the Paper Wasp *Polistes Metricus*. Mol Ecol **20**(24): 5337-5347.
De Araujo, C., J. Lachaud and D. Fresneua (1990). Reproductive-System of Queenless Ponerine Ants, *Dinoponera-Quadriceps* Santschi. Behav Processes **22**(1-2): 101-111.
Debout, G., B. Schatz, M. Elias and D. McKey (2007). Polydomy in Ants: What We Know, What We Think We Know, and What Remains to Be Done. Biol J Linn Soc **90**(2): 319-348.
Dickman, M. J., R. Kucharski, R. Maleszka and P. J. Hurd (2013). Extensive Histone Post-Translational Modification in Honey Bees. Insect Biochem Mol Biol **43**(2): 125-137.
Dornhaus, A. (2008). Specialization Does Not Predict Individual Efficiency in an Ant. PLoS Biol **6**(11): 2368-2375.
Dukas, R. (2008). Life History of Learning: Performance Curves of Honeybees in the Wild. Ethology **114**(12): 1195-1200.
Durier, V. and C. Rivault (2000). Learning and Foraging Efficiency in German Cockroaches, *Blattella Germanica* (L.)(Insecta: Dictyoptera). Anim Cogn **3**(3): 139-145.
Ferreira, P. G., S. Patalano, R. Chauhan, R. Ffrench-Constant, T. Gabaldón, R. Guigó and S. Sumner (2013). Transcriptome Analyses of Primitively Eusocial Wasps Reveal Novel Insights into the Evolution of Sociality and the Origin of Alternative Phenotypes. Genome Biol **14**(2): R20.
Feyereisen, R. (1999). Insect P450 Enzymes. Annu Rev Entomol **44**(1): 507-533.
Field, J., A. L. Cronin and C. Bridge (2006). Future Fitness and Helping in Social Queues. Nature **441**(7090): 214-217.

Field, J. and M. Cant (2009). Social Stability and Helping in Small Animal Societies. Philos T R Soc B **364**: 3181-3189.
Fishwild, T. G. and G. J. Gamboa (1992). Colony Defence against Conspecifics: Caste-Specific Differences in Kin Recognition by Paper Wasps, *Polistes Fuscatus*. Anim Behav **43**(1): 95-102.
Foster, K. R. and F. L. W. Ratnieks (2001a). Convergent Evolution of Worker Policing by Egg Eating in the Honeybee and Common Wasp. P Roy Soc B-Biol Sci **268**(1463): 169-174.
Foster, K. R. and F. L. W. Ratnieks (2001b). The Effect of Sex-Allocation Biasing on the Evolution of Worker Policing in Hymenopteran Societies. Am Nat **158**(6): 615-623.
Frishman, D. (2007). Protein Annotation at Genomic Scale: The Current Status. Chem Rev **107**(8): 3448-3466
Fourcassie, V. and P. Oliveira (2002). Foraging Ecology of the Giant Amazonian Ant *Dinoponera Gigantea* (Hymenoptera, Formicidae, Ponerinae): Activity Schedule, Diet and Spatial Foraging Patterns. J Nat Hist **36**(18): 2211-2227.
Galindo-Leal, C. and I. D. G. Camara (2003). The Atlantic Forest of South America: Biodiversity Status, Threats, and Outlook (State of the Hotspots), Island Press.
Gardner, K. E., R. L. Foster and S. O'Donnell (2007). Experimental Analysis of Worker Division of Labor in Bumblebee Nest Thermoregulation (*Bombus Huntii*, Hymenoptera: Apidae). Behav Ecol Sociobiol **61**(5): 783-792.
Gayral, P., L. Weinert, Y. Chiari, G. Tsagkogeorga, M. Ballenghien and N. Galtier (2011). Next-Generation Sequencing of Transcriptomes: A Guide to RNA Isolation in Nonmodel Animals. Mol Ecol Resour **11**(4): 650-661.
Gill, R. J., O. Ramos-Rodriguez and N. E. Raine (2012). Combined Pesticide Exposure Severely Affects Individual- and Colony-Level Traits in Bees. Nature **491**(7422): 105-108.
Giray, T., M. Giovanetti and M. West-Eberhard (2005). Juvenile Hormone, Reproduction, and Worker Behavior in the Neotropical Social Wasp *Polistes Canadensis*. Proc Natl Acad Sci USA **102**(9): 3330-3335.
Glaus, P., A. Honkela and M. Rattray (2012). Identifying Differentially Expressed Transcripts from RNA-Seq Data with Biological Variation. Bioinformatics **28**(13): 1721-1728.
Gobin, B., C. Peeters and J. Billen (1997). Colony Reproduction and Arboreal Life in the Ponerine Ant *Gnamptogenys Menadensis* (Hymenoptera: Formicidae). Neth J Zool **48**(1): 53-63.
Graff, J., S. Jemielity, J. Parker, K. Parker and L. Keller (2007). Differential Gene Expression between Adult Queens and Workers in the Ant *Lasius Niger*. Mol Ecol **16**: 675-683.
Grozinger, C. M., Y. Fan, S. E. R. Hoover and M. L. Winston (2007). Genome-Wide Analysis Reveals Differences in Brain Gene Expression Patterns Associated with Caste and Reproductive Status in Honey Bees (*Apis Mellifera*). Mol Ecol **16**(22): 4837-4848.
Halestrap, A. and N. Price (1999). The Proton-Linked Monocarboxylate Transporter (Mct) Family: Structure, Function and Regulation. Biochem J **343**: 281-299.
Halestrap, A. P. and D. Meredith (2004). The Slc16 Gene Family - from Monocarboxylate Transporters (Mcts) to Aromatic Amino Acid Transporters and Beyond. Pflug Arch Eur J Phy **447**(5): 619-628.
Hamilton, W. D. (1964). The Genetical Evolution of Social Behaviour. I. J Theor Biol **7**(1): 1-16.
Hart, A. G. and F. L. W. Ratnieks (2005). Crossing the Taxonomic Divide: Conflict and Its Resolution in Societies of Reproductively Totipotent Individuals. J Evol Biol **18**: 383-395.
Hartmann, A., J. Wantia, J. Torres and J. Heinze (2003). Worker Policing without Genetic Conflicts in a Clonal Ant. Proc Natl Acad Sci USA **100**(22): 12836-12840.
Hattori, A., Y. Sugime, C. Sasa, H. Miyakawa, Y. Ishikawa, S. Miyazaki, Y. Okada, R. Cornette, L. Lavine and D. Emlen (2013). Soldier Morphogenesis in the Damp-Wood Termite Is Regulated by the Insulin Signaling Pathway. J Exp Zool B Mol Dev Evol **320B**: 295-306.
Helton, W. S. (2007). Expertise Acquisition as Sustained Learning in Humans and Other Animals: Commonalities across Species. Anim Cogn **11**(1): 99-107.
Hewes, R. (2008). The Buzz on Fly Neuronal Remodeling. Trends Endocrinol Met **19**(9): 317-323.
Hijmans, R. J., S. E. Cameron, J. L. Parra, P. G. Jones and A. Jarvis (2005). Very High Resolution Interpolated Climate Surfaces for Global Land Areas. Int J Climatol **25**: 1965-1978.
Hines, H. M., J. H. Hunt, T. K. O'Connor, J. J. Gillespie and S. A. Cameron (2007). Multigene Phylogeny Reveals Eusociality Evolved Twice in Vespid Wasps. Proc Natl Acad Sci USA **104**: 3295-3299.
Hobbs, A. (1997). Soluble Guanylate Cyclase: The Forgotten Sibling. Trends Pharmacol Sci **18**(4): 484-491.
Hoffman, E. A. and M. A. Goodisman (2007). Gene Expression and the Evolution of Phenotypic Diversity in Social Wasps. BMC Biol **5**(23).

Holldobler, B. and E. Wilson (1990). The Ants, Harvard University Press.
Huang, Z. Y. and G. E. Robinson (1992). Honeybee Colony Integration: Worker-Worker Interactions Mediate Hormonally Regulated Plasticity in Division of Labor. Proc Natl Acad Sci USA **89**(1465390): 11726-11729.
Hughes, W., B. Oldroyd, M. Beekman and F. Ratnieks (2008). Ancestral Monogamy Shows Kin Selection Is Key to the Evolution of Eusociality. Science **320**(5880): 1213-1216.
Hunt, J. H., N. S. Mutti, H. Havukainen, M. T. Henshaw and G. V. Amdam (2011). Development of an RNA Interference Tool, Characterization of Its Target, and an Ecological Test of Caste Differentiation in the Eusocial Wasp *Polistes*. PLoS ONE **6**(11): e26641.
Ibm (2003). Food Preferences and Foraging Activity of the Odorous House Ant (*Tapinoma Sessile Say*) (Hymenoptera: Formicidae). Life Sciences / Entomology, Virginia Polytechnic Institute and State University. **Master of Science:** 1-87.
Ingram, K., P. Oefner and D. Gordon (2005). Task-Specific Expression of the Foraging Gene in Harvester Ants. Mol Ecol **14**(3): 813-818.
Ito, F. (1993). Observation of Group Recruitment to Prey in a Primitive Ponerine Ant, *Amblyopone* Sp.(Reclinata Group)(Hymenoptera: Formicidae). Insect Soc **40**(2): 163-167.
Jayatilaka, P., A. Narendra, S. F. Reid, P. Cooper and J. Zeil (2011). Different Effects of Temperature on Foraging Activity Schedules in Sympatric *Myrmecia Ants*. J Exp Biol **214**(16): 2730-2738.
Jeanson, R., J. H. Fewell, R. Gorelick and S. M. Bertram (2007). Emergence of Increased Division of Labor as a Function of Group Size. Behav Ecol Sociobiol **62**(2): 289-298.
Johnson, B. R., T. Linksvayer and L. Chittka (2010). Deconstructing the Superorganism: Social Physiology, Groundplans, and Sociogenomics. Q Rev Biol **85**: 57-79.
Johnson, B. R. and N. D. Tsutsui (2011). Taxonomically Restricted Genes Are Associated with the Evolution of Sociality in the Honey Bee. BMC Genomics **12**(1): 164.
Johnson, R. A. (1991). Learning, Memory, and Foraging Efficiency in Two Species of Desert Seed-Harvester Ants. Ecology **72**(4): 1408-1419.
Judice, C., K. Hartfelder and G. A. G. Pereira (2004). Caste-Specific Gene Expression in the Stingless Bee *Melipona Quadrifasciata*? Are There Common Patterns in Highly Eusocial Bees? Insect Soc **51**(4): 352-358.
Kamikouchi, A., H. Takeuchi, M. Sawata, K. Ohashi, S. Natori and T. Kubo (1998). Preferential Expression of the Gene for a Putative Inositol 1, 4, 5-Trisphosphate Receptor Homologue in the Mushroom Bodies of the Brain of the Worker Honeybee *Apis Mellifera* L. Biochem Biophys Res Commun **242**(1): 181-186.
Keller, L. (1997). Indiscriminate Altruism: Unduly Nice Parents and Siblings. Trends Ecol Evol **12**(3): 99-103.
Kucharski, R. and R. Maleszka (2002). Evaluation of Differential Gene Expression During Behavioral Development in the Honeybee Using Microarrays and Northern Blots. Genome Biol **3**(2).
Lacey, E. and P. Sherman (1991). Social Organisation of Naked Mole-Rat Colonies: Evidence for Divisions of Labor. The Biology of the Naked Mole-Rat. R. D. Alexander. Princeton, New Jersey, Princeton University Press**:** 275-336.
Langridge, E. A., A. B. Sendova-Franks and N. R. Franks (2008). How Experienced Individuals Contribute to an Improvement in Collective Performance in Ants. Behav Ecol Sociobiol **62**(3): 447-456.
Lengyel, S., A. D. Gove, A. M. Latimer, J. D. Majer and R. R. Dunn (2010). Convergent Evolution of Seed Dispersal by Ants, and Phylogeny and Biogeography in Flowering Plants: A Global Survey. Perspect Plant Ecol Evol Syst **12**(1): 43-55.
Lewis, T., G. Pollard and G. Dibley (1974). Micro-Environmental Factors Affecting Diel Patterns of Foraging in Leaf-Cutting Ant *Atta Cephalotes* (L) (Formicidae-Attini). J Anim Ecol **43**(1): 143-153.
Li-Byarlay, H., Y. Li, H. Stroud, S. Feng, T. C. Newman, M. Kaneda, K. K. Hou, K. C. Worley, C. G. Elsik, S. A. Wickline, S. E. Jacobsen, J. Ma and G. E. Robinson (2013). RNA Interference Knockdown of DNA Methyl-Transferase 3 Affects Gene Alternative Splicing in the Honey Bee. Proc Natl Acad Sci USA **110**(31): 1-6.
Liebig, J., C. Peeters, N. Oldham, C. Markstadter and B. Holldobler (2000). Are Variations in Cuticular Hydrocarbons of Queens and Workers a Reliable Signal of Fertility in the Ant *Harpegnathos Saltator*? Proc Natl Acad Sci USA **97**(8): 4124-4131.
Lockett, G. A., R. Kucharski and R. Maleszka (2011). DNA Methylation Changes Elicited by Social Stimuli in the Brains of Worker Honey Bees. Genes, Brain and Behav **11**(2): 235-242.
Lucas, K., G. Pitari, S. Kazerounian, I. Ruiz-Stewart, J. Park, S. Schulz, K. Chepenik and S. Waldman (2000). Guanylyl Cyclases and Signaling by Cyclic Gmp. Pharmacol Rev **52**(3): 375-414.

Luco, R. F., Q. Pan, K. Tominaga, B. J. Blencowe, O. M. Pereira-Smith and T. Misteli (2010). Regulation of Alternative Splicing by Histone Modifications. Science **327**(5968): 996-1000.
Lyko, F., S. Foret, R. Kucharski, S. Wolf, C. Falckenhayn and R. Maleszka (2010). The Honey Bee Epigenomes: Differential Methylation of Brain DNA in Queens and Workers. PLoS Biol **8**(11): e1000506.
Martins, J., F. Nunes, A. Cristino, Z. Simões and M. Bitondi (2010). The Four Hexamerin Genes in the Honey Bee: Structure, Molecular Evolution and Function Deduced from Expression Patterns in Queens, Workers and Drones. BMC Mol Biol **11**(1): 23.
Marygold, S., J. Roote, G. Reuter, A. Lambertsson, M. Ashburner, G. Millburn, P. Harrison, Z. Yu, N. Kenmochi and T. Kaufman (2007). The Ribosomal Protein Genes and Minute Loci of *Drosophila Melanogaster*. Genome Biol **8**(10): R216.
Maynard Smith, J. and E. Szathmary (1995). The Major Transitions in Evolution, Oxford University Press.
Mehdiabadi, N. J., H. K. Reeve and U. G. Mueller (2003). Queens Versus Workers: Sex-Ratio Conflict in Eusocial Hymenoptera. Trends Ecol Evol **18**(2): 88-93.
Mitra, A., P. Saha, M. E. Chaoulideer, A. Bhadra and R. Gadagkar (2011). Chemical Communication in *Ropalidia Marginata*: Dufour's Gland Contains Queen Signal That Is Perceived across Colonies and Does Not Contain Colony Signal. J Insect Physiol **57**(2): 280-284.
Monnin, T. and C. Peeters (1997). Cannibalism of Subordinates' Eggs in the Monogynous Queenless Ant *Dinoponera Quadriceps*. Naturwissenschaften **84**(11): 499-502.
Monnin, T. and C. Peeters (1998). Monogyny and Regulation of Worker Mating in the Queenless Ant *Dinoponera Quadriceps*. Anim Behav **55**(2): 299-306.
Monnin, T. and C. Peeters (1999). Dominance Hierarchy and Reproductive Conflicts among Subordinates in a Monogynous Queenless Ant. Behav Ecol **10**(3): 323-332.
Monnin, T., F. L. W. Ratnieks, G. Jones and R. Beard (2002). Pretender Punishment Induced by Chemical Signalling in a Queenless Ant. Nature **419**(6902): 61-65.
Monnin, T. and C. Peeters (2008). How Many Gamergates Is an Ant Queen Worth? Naturwissenschaften **95**(2): 109-116.
Nielsen, M., S. Gadagkar and L. Gutzwiller (2010). Tubulin Evolution in Insects: Gene Duplication and Subfunctionalization Provide Specialized Isoforms in a Functionally Constrained Gene Family. BMC Evol Biol **10**(1): 113-134.
Nowak, M. A., C. E. Tarnita and E. O. Wilson (2010). The Evolution of Eusociality. Nature **466**(7310): 1057-1062.
O'Donnell, S. and R. Jeanne (1992). Forager Success Increases with Experience in *Polybia Occidentalis* (Hymenoptera: Vespidae). Insect Soc **39**(4): 451-454.
O'Riain, M. J. and J. U. M. Jarvis (1997). Colony Member Recognition and Xenophobia in the Naked Mole-Rat. Anim Behav **53**: 487-498.
Olson, D. M., E. Dinerstein, E. D. Wikramanayake, N. D. Burgess, G. V. N. Powell, E. C. Underwood, J. A. D'Amico, I. Itoua, H. E. Strand and J. C. Morrison (2001). Terrestrial Ecoregions of the World: A New Map of Life on Earth. BioScience **51**(11): 933-938.
Ometto, L., D. Shoemaker, K. G. Ross and L. Keller (2010). Evolution of Gene Expression in Fire Ants: The Effects of Developmental Stage, Caste, and Species. Mol Biol Evol **28**(4): 1381-1392.
Orr, A. and J. Charles (1994). Foraging in the Giant Forest Ant, *Camponotus Gigas* (Smith)(Hymenoptera: Formicidae): Evidence for Temporal and Spatial Specialization in Foraging Activity. J Nat Hist **28**(4): 861-872.
Oster, G. and E. Wilson (1978). Caste and Ecology in the Social Insects, Princeton University Press.
Paiva, R. and C. Brandao (1995). Nests, Worker Population, and Reproductive Status of Workers, in the Giant Queenless Ponerine Ant *Dinoponera Roger* (Hymenoptera Formicidae). Ethol Ecol Evol **7**(4): 297-312.
Parr, L., M. Matheson, I. Bernstein and F. de Waal (1997). Grooming Down the Hierarchy: Allogrooming in Captive Brown Capuchin Monkeys, *Cebus Apella*. Anim Behav **54**(2): 361-367.
Parra, G., K. Bradnam and I. Korf (2007). Cegma: A Pipeline to Accurately Annotate Core Genes in Eukaryotic Genomes. Bioinformatics **23**(9): 1061-1067.
Parra, G., K. Bradnam, Z. Ning, T. Keane and I. Korf (2009). Assessing the Gene Space in Draft Genomes. Nucleic Acids Res **37**(1): 289-297.
Passos, L. and P. S. Oliveira (2002). Ants Affect the Distribution and Performance of Seedlings of *Clusia Criuva*, a Primarily Bird-Dispersed Rain Forest Tree. J Ecol **90**(3): 517-528.
Passos, L. and P. S. Oliveira (2003). Interactions between Ants, Fruits and Seeds in a Restinga Forest in South-Eastern Brazil. J Trop Ecol **19**(3): 261-270.

Patalano, S., T. A. Hore, W. Reik and S. Sumner (2012). Shifting Behaviour: Epigenetic Reprogramming in Eusocial Insects. Curr Opin Cell Biol **24**(3): 367-373.

Peeters, C. and R. Crewe (1984). Insemination Controls the Reproductive Division of Labor in a Ponerine Ant. Naturwissenschaften **71**(1): 50-51.

Peeters, C. (1991). The Occurrence of Sexual Reproduction among Ant Workers. Biol J Linn Soc **44**(2): 141-152.

Peeters, C., T. Monnin and C. Malosse (1999). Cuticular Hydrocarbons Correlated with Reproductive Status in a Queenless Ant. P Roy Soc B-Biol Sci **266**(1426): 1323-1327.

Pereboom, J. J., W. C. Jordan, S. Sumner, R. L. Hammond and A. F. Bourke (2005). Differential Gene Expression in Queen-Worker Caste Determination in Bumble-Bees. P Roy Soc B-Biol Sci **272**: 1145-1152.

Pie, M. (2004). Foraging Ecology and Behaviour of the Ponerine Ant *Ectatomma Opaciventre Roger* in a Brazilian Savannah. J Nat Hist **38**(6): 717-729.

Queller, D. C. (2000). Relatedness and the Fraternal Major Transitions. Philos T R Soc B **355**(1403): 1647-1655.

Radford, A. N. (2012). Post-Allogrooming Reductions in Self-Directed Behaviour Are Affected by Role and Status in the Green Woodhoopoe. Biol Lett **8**(1): 24-27.

Ratnieks, F. L. W. (1988). Reproductive Harmony Via Mutual Policing by Workers in Eusocial Hymenoptera. Am Nat **132**(2): 217-236.

Ratnieks, F. L. W. and P. K. Visscher (1989). Worker Policing in the Honeybee. Nature **342**(6251): 796-797.

Ratnieks, F. L. W., T. Monnin and K. R. Foster (2001). Inclusive Fitness Theory: Novel Predictions and Tests in Eusocial Hymenoptera. Ann Zool Fenn **38**(3-4): 201-214.

Ratnieks, F. L. W., K. R. Foster and T. Wenseleers (2006). Conflict Resolution in Insect Societies. Annu Rev Entomol **51**: 581-608.

Ravary, F., E. Lecoutey, G. Kaminski, N. Chaline and P. Jaisson (2007). Individual Experience Alone Can Generate Lasting Division of Labor in Ants. Curr Biol **17**(15): 1308-1312.

Robinson, G. E., R. Page and Z. Huang (1994). Temporal Polyethism in Social Insects Is a Developmental Process. Anim Behav **48**: 467-469.

Robinson, G. E. (1992). Regulation of Division of Labour in Insect Societies. Annu Rev Entomol **37**: 637-665.

Robinson, G. E. and Y. Ben-Shahar (2002). Social Behavior and Comparative Genomics: New Genes or New Gene Regulation? Genes, Brain and Behav **1**(4): 197-203.

Robinson, G. E., C. M. Grozinger and C. W. Whitfield (2005). Sociogenomics: Social Life in Molecular Terms. Nat Rev Genet **6**(4): 257-270.

Robinson, M. D., D. J. McCarthy and G. K. Smyth (2010). Edger: A Bioconductor Package for Differential Expression Analysis of Digital Gene Expression Data. Bioinformatics **25**(1): 139-140.

Rust, A.G., E. Mongin, E. Birney (2002). Genome Annotation Techniques: New Approaches and Challenges. Drug Discovery Today **7**(11): S70-S76

Sayers, E. W., T. Barrett, D. A. Benson, S. H. Bryant, K. Canese, V. Chetvernin, D. M. Church, M. Dicuccio, R. Edgar, S. Federhen, M. Feolo, L. Y. Geer, W. Helmberg, Y. Kapustin, D. Landsman, D. J. Lipman, T. L. Madden, D. R. Maglott, V. Miller, I. Mizrachi, J. Ostell, K. D. Pruitt, G. D. Schuler, E. Sequeira, S. T. Sherry, M. Shumway, K. Sirotkin, A. Souvorov, G. Starchenko, T. A. Tatusova, L. Wagner, E. Yaschenko and J. Ye (2009). Database Resources of the National Center for Biotechnology Information. Nucleic Acids Res **37**(Database): D5-D15.

Schmidt, C. (2009). Molecular Phylogenetics and Taxonomic Revision of Ponerine Ants (Hymenoptera: Formicidae: Ponerinae). Graduate Interdisciplinary Program in Insect Science, University of Arizona. **Doctor of Philosophy**.

Schmidt, C. (2013). Molecular Phylogenetics of Ponerine Ants (Hymenoptera: Formicidae: Ponerinae). Zootaxa **3647**(2): 201-250.

Scott, J. and Z. Wen (2001). Cytochromes P450 of Insects: The Tip of the Iceberg. Pest Manage Sci **57**(10): 958-967.

Shapira, M., C. K. Thompson, H. Soreq and G. E. Robinson (2001). Changes in Neuronal Acetylcholinesterase Gene Expression and Division of Labor in Honey Bee Colonies. J Mol Neurosci **17**: 1-12.

Sherman, P., E. Lacey, H. Reeve and L. Keller (1995). The Eusociality Continuum. Behav Ecol **6**(1): 102-108.

Shorter, J. and E. Tibbetts (2009). The Effect of Juvenile Hormone on Temporal Polyethism in the Paper Wasp *Polistes Dominulus*. Insect Soc **56**: 7-13.
Sigel, A., H. Sigel and R. K. O. Sigel (2007). The Ubiquitous Roles of Cytochrome P450 Proteins: Metal Ions in Life Sciences, John Wiley & Sons 2007.
Simola, D. F., C. Ye, N. S. Mutti, K. Dolezal, R. Bonasio, J. Liebig, D. Reinberg and S. L. Berger (2013). A Chromatin Link to Caste Identity in the Carpenter Ant *Camponotus Floridanus*. Genome Res **23**(3): 486-496.
Sjöström, H., O. Norén and J. Olsen (2002). Structure and Function of Aminopeptidase N. Adv Exp Med Biol. **477:** 25-34.
Sokolowski, M. B. (1980). Foraging Strategies of *Drosophila Melanogaster* - a Chromosomal Analysis. Behav Genet **10**: 291-302.
Soro, A., M. Ayasse, M. Zobel and R. Paxton (2011). Kin Discriminators in the Eusocial Sweat Bee *Lasioglossum Malachurum*: The Reliability of Cuticular and Dufour's Gland Odours. Behav Ecol Sociobiol **65**(4): 641-653.
Soroker, V., D. Fresneau and A. Hefetz (1998). Formation of Colony Odor in Ponerine Ant *Pachycondyla Apicalis*. J Chem Ecol **24**(6): 1077-1090.
Storey, J. and R. Tibshirani (2003). Statistical Significance for Genomewide Studies. Proc Natl Acad Sci USA **100**(16): 9440-9445.
Stow, A., D. Briscoe, M. Gillings, M. Holley, S. Smith, R. Leys, T. Silberbauer, C. Turnbull and A. Beattie (2007). Antimicrobial Defences Increase with Sociality in Bees. Biol Lett **3**(4): 422-424.
Streit, S., F. Bock, C. Pirk and J. Tautz (2003). Automatic Life-Long Monitoring of Individual Insect Behaviour Now Possible. Zoology **106**(3): 169-171.
Sumner, S., J. J. M. Pereboom and W. C. Jordan (2006). Differential Gene Expression and Phenotypic Plasticity in Behavioural Castes of the Primitively Eusocial Wasp, *Polistes Canadensis*. P Roy Soc B-Biol Sci **273**(1582): 19-26.
Sumner, S., E. Lucas, J. Barker and N. Isaac (2007). Radio-Tagging Technology Reveals Extreme Nest-Drifting Behavior in a Eusocial Insect. Curr Biol **17**(2): 140-145.
Sutherland, T., G. Unnithan, J. Andersen, P. Evans, M. Murataliev, L. Szabo, E. Mash, W. Bowers and R. Feyereisen (1998). A Cytochrome P450 Terpenoid Hydroxylase Linked to the Suppression of Insect Juvenile Hormone Synthesis. Proc Natl Acad Sci USA **95**(22): 12884-12889.
Tannure-Nascimento, I. C., F. S. Nascimento, J. O. Dantas and R. Zucchi (2009). Decision Rules for Egg Recognition Are Related to Functional Roles and Chemical Cues in the Queenless Ant Dinoponera Quadriceps. Naturwissenschaften **96**(7): 857-861.
Tarver, M. R., M. R. Coy and M. E. Scharf (2012). Cyp15f1: A Novel Cytochrome P450 Gene Linked to Juvenile Hormone-Dependent Caste Differentiation in the Termite *Reticulitermes Flavipes*. Arch Insect Biochem Physiol **80**(2): 92-108.
Taylor, A. (1993). Aminopeptidases: Structure and Function. FASEB J **7**(2): 290-298.
Teseo, S., D. Kronauer, P. Jaisson and N. Châline (2013). Enforcement of Reproductive Synchrony Via Policing in a Clonal Ant. Curr Biol **23**: 328-332.
Thompson, G. J., R. Kucharski, R. Maleszka and B. P. Oldroyd (2006). Towards a Molecular Definition of Worker Sterility: Differential Gene Expression and Reproductive Plasticity in Honey Bees. Insect Mol Biol **15**(5): 637-644.
Toth, A. L., K. Varala, M. T. Henshaw, S. L. Rodriguez-Zas, M. E. Hudson and G. E. Robinson (2010). Brain Transcriptomic Analysis in Paper Wasps Identifies Genes Associated with Behaviour across Social Insect Lineages. P Roy Soc B-Biol Sci **277**(1691): 2139-2148.
Trivers, R. (1971). The Evolution of Reciprocal Altruism. Q Rev Biol **46**(1): 35.
Trivers, R. and H. Hare (1976). Haplodiploidy and Evolution of Social Insects. Science **191**(4224): 249-263.
Truman, J., J. De Vente and E. Ball (1996). Nitric Oxide-Sensitive Guanylate Cyclase Activity Is Associated with the Maturational Phase of Neuronal Development in Insects. Development **122**(12): 3949-3958.
Trumbo, S. T. and G. E. Robinson (1997). Learning and Task Interference by Corpse-Removal Specialists in Honey Bee Colonies. Ethology **103**(11): 966-975.
Van Zweden, J. S., M. A. Fuerst, J. Heinze and P. D'Ettorre (2007). Specialization in Policing Behaviour among Workers in the Ant *Pachycondyla Inversae*. P Roy Soc B-Biol Sci **274**(1616): 1421-1428.
Velloso, A. D. L. (2010). Fourth National Report to the Convention on Biological Diversity: Brazil, Convention on Biological Diversity**:** 286.

Vieira, A., W. Antonialli-Junior and W. Fernandes (2007). Nest Architecture of the Ant *Ectatomma Vizottoi* Almeida (Hymenoptera, Formicidae). Rev Bras Entomol **51**(4): 489-493.

Waddington, S. J. and W. O. H. Hughes (2010). Waste Management in the Leaf-Cutting Ant *Acromyrmex Echinatior*: The Role of Worker Size, Age and Plasticity. Behav Ecol Sociobiol **64**(8): 1219-1228.

Walter, B. (2012). Reduction of Potential Fecundity Leads to Premature Foraging in Ants - an Ancestral Trait in the Evolution of Eusociality? 5th Congress of the European Sections of the International Union for the Study of Social Insects.

Watson, J. (1985). Caste Differentiation in Social Insects, Pergamon Press.

Weil, T., M. Rehli and J. Korb (2007). Molecular Basis for the Reproductive Division of Labour in a Lower Termite. BMC Genomics **8**: 198.

Weil, T., J. Korb and M. Rehli (2009). Comparison of Queen-Specific Gene Expression in Related Lower Termite Species. Mol Biol Evol **26**(8): 1841-1850.

West-Eberhard, M. J. (1987). Flexible Strategy and Social Evolution. Animal Societies: Theories and Facts. Y. Ito, L. Brown and J. Kikkawa. Tokyo, Japan Scientific Societies Press, Ltd**:** 35-51.

Whitfield, C. W. (2003). Gene Expression Profiles in the Brain Predict Behavior in Individual Honey Bees. Science **302**(5643): 296-299.

Whitfield, C. W., Y. Ben-Shahar, C. Brillet, I. Leoncini, D. Crauser, Y. Leconte, S. Rodriguez-Zas and G. E. Robinson (2006). Genomic Dissection of Behavioral Maturation in the Honey Bee. Proc Natl Acad Sci USA **103**(44): 16068-16075.

Wilson, E. O. (1974). The Insect Societies, Harvard University Press.

Wilson, E. O. (1985). The Sociogenesis of Insect Colonies. Science **228**(4707): 1489-1495.

Wilson, E. O. (2000). Sociobiology: The New Synthesis, Harvard University Press.

Winston, M. L. (1987). The Biology of the Honey Bee, Harvard University Press.

Wolschin, F., N. S. Mutti and G. V. Amdam (2011). Insulin Receptor Substrate Influences Female Caste Development in Honeybees. Biol Lett **7**(1): 112-115.

Woltereck, W. (1909). Experimentelle Untersuchungen Fiber Artverinderung, Speziell Fiber Das Wesen Quantitativer Artunterschiede Bei Daphniden. Verh Dtsch Zool Ges **19**: 110-172.

Woodard, S. H., B. J. Fischman, A. Venkat, M. E. Hudson, K. Varala, S. A. Cameron, A. G. Clark and G. E. Robinson (2011). Genes Involved in Convergent Evolution of Eusociality in Bees. Proc Natl Acad Sci USA **108**(18): 7472-7477.

Wurm, Y., J. Wang, O. Riba-Grognuz, M. Corona, S. Nygaard, B. G. Hunt, K. K. Ingram, L. Falquet, M. Nipitwattanaphon, D. Gotzek, M. B. Dijkstra, J. Oettler, F. Comtesse, C. J. Shih, W. J. Wu, C. C. Yang, J. Thomas, E. Beaudoing, S. Pradervand, V. Flegel, E. D. Cook, R. Fabbretti, H. Stockinger, L. Long, W. G. Farmerie, J. Oakey, J. J. Boomsma, P. Pamilo, S. V. Yi, J. Heinze, M. A. D. Goodisman, L. Farinelli, K. Harshman, N. Hulo, L. Cerutti, I. Xenarios, D. Shoemaker and L. Keller (2011). The Genome of the Fire Ant *Solenopsis Invicta*. Proc Natl Acad Sci USA **108**(14): 5679-5684.

Zhou, X., M. R. Tarver and M. E. Scharf (2007). Hexamerin-Based Regulation of Juvenile Hormone-Dependent Gene Expression Underlies Phenotypic Plasticity in a Social Insect. Development **134**(3): 601-610

Appendix

A1 Field and Laboratory Colonies

A1.1 Nest Characteristics

Table A1

Nests located at three sites in Bahia and Sergipe in 2010 and 2011. For each colony, the site and year it was located are shown, as well as the types of measurements recorded. Nest measurements including diameter of the nest mound, coverage of twigs at the entrance, number of entrances and direction of entrances (*chapter 2.3.2.1*). Foraging observations involved monitoring the activities of marked foragers (*chapter 2.3.3.2*). Whether or not the nest was subsequently excavated is also recorded, and the colony size recorded for excavated nests

Nest	Year	Site	Nest Characteristics?	Foraging Observations?	Excavated?	Colony size
1a	2010	São Cristavão (site 3)	No	No	Yes	74
2a	2010	São Cristavão (site 3)	No	No	Yes	12
3a	2010	São Cristavão (site 3)	No	No	Yes	79
4a	2010	São Cristavão (site 3)	No	No	Yes	117
5a	2010	São Cristavão (site 3)	No	No	Yes	14
6a	2010	São Cristavão (site 3)	No	No	Yes	22
7a	2010	São Cristavão (site 3)	No	No	Yes	16
8a	2010	São Cristavão (site 3)	No	No	Yes	42
9a	2010	São Cristavão (site 3)	No	No	Yes	4
10a	2010	São Cristavão (site 3)	No	No	Yes	41
11a	2010	São Cristavão (site 3)	No	No	Yes	25
12a	2010	São Cristavão (site 3)	No	No	Yes	25
1b	2011	Campo Formoso (site 1)	Yes	Yes	No	NA
2b	2011	Campo Formoso (site 1)	Yes	Yes	No	NA
3b	2011	Campo Formoso (site 1)	Yes	Yes	Yes	108
4b	2011	Campo Formoso (site 1)	Yes	Yes	No	NA
5b	2011	Campo Formoso (site 1)	Yes	Yes	Yes	75
6b	2011	Campo Formoso (site 1)	Yes	No	No	NA
7b	2011	Campo Formoso (site 1)	Yes	Yes	No	NA

8b	2011	Campo Formoso (site 1)	Yes	No	Yes	52
9b	2011	Campo Formoso (site 1)	Yes	No	Yes	50
10b	2011	Campo Formoso (site 1)	Yes	Yes	Yes	80
11b	2011	Campo Formoso (site 1)	Yes	No	No	NA
12b	2011	Campo Formoso (site 1)	Yes	No	Yes	54
13b	2011	Campo Formoso (site 1)	Yes	No	Yes	75
14b	2011	Campo Formoso (site 1)	Yes	No	Yes	124
15b	2011	Campo Formoso (site 1)	Yes	No	Yes	44
16b	2011	Campo Formoso (site 1)	Yes	No	No	NA
17b	2011	Campo Formoso (site 1)	Yes	No	No	NA
18b	2011	Campo Formoso (site 2)	Yes	No	No	NA
19b	2011	Campo Formoso (site 2)	Yes	No	Yes	63
20b	2011	Campo Formoso (site 2)	Yes	No	Yes	25
21b	2011	Campo Formoso (site 2)	Yes	No	No	NA
22b	2011	Campo Formoso (site 2)	Yes	No	No	NA
23b	2011	Campo Formoso (site 2)	Yes	No	Yes	50
24b	2011	Campo Formoso (site 2)	Yes	No	No	NA
25b	2011	Campo Formoso (site 2)	Yes	No	Yes	37
26b	2011	Campo Formoso (site 2)	Yes	No	No	NA
27b	2011	Campo Formoso (site 2)	Yes	No	No	NA
28b	2011	Campo Formoso (site 2)	Yes	No	No	NA
29b	2011	Campo Formoso (site 2)	Yes	No	No	NA
30b	2011	Campo Formoso (site 2)	Yes	No	No	NA
31b	2011	Campo Formoso (site 2)	Yes	No	Yes	31
33b	2011	Campo Formoso (site 2)	Yes	No	Yes	26
34b	2011	Campo Formoso (site 2)	Yes	No	Yes	68
35b	2011	São Cristavão (site 3)	Yes	No	Yes	68
36b	2011	São Cristavão (site 3)	Yes	No	Yes	106

A1.2 Comparison of Field and Laboratory Colonies

To help inform future laboratory studies of *D. quadriceps*, I compared foraging behaviour as measured in the laboratory and the field. Number of foragers was lowest

for field observed colonies, and highest for laboratory colonies (*table 2.2*), despite larger colonies sizes in the field. RFID monitoring showed lower levels of foraging compared to behavioural observations. It may have underestimated the number of individuals foraging if interference prevented detection, or if individuals spent long periods sitting in at the nest entrance before departing.

Table A2 Comparison of Field and Laboratory Colonies

The mean number of foragers at different times of day (09:00 – 17:00) observed for field colonies (n = 7) and laboratory colonies monitored with RFID (n = 10) and behavioural observations (n = 11). Mean colony size is reported for 3 excavated colonies (4 observed colonies were not excavated), and for 21 all laboratory colonies.

	Field Colonies	**Laboratory Colonies** RFID	**Laboratory Colonies** Behavioural
Mean Colony Size	87.67 ± 10.27	38.67 ± 9.14	30.64 ± 6.55
Number of Foragers			
Mean Diurnal	3.65 ± 0.26	5.10 ± 0.34	9.7 ± 0.49
09:00 – 10:00	2.6 ± 0.77	5.2 ± 1.24	9.2 ± 1.03
10:00 – 11:00	3.18 ± 0.88	3.2 ± 0.83	9.4 ± 1.05
11:00 – 12:00	3.69 ± 0.68	2.2 ± 0.75	9.4 ± 1.00
12:00 – 13:00	3.14 ± 0.59	3.1 ± 1.27	9.4 ± 1.06
13:00 – 14:00	3.58 ± 0.65	4.1 ± 1.30	9.4 ± 1.43
14:00 – 15:00	2.83 ± 0.41	4 ± 1.12	10.2 ± 1.56
15:00 – 16:00	5.42 ± 0.73	4.3 ± 1.32	9.9 ± 1.47
16:00 – 17:00	4.54 ± 0.81	3.8 ± 0.98	10.6 ± 1.36

A2 Chi-Squared Distributions for Allogrooming Data

The frequency of allogrooming events was compared statistically, using a chi-squared test, to both an even distribution of allogrooming across all categories and also a more realistic distribution taking into account the relative abundance of members of each rank. Chi-squared tests were performed for both the direction of allogrooming events and their type.

Table A3 Chi-Squared Distributions

Observed occurrence of allogrooming events, and expected values from an even distribution, and from a distribution based upon the proportion of colony members of each rank.

	Type								
	HH	HM	HL	MM	ML	LL	MH	LM	LH
Observed	105	69	108	10	14	46	6	10	5
Expected	6.1	12.1	29.5	24.1	58.6	142.5	12.1	58.6	29.5

	Direction				
	+2	+1	0	-1	-2
Observed	5	16	160	84	108
Expected	29.5	70.7	172.7	70.7	29.5

A3 Full Results for Egg Policing

Table A4

Comparison of results obtained from separate analyses of individual responses to foreign eggs during 25-introduction egg-policing trials (25 foreign eggs introduced over 5 days) and 15-introduction egg-policing trials (15 foreign eggs introduced over 5 days)(*chapter 5.31*). One colony was excluded from the individual-level analysis 25-introduction dataset because none of the introduced eggs were policed.

	25 Introductions	15 Introductions
No. Colonies	6	4
Mean No. Encounters	4.58	2.60
Statistical Analyses		
Police Current Encounter? (Yes / No) ~		
Introduction Number	NS p = 0.30	** p < 0.01
Encounter Number	NS p = 0.14	NS p = 0.36
Rank	NS p = 0.16	
Donator Colony	NS p = 0.18	NS p = 0.09
Egg Age	NS p = 0.12	NS p = 0.43
Proportion of Previous Egg Encounters Policed	** p < 0.01	** p < 0.01
Time Since Last Egg Encounter	NS p = 0.68	NS p = 0.25
Processing Time ~		
Introduction Number	NS p = 0.42	** p < 0.01
Encounter Number	NS p = 0.74	** p < 0.01
Rank	NS p = 0.07	NS p = 0.39
Donator Colony	** p < 0.01	** p < 0.01
Egg Age	NS p = 0.85	* p = 0.02
Proportion of Previous Egg Encounters Policed	NS p = 0.92	NS p = 0.70
Time Since Last Egg Encounter	** p < 0.01	** p < 0.01
Response (Accept, None, Eat, Waste) ~		
Introduction Number	NS p = 0.49	* p = 0.05
Encounter Number	* p = 0.04	NS p = 0.68
Rank	* p = 0.02	NS p = 0.14
Donator Colony	NS p = 0.20	NS p = 0.57
Egg Age	NS p = 0.78	NS p = 0.85
Proportion of Previous Egg Encounters Policed	** p < 0.01	NS p = 0.06
Time Since Last Egg Encounter	NS p = 0.15	** p < 0.01

A4 Additional Methods for Genome and Transcriptome Sequence

A*4.1 RFID Data Manipulation*

Multiple consecutive records for the same individual within a 30 second period were removed, as this likely represents an extended pause in the tunnel. *D. quadriceps* workers are frequently observed sitting in the tunnel for up to several minutes, and during RFID monitoring this behaviour would result in multiple records for the same tag number throughout the period of time it remained within range of the antenna. Records from each antenna were then aligned to enable the direction of movement to be discerned.

A*4.2 DNA Extraction optimisation*

Initial DNA extractions using several protocols (Qiagen DNeasy blood & tissue kit, Trizol) yielded very low quantities of DNA. Extractions were trialled with different tissues (whole bodies, legs) in different storage mediums (-80°C / room temperature ethanol) and several protocol modifications were attempted in order to improve yield for extracting DNA from this non-model organism. Using the Qiagen DNeasy blood and tissue kit, which utilises a column-based extraction system and attempts at achieving increased yield involved splitting a single male across several columns, and increasing the number of elution steps. These modifications, however, failed to improve yield sufficiently for DNA sequencing. Finally, a phenol-chloroform protocol was attempted that successfully extracted DNA of high quantity and quality.

A4.3 Phenol Chloroform DNA Extraction Methodology

A single whole body was stored at -80°C prior to extraction. The body was then cut into small pieces, and further homogenised using a TissueLyser. One body was split across 4 extractions performed in separate 1.5ml eppendorf tubes and subject to the following protocol. 250μl 10X buffer, 25μl proteinase K and 25μl of SDS were added to the homogenised tissue, being mixed by vortexing at each stage. This mixture was incubated at 55°C for 1 hour on a rocking platform, after which 4μl of RNase was added and the incubated for a further 30 minutes at 37°C.

Next, 300μl of 25:24:1 Phenol:Chloroform:Isoamyl was added and centrifuged for 5

minutes at 4°C, 15,000 rpm. The aqueous phase was transferred to a new 1.5ml eppendorf, and this process repeated from the addition of Phenol:Chloroform:Isoamyl. Having repeated this process, 300μl of chloroform was added and the mixture centrifuged for 5 minutes at 4°C, 15,000 rpm. Again, the aqueous phase was transferred to a new eppendorf, and the process repeated from the addition of Chloroform. 2x volume of 4°C Ethanol 100% was added, in addition to 1μl glycogen, mixed gently and left to incubate at -20°C overnight.

Following incubation, the mixture was centrifuged at 4°C, 15,000g for 30 minutes. The supernatant was removed, and 1ml ethanol 70% added. At this point a pellet is visible, and this was dislodged by vortexing, before centrifuging at 8,000g for 10 minutes. As much as possible of the ethanol was then removed by pipetting, and the remaining ethanol left to evaporate by air-drying. The dry pellet was re-suspended in 30μl water.

A4.4 Direct-Zol RNA Extraction Protocol

Tissue homogenisation was achieved using a TissueLyser LT at 50Hz for 3 minutes and then centrifuged at 4°C, 15,000rpm for 1 minute. The supernatant was transferred to a new eppendorf and 500μl of ethanol 95% was added. This mixture was then loaded into the Direct-zol spin column, and centrifuged at 15,000rpm for 1 minute. The flow-through was discarded and the column transferred to a new collection tube, where 400μl DirectZol RNA wash buffer was added and the column centrifuged at 4°C, 15,000 rpm for 1 minute. The flow-through was again discarded and 80μl of DNase cocktail (DNase I, DNase I reaction buffer, distilled water, RNA wash buffer) added and incubated at 37°C for 15 minutes. The column was then centrifuged again at 4°C, 15,000rpm for 30 seconds, followed by the addition of 400μl RNA pre-wash, and centrifuged again at 4°C, 15,000rpm for 1 minutes. The flow-through was discarded and the column transferred to a new collection tube. These steps were then repeated from the addition of RNA pre-wash. Next, 700μl of RNA wash buffer was added and the column centrifuged at 4°C, 15,000rpm for 1 minute. Finally, the column was transferred to an RNAse-free eppendorf, 25μl of RNase-free distilled water was added and centrifuged at 4°C, 14,000rpm speed for 1 minute.

A4.5 mRNA Sequencing Methodology

From 0.8 – 2 µg of total RNA were used for poly(A)-mRNA selection using streptavidin-coated magnetic beads and were subsequently fragmented to approximately 300bp. cDNA was synthesized using reverse transcriptase (18064-014, Invitrogen) and random primers. The cDNA was further converted into double stranded DNA that was used for library preparation. dsDNA was subjected to end repair, addition of "A" bases to 3′ ends and ligation of the barcoded Truseq adapters. All purification steps were performed using Qiagen PCR purification columns (50928106 and 50928006, Qiagen). Library size selection was done with 2% low-range agarose gels. Fragments with insert sizes of 180 to 280 bp were cut from the gel, and DNA was extracted using QIAquick Gel extraction kit (50928706, Qiagen) and eluted in 20 µl EB. Library amplification was performed by PCR on the size selected fragments using the primer cocktail supplied in the kit.

A4.6 EVM and PASA Pipeline Methodology

As sources of evidence for the evidence modeller, four *ab initio* gene prediction programs were used: Geneid, GlimmerHMM, Augustus and GeneMark. These prediction programs were trained with long *D.quadriceps* full length sequences obtained from within the PASA assemblies. EVM was also provided with predictions from two homology-based gene prediction tools: SGP2, trained with *D.quadriceps* sequences and using *Apis mellifera* as an informant genome, and Augusts plus hints, trained with *D.quadriceps* sequences and using the PASA transcripts as evidence. The EVM program also used spliced-protein alignments from three sources: genewise / exonerate of the SwissProt highly curated non-redundant invertebrate protein set, genewise *Harpegnathos saltator* protein coding models and genewise / exonerate of the *Apis mellifera* ensemble proteins. Finally, 26,944 PASA assemblies were used as an input to the EVM.

A5 Additional Results for Transcriptome-Wide Gene Expression

A5.1 Overlap in Differentially Expressed Genes

Table A5

List of genes (as homologues with > 30% sequence identity and e = 10^{-2}) identified as being up-regulated in more than one comparison (rank; alpha, beta, low, mating status, ovarian activity)

Gi_number of Closest Homologue	Definition
Up-regulated in both Alpha and Beta	
gi_307168970	Nucleoporin NUP188-like protein
gi_307183300	hypothetical protein
gi_322800476	hypothetical protein
gi_307196324	Sushi, von Willebrand factor type A, EGF and
Up regulated in both Beta and Low	
gi_307170754	HEAT repeat-containing protein KIAA1833-like
gi_307183300	hypothetical protein
gi_307207995	Cat eye syndrome critical region protein 1
gi_307185662	Calmodulin-like protein 4
gi_307175382	Solute carrier organic anion transporter family
gi_332026181	Putative ribosome production factor 1
gi_307165887	UPF0518 protein CG3558
gi_211904138	APAF1 interacting protein
gi_307187368	hypothetical protein
gi_332030044	Sodium-coupled monocarboxylate transporter 1
gi_307186806	Putative octanoyltransferase, mitochondrial
gi_307175251	Cystinosin-like protein
gi_166865186	homeotic protein distal-less
gi_307168596	Homocysteine S-methyltransferase 3
gi_307181693	Probable cytochrome P450 6a13
gi_307186415	hypothetical protein
gi_307187729	C2 domain-containing protein 3
gi_307176281	Leucine-rich repeat serine/threonine-protein
gi_307206355	Retinoic acid-induced protein 1
gi_307191291	Ring canal kelch-like protein
gi_110757982	hypothetical protein LOC725481
gi_307179455	40S ribosomal protein SA
gi_307212873	hypothetical protein
gi_307181233	hypothetical protein
gi_307171292	hypothetical protein
gi_307183653	Uncharacterized protein KIAA2013-like protein
gi_307188369	CTTNBP2 N-terminal-like protein
gi_307175290	DE-cadherin
gi_307206030	Dipeptidase 1
gi_307196840	Sushi, von Willebrand factor type A, EGF and
gi_307193796	Peptidyl-prolyl cis-trans isomerase,
gi_340711403	protein transport protein SFT2-like
gi_307185086	Calsyntenin-1
gi_119114103	AGAP009919-PA
gi_307171173	Transient receptor potential channel pyrexia
gi_307174707	Uncharacterized protein F44E2.2
gi_307185865	WD repeat-containing protein LOC51057-like

gi_307168970	Nucleoporin NUP188-like protein
gi_219686085	hypothetical protein
gi_307203440	Microtubule-associated protein futsch
gi_307182790	Zinc finger protein 800
gi_118150484	histamine-gated chloride channel 1 precursor
gi_307169240	Acyl-CoA Delta(11) desaturase
gi_307179799	Rho GTPase-activating protein 100F
gi_156336942	hypothetical protein
gi_242022031	protein toll precursor, putative
gi_254548011	Gram-negative bacteria binding-protein
gi_307196663	Protein lingerer
gi_307176763	Guanine nucleotide-binding protein G(o) subunit
gi_307181908	Probable tyrosine-protein phosphatase F54C8.4
gi_307168935	U11/U12 small nuclear ribonucleoprotein 35 kDa
gi_156554918	hypothetical protein LOC100116880
Up Regulated in Alpha and Low	
gi_307195685	Protein Fer3
gi_156542283	tubulin gamma-1 chain-like
gi_307168970	Nucleoporin NUP188-like protein
gi_322797357	hypothetical protein
gi_307183300	hypothetical protein
gi_307169884	Elongation factor 1-gamma
Up Regulated For Alpha and Active Ovaries	
gi_194864392	Dere\GG10911 PA
gi_17647611	masquerade, isoform B
gi_307173918	Zinc finger CCCH domain-containing protein 13
gi_156402337	hypothetical protein
gi_156543967	FAD-dependent oxidoreductase domain-containing
gi_307188538	Putative EGF-like domain-containing protein
gi_307166490	hypothetical protein
Up Regulated For Beta and Active Ovaries	
gi_166865186	homeotic protein distal-less
Up Regulated For Low and Active Ovaries	
gi_166865186	homeotic protein distal-less
Up Regulated for Beta and Mated	
gi_307207997	Structural maintenance of chromosomes protein 5
Up Regulated for Low and Mated	
gi_307184775	ATP-binding cassette sub-family G member 1

A5.2 Comparison of Different PPLR Thresholds

Table A6
Numbers of significantly differentially expressed genes at different pplr thresholds: abs(pplr)> 0.03, 0.04 and 0.05.

Comparison	**Significant** 0.05	**Up-regulated** 0.05	**Significant** 0.04	**Up-regulated** 0.04	**Significant** 0.03	**Up-regulated** 0.03
Alpha vs Beta	31	23	173	115	850	548
Beta vs Low	22	6	158	57	860	350
Alpha vs Low	26	16	137	72	830	458
Ovarian Activity	23	11	170	87	837	420
Mating Status	32	6	203	48	980	237

A5.3 Comparison of EdgeR and Bitseq

In addition to the main analyses performed using the Bayesian analysis package BitSeq, I also performed pairwise comparisons using the R package EdgeR for alpha vs. beta, beta vs. low and alpha vs. low, and for ovarian activity, in order to provide a comparison with, and verify the results of BitSeq (table 5). However, this analysis yielded no significantly differentially expressed genes for any comparison. The smallest p-value obtained was p = 0.115 (alpha vs low), and all genes were found to be non-significant after false discovery rate correction (q = 1). The magnitude of differences between groups was generally small, with the largest fold change observed between the beta and low comparison (fold change = 1.0764). Using a fold-change threshold of 0.1, as above, no genes are identified as being differentially expressed in the EdgeR analysis. An EdgeR glm approach, yielded similar results. No genes were identified as significantly differentially expressed between ranks before (smallest p-value = 0.1107) or after FDR correction (smallest q = 1).

Table A7

Comparison of BitSeq and EdgeR results for the top 10 most differentially expressed genes as highlighted in the EdgeR pair-wise comparisons. BitSeq absPPLR values above 0.04 are considered significant. For fold change, values between 0 and 1 indicate the gene is down-regulated I the first comparison group, values above 1 indicate it is up-regulated.

Gene ID	Description	BitSeq		Edge R exact test		EdgeR glmLRT
		absPPLR	Fold Change	p-value (FDR)	Fold Change	p-value (FDR)
Alpha vs. Beta						
cuff.2086	unannotated	0.069 **	0.9224	0.1387 (1)	1.0626	0.1335 (1)
cuff.12295	unannotated	0.019	1.0402	0.2024 (1)	0.9520	0.1959 (1)
cuff.1041	unannotated	0.023	1.0179	0.2249 (1)	0.9468	0.2228 (1)
cuff.4832	Zinc finger protein 687	0.03	0.9503	0.2361 (1)	1.0547	0.2232 (1)
cuff.9448	unannotated	0.011	1.0154	0.2386 (1)	0.96191	0.2324 (1)
cuff.3281	unannotated	0.019	0.9747	0.2561 (1)	1.0444	0.2481 (1)
cuff.5863	unannotated	0.024	1.0189	0.2664 (1)	0.9421	0.2523 (1)
cuff.653	unannotated	0.009	0.9741	0.2671 (1)	1.0569	0.2625 (1)
cuff.11768	unannotated	0.039	0.9133	0.2737 (1)	1.0610	0.2626 (1)
cuff.13317	unannotated	0.018	1.0190	0.2771 (1)	0.9553	0.2685 (1)
Alpha vs. Low						
cuff.15624	gi_307166707	0.016	1.0268	0.1148 (1)	0.9441	0.1107 (1)
cuff.12926	unannotated	0.05 *	1.0740	0.1826 (1)	0.9516	0.1767 (1)
cuff.3281	unannotated	0.006	0.9836	0.1847 (1)	1.0492	0.1788 (1)
cuff.1007	Probable cytochrome P450 6a14	0.017	0.9709	0.2244 (1)	1.0529	0.2128 (1)
cuff.2491	Cytochrome P450 6j1	0.015	1.0347	0.2264 (1)	0.9313	0.2165 (1)
cuff.12862	unannotated	0.011	0.9617	0.2321 (1)	1.0594	0.2239 (1)
cuff.12817	unannotated	0.01	0.9647	0.2609 (1)	1.0418	0.2532 (1)
cuff.12294	unannotated	0.017	0.9599	0.2753 (1)	1.0550	0.2614 (1)
cuff.9448	unannotated	0.027	1.0236	0.2861 (1)	0.9671	0.2794 (1)
cuff.6941	gi_307166386	0.015	0.9758	0.2964 (1)	1.0350	0.2828 (1)
Beta vs. Low						
cuff.653	unannotated	0.024	1.0741	0.2607 (1)	0.9431	0.2512 (1)
cuff.15149	gi_328790167	0.014	1.0389	0.2702 (1)	0.9495	0.2605 (1)
cuff.1938	ATP-binding cassette sub-family G member 1	0.005	0.9761	0.3049 (1)	1.0656	0.2869 (1)
cuff.13317	unannotated	0.031	0.9563	0.3121 (1)	1.0451	0.3020 (1)
cuff.11456	SCY1-like protein 2	0.001	0.9796	0.3207 (1)	1.0619	0.3022 (1)
cuff.14716	Eukaryotic peptide chain release factor	0.019	1.0385	0.3220 (1)	0.9411	0.3065 (1)
cuff.7647	ER lumen protein retaining receptor	0.03	1.0231	0.3227 (1)	1.0520	0.3097 (1)
cuff.1041	unannotated	0.019	0.9728	0.3231 (1)	1.0477	0.3098 (1)
cuff.11905	JmjC domain-containing histone demethylation	0.013	1.0411	0.3231 (1)	0.9442	0.3141 (1)
Cuff.6818	FGGY carbohydrate kinase domain-containing	0.027	0.9612	0.3448 (1)	1.0492	0.3324 (1)

A6 Additional Methods for Toolkit Analysis

A literature search yielded 19 academic papers which provided information about genes that have been identified as differentially expressed in relation to caste, reproductive physiology or worker task specialisation in other eusocial species. This list covered the following species: *Apis mellifera* (Ament *et al.* 2010; Ben-Shahar *et al.* 2004; Ben-Shahar *et al.* 2002; Bloch 2010; Cardoen *et al.* 2011; Grozinger *et al.* 2007; Kucharski and Maleszka 2002; Shapira *et al.* 2001; Whitfield *et al.* 2006)*, Bombus terrestris* (Colgan *et al.* 2011; Corona *et al.* 2007; Pereboom *et al.* 2005), *Melipona quadrifasciata* (Judice *et al.* 2004; Thompson *et al.* 2006)*, Polistes canadensis* (Sumner *et al.* 2006)*, Polistes dominulus* (Shorter and Tibbetts 2009)*, Polistes metricus* (Daugherty *et al.* 2011; Toth *et al.* 2010), *Vespula squamata* (Hoffman and Goodisman 2007), *Camponotus floridanus* (Simola *et al.* 2013)*, Lasius Niger* (Graff *et al.* 2007), *Solenopsis invicta* (Wurm *et al.* 2011)*,* Cryptotermes secundus (Weil *et al.* 2007)*, Cryptotermes cynocephalus* (Weil *et al.* 2009)*, Homotermopsis sjostedti* (Hattori *et al.* 2013)*, Reticulitermes flavipes* (Tarver *et al.* 2012).

References

Ament, S., Y. Wang and G. E. Robinson (2010). Nutritional Regulation of Division of Labor in Honey Bees: Toward a Systems Biology Perspective. Wiley Interdisciplinary Reviews: Systems Biology and Medicine **2**: 566-576.

Ben-Shahar, Y., A. Robichon, M. B. Sokolowski and G. E. Robinson (2002). Influence of Gene Action across Different Time Scales on Behavior. Science **296**(5568): 741-744.

Ben-Shahar, Y., N. Dudek and G. Robinson (2004). Phenotypic Deconstruction Reveals Involvement of Manganese Transporter Malvolio in Honey Bee Division of Labor. J Exp Biol **207**(19): 3281-3288.

Bloch, G. (2010). The Social Clock of the Honeybee. J Biol Rhythms **25**: 307.

Cardoen, D., T. Wenseleers, U. Ernst, E. Danneels, D. Laget, D. De Graaf, L. Schoofs and P. Verleyen (2011). Genome-Wide Analysis of Alternative Reproductive Phenotypes in Honeybee Workers. Mol Ecol **20**(19): 4070-4084.

Colgan, T. J., J. C. Carolan, S. J. Bridgett, S. Sumner, M. L. Blaxter and M. J. Brown (2011). Polyphenism in Social Insects: Insights from a Transcriptome-Wide Analysis of Gene Expression in the Life Stages of the Key Pollinator, *Bombus Terrestris*. BMC Genomics **12**(1): 623-643.

Corona, M., R. Velarde, S. Remolina, A. Moran-Lauter, Y. Wang, K. Hughes and G. E. Robinson (2007). Vitellogenin, Juvenile Hormone, Insulin Signaling, and Queen Honey Bee Longevity. Proc Natl Acad Sci USA **104**(17): 7128-7133.

Daugherty, T., A. Toth and G. E. Robinson (2011). Nutrition and Division of Labor: Effects on Foraging and Brain Gene Expression in the Paper Wasp *Polistes Metricus*. Mol Ecol **20**(24): 5337-5347.

Graff, J., S. Jemielity, J. Parker, K. Parker and L. Keller (2007). Differential Gene Expression between Adult Queens and Workers in the Ant *Lasius Niger*. Mol Ecol **16**: 675-683.

Grozinger, C. M., Y. Fan, S. E. R. Hoover and M. L. Winston (2007). Genome-Wide Analysis Reveals Differences in Brain Gene Expression Patterns Associated with Caste and Reproductive Status in Honey Bees (*Apis Mellifera*). Mol Ecol **16**(22): 4837-4848.

Hattori, A., Y. Sugime, C. Sasa, H. Miyakawa, Y. Ishikawa, S. Miyazaki, Y. Okada, R. Cornette, L. Lavine and D. Emlen (2013). Soldier Morphogenesis in the Damp-Wood Termite Is Regulated by the Insulin Signaling Pathway. J Exp Zool B Mol Dev Evol **320B**: 295-306.

Hoffman, E. A. and M. A. Goodisman (2007). Gene Expression and the Evolution of Phenotypic Diversity in Social Wasps. BMC Biol **5**(23).

Judice, C., K. Hartfelder and G. A. G. Pereira (2004). Caste-Specific Gene Expression in the Stingless Bee *Melipona Quadrifasciata*? Are There Common Patterns in Highly Eusocial Bees? Insect Soc **51**(4): 352-358.

Kucharski, R. and R. Maleszka (2002). Evaluation of Differential Gene Expression During Behavioral Development in the Honeybee Using Microarrays and Northern Blots. Genome Biol **3**(2).

Pereboom, J. J. M., W. C. Jordan, S. Sumner, R. L. Hammond and A. F. Bourke (2005). Differential Gene Expression in Queen-Worker Caste Determination in Bumble-Bees. P Roy Soc B-Biol Sci **272**: 1145-1152.

Shapira, M., C. K. Thompson, H. Soreq and G. E. Robinson (2001). Changes in Neuronal Acetylcholinesterase Gene Expression and Division of Labor in Honey Bee Colonies. J Mol Neurosci **17**: 1-12.

Shorter, J. and E. Tibbetts (2009). The Effect of Juvenile Hormone on Temporal Polyethism in the Paper Wasp *Polistes Dominulus*. Insect Soc **56**: 7-13.

Simola, D. F., C. Ye, N. S. Mutti, K. Dolezal, R. Bonasio, J. Liebig, D. Reinberg and S. L. Berger (2013). A Chromatin Link to Caste Identity in the Carpenter Ant *Camponotus Floridanus*. Genome Res **23**(3): 486-496.

Sumner, S., J. J. M. Pereboom and W. C. Jordan (2006). Differential Gene Expression and Phenotypic Plasticity in Behavioural Castes of the Primitively Eusocial Wasp, *Polistes Canadensis*. P Roy Soc B-Biol Sci **273**(1582): 19-26.

Tarver, M. R., M. R. Coy and M. E. Scharf (2012). Cyp15f1: A Novel Cytochrome P450 Gene Linked to Juvenile Hormone-Dependent Caste Differentiation in the Termite *Reticulitermes Flavipes*. Arch Insect Biochem Physiol **80**(2): 92-108.

Thompson, G. J., R. Kucharski, R. Maleszka and B. P. Oldroyd (2006). Towards a Molecular Definition of Worker Sterility: Differential Gene Expression and Reproductive Plasticity in Honey Bees. Insect Mol Biol **15**(5): 637-644.

Toth, A. L., K. Varala, M. T. Henshaw, S. L. Rodriguez-Zas, M. E. Hudson and G. E. Robinson (2010). Brain Transcriptomic Analysis in Paper Wasps Identifies Genes Associated with Behaviour across Social Insect Lineages. P Roy Soc B-Biol Sci **277**(1691): 2139-2148.

Weil, T., M. Rehli and J. Korb (2007). Molecular Basis for the Reproductive Division of Labour in a Lower Termite. BMC Genomics **8**: 198.

Weil, T., J. Korb and M. Rehli (2009). Comparison of Queen-Specific Gene Expression in Related Lower Termite Species. Mol Biol Evol **26**(8): 1841-1850.

Whitfield, C. W., Y. Ben-Shahar, C. Brillet, I. Leoncini, D. Crauser, Y. Leconte, S. Rodriguez-Zas and G. E. Robinson (2006). Genomic Dissection of Behavioral Maturation in the Honey Bee. Proc Natl Acad Sci USA **103**(44): 16068-16075.

Wurm, Y., J. Wang, O. Riba-Grognuz, M. Corona, S. Nygaard, B. G. Hunt, K. K. Ingram, L. Falquet,
M. Nipitwattanaphon, D. Gotzek, M. B. Dijkstra, J. Oettler, F. Comtesse, C. J. Shih, W. J. Wu,
C. C. Yang, J. Thomas, E. Beaudoing, S. Pradervand, V. Flegel, E. D. Cook, R. Fabbretti, H. Stockinger, L. Long, W. G. Farmerie, J. Oakey, J. J. Boomsma, P. Pamilo, S. V. Yi, J. Heinze,
M. A. D. Goodisman, L. Farinelli, K. Harshman, N. Hulo, L. Cerutti, I. Xenarios, D. Shoemaker and L. Keller (2011). The Genome of the Fire Ant Solenopsis Invicta. Proc Natl Acad Sci USA **108**(14): 5679-5684.