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Genetic Structure, Reproductive Skew, and the Evolution of Sociality in the Hover Wasp *Liostenogaster flavolineata*.

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A dissertation submitted to the University of Sussex in accordance with the requirements of the degree of DOCTOR OF PHILOSOPHY in the Faculty of Life Sciences.

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AUTHOR'S DECLARATION

I declare that the work in this thesis has not been, and will not be submitted in whole or in part to another University for any other academic award.

Chapters 5 and 6 contain unpublished data collected by Dr. Catherine Bridge in 2001. Chapter 4 incorporates data from Sumner et al. (2002). However, the particular analyses undertaken on these data are entirely my own, as well as the interpretations drawn.

I certify that, with the above qualification, the work carried out in my thesis is entirely my own, and that any help provided by other individuals with data collection and analysis is fully acknowledged.

SIGNED: ..... DATE: .....

DEDICATION

To my Mother.
Thank you for your love and support.

"Nature uses only the longest threads to weave her patterns, so each small piece of her fabric reveals the organization of the entire tapestry."

- Richard Feynman (1965)

SUMMARY OF THESIS

UNIVERSITY OF SUSSEX

LAUREN ADELE HOLT, DOCTOR OF PHILOSOPHY

GENETIC STRUCTURE, REPRODUCTIVE SKEW AND THE EVOLUTION OF SOCIALITY IN THE HOVER WASP *LIOSTENOGASTER FLAVOLINEATA*

SUMMARY

In this thesis, I use field experiments to test whether a potentially universal benefit of sociality operates in the facultatively eusocial hover wasp *Liostenogaster flavolineata* (Stenogastrinae). The Central Limit Theorem (CLT) is a potential reason why individuals come together to provision offspring. I report the results of an original experiment designed to test the central tenets of the CLT by manipulating the variance of food inputs to *L. flavolineata* nests. I show that with increased variance there was no effect on levels of brood abortion, or larval development rates. However, some assumptions of the CLT were found to hold, such as smaller groups producing more brood per-capita with higher rates of brood removal.

Theoretically, genetic relatedness between adult nest-mates should have an important influence on how reproduction is partitioned (reproductive skew). However, reproductive skew in newly initiated colonies of *L. flavolineata*, where nest-mate relatedness is low, is not significantly different to that in mature colonies, and is unaffected by group size.

Genetic relatedness between subordinates and the dominant egg-layer could potentially vary systematically according to position in the age-based queue to inherit the egg-laying role in *L. flavolineata* groups. Using newly developed microsatellite markers, in combination with manipulations to determine queue position, I test for systematic associations between a worker's relationship to the dominant, inheritance rank and group size. I find that there is an increasing likelihood for daughters and nieces to occur at the lowest ranks, i.e. those with the greatest indirect fitness pay-offs and greatest foraging effort.

I also investigate how genetic relatedness and group size influence foraging effort, and suggest that smaller groups might experience effects of the CLT, but work harder to compensate. I integrate theories of reproductive skew and my findings from *L. flavolineata* into a framework of potential behavioural concessions in larger groups.

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TABLE OF CONTENTS

Page

List of Tables **xi**

List of Figures **xii**

1 Introduction **1**

1.1 Introduction to eusociality . . . 1
1.2 Research questions . . . 3
1.2.1 Productivity . . . 3
1.2.2 Reproductive partitioning . . . 4
1.2.3 Systematic patterns throughout inheritance queues . . . 4
1.2.4 Differential foraging effort and group size . . . 4
1.3 Classification and life history . . . 5
1.3.1 Classification . . . 5
1.3.2 Distribution . . . 5
1.3.3 Nest architecture . . . 6
1.3.4 Ovipositon and abdominal substance . . . 8
1.3.5 Larval development and offspring provisioning . . . 8
1.3.6 Nest initiation and seasonal variation . . . 9
1.3.7 Social organization and behaviour . . . 9
1.3.8 Colony genetic structure . . . 10
1.3.9 Forging and larval provisioning . . . 10
1.3.10 Male behaviour and mating . . . 11
1.4 An overview of group living . . . 11
1.5 Benefits of group living . . . 12
1.5.1 Benefits of a central location . . . 12
1.5.2 Inheritance benefits . . . 13
1.5.3 Insurance benefits . . . 13
1.6 Universal benefits to increasing group size . . . 13
1.6.1 The Central Limit Theorem . . . 14
1.6.2 Benefits of task differentiation including reproductive division of labour 15
1.6.3 Risk management . . . 15
1.6.4 Benefits of social complexity . . . 16
1.6.5 Optimization principle . . . 16
1.7 Costs of group living . . . 18

1.7.1 The costs of conflict and aggression . . . 18
1.7.2 The costs of task differentiation and reproductive division of labour . 18
1.7.3 Rank and foraging effort - the costs of being a worker . . . 20
1.7.4 Universal energetic costs to increasing social complexity . . . 20
1.8 Genetic structure of groups . . . 20
1.8.1 Genetic relatedness at nest initiation - a special case? . . . 21
1.9 Aims of Thesis . . . 22
1.10 Summary of Thesis . . . 24

2 General Methods **25**
2.1 Field Techniques . . . 25
2.1.1 Sites . . . 25
2.1.2 Experimental preparation . . . 25
2.1.3 Monitoring foraging returns . . . 26
2.1.4 Isolating nests . . . 26
2.1.5 Marking procedure . . . 28
2.1.6 Monitoring adult wasps . . . 28
2.1.7 Determining inheritance rank . . . 28
2.1.8 Behavioural observations . . . 29
2.1.9 Censusing brood . . . 29
2.1.10 Collection methods . . . 30
2.1.10.1 Adults . . . 30
2.1.10.2 Brood . . . 31
2.2 Molecular genetic methods . . . 31
2.2.1 Extraction of DNA . . . 31
2.2.2 Development of microsatellites from a genomic library . . . 32
2.2.3 Amplification using PCR . . . 32
2.2.4 Post-PCR optimization and analysis . . . 32
2.2.5 Optimisation, multiplexes, and fluorescent labels . . . 32
2.3 Analysis . . . 33
2.3.1 Relatedness values . . . 33
2.3.2 Genetic Relationships (using KINSHIP) . . . 33
2.3.3 Measuring reproductive skew . . . 35
2.3.4 Identification of males and calculation of male brood skew . . . 36
2.4 General Statistics . . . 36

3 Group Size Dependent Foraging Returns. A Test of The Central Limit Theorem **37**
3.1 Abstract: . . . 37
3.2 Introduction . . . 38
3.2.1 Study species . . . 42
3.3 Methods . . . 44
3.3.1 Experimental procedure . . . 44

3.3.2 Calculating average number of food returns throughout the day . . . 44
3.3.3 Manipulation of variance in food returns . . . 45
3.3.4 Measuring brood development and abortions . . . 47
3.3.5 Abortions . . . 47
3.3.6 The effect of ants . . . 48
3.3.7 Data analysis . . . 48
3.4 Results . . . 49
3.4.1 Do *L. flavolineata* display higher per-capita productivity in smaller groups? . . . 49
3.4.2 Brood composition . . . 50
3.4.3 Empirical manipulation of variance in food returns . . . 52
3.4.4 Effect of the manipulation on number of detected abortions . . . 52
3.4.5 Effect of the manipulation on larval transitions . . . 54
3.5 Discussion . . . 55
3.5.1 Do *L. flavolineata* display Michener's paradox? . . . 56
3.5.2 Do smaller groups of *L. flavolineata* have greater overall success? . . . 56
3.5.3 Empirical manipulation of variance in food returns . . . 57
3.5.4 Effect of the manipulation on number of all detected abortions . . . 57
3.5.5 The effect of the manipulation on larval transitions/ brood development rates . . . 58
3.5.6 Does the CLT affect *L. flavolineata*? . . . 58
3.6 Conclusion . . . 60

4 Reproductive skew and genetic relatedness at nest initiation 62
4.1 Abstract: . . . 62
4.2 Introduction . . . 63
4.2.1 Study species . . . 68
4.3 Methods . . . 69
4.3.1 Experimental procedure . . . 70
4.3.2 Nest collections . . . 70
4.3.3 Molecular genetic methods . . . 71
4.3.4 Assignment of sex . . . 72
4.3.5 Identifying sib-groups . . . 72
4.3.6 Relatedness values . . . 73
4.3.7 Measuring skew . . . 73
4.3.8 Calculation of male brood skew . . . 74
4.3.9 Data analysis . . . 74
4.4 Results . . . 76
4.4.1 Relatedness profiles of new and established nests . . . 76
4.4.2 Developmental stage and sex of brood not belonging to the primary egg layer on new nests . . . 76
4.4.3 Reproductive skew in newly established nests . . . 78

4.4.4 Factors affecting the proportion of brood laid by the primary egg-layer on new nests . . . 78
4.4.5 Proportion of males from the primary or secondary egg-layer . . . 80
4.4.6 Comparing between new and established nests . . . 81
4.5 Discussion . . . 81
4.5.1 Relatedness profiles of new and established nests . . . 82
4.5.2 Reproductive skew on new nests . . . 83
4.5.3 Factors affecting the proportion of brood laid by the primary egg-layer on new nests . . . 84
4.5.4 Proportion of male brood . . . 85
4.5.5 Comparing the proportion of small female brood laid by the primary egg-layer between new and established nests, and factors which may affect it . . . 85
4.5.6 Why might high skew be maintained in *L. flavolineata* even when there is low relatedness? . . . 87
4.5.7 Why might high skew be a universal feature of *L. flavolineata* groups? 88
4.6 Conclusion . . . 89

5 Systematic patterns of in intra-nest relationships and nest-size dynamics in Liostenogaster flavolineata groups. 90
5.1 Abstract . . . 90
5.2 Introduction . . . 91
5.2.1 Study Species . . . 94
5.3 Methods . . . 95
5.3.1 Field site and preparation . . . 95
5.3.2 Collecting adults . . . 95
5.3.3 Identifying rank . . . 96
5.3.4 Molecular work and genotyping . . . 97
5.3.5 Relatedness values and relationships . . . 97
5.3.6 Data analysis . . . 98
5.4 Results . . . 100
5.4.1 Group size and relatedness . . . 100
5.4.2 Relatedness values within groups . . . 101
5.4.3 Relationships within groups . . . 101
5.4.4 Worker relatedness to the dominant's offspring . . . 104
5.5 Discussion . . . 105
5.5.1 Relatedness and group size . . . 106
5.5.2 Relatedness values within different sized groups . . . 107
5.5.3 Relationships within different sized groups . . . 107
5.5.4 Subordinate relatedness to brood . . . 108
5.5.5 Could group size and/or rank be used to indicate relatedness? . . . 109
5.6 Conclusion . . . 110

6 Foraging effort and group size in Liostenogaster flavolineata. 111
6.1 Abstract: . . . 111
6.2 Introduction . . . 112
6.2.1 Study species . . . 120
6.3 Methods . . . 120
6.3.1 Determining rank and foraging effort . . . 121
6.3.2 Standardizing foraging effort . . . 121
6.3.3 Adult removal . . . 122
6.3.4 Molecular genetic methods . . . 122
6.3.5 Relatedness values . . . 122
6.3.6 Data analysis . . . 122
6.4 Results . . . 123
6.4.1 Does total foraging effort increase linearly with group size? . . . 123
6.4.2 Foraging effort in *L. flavolineata* groups . . . 124
6.4.3 Factors affect foraging effort within ranks . . . 125
6.4.4 Does variation in foraging returns in *L. flavolineata* meet assumptions of the CLT? . . . 128
6.5 Discussion . . . 129
6.5.1 Does total foraging effort increase linearly with group size? . . . 129
6.5.2 Factors which affect foraging effort within ranks . . . 129
6.5.3 Does variation in foraging returns in *L. flavolineata* meet assumptions of the CLT? . . . 130
6.5.4 What are the potential reasons for a non-linear relationship between total foraging effort and group size? . . . 130
6.6 Conclusion . . . 134

7 Summary and concluding comments 136
7.1 Summary of results . . . 136
7.2 Concluding comments . . . 138

A Appendix 142
A.1 Appendix Chapter 1 . . . 142
A.2 Appendix Chapter 2 . . . 142
A.2.1 Protocol for tissue preparation: procedures for eggs, larvae, adults and library tissue . . . 142
A.2.2 Protocol for DNA extraction using ammonium acetate precipitation method . . . 143
A.2.3 Microsatellite amplification: PCR of individual or multiple loci using Quiagen PCR master mix. . . . 144
A.2.4 Genotyping: ethanol/EDTA/sodium acetate precipitation 96 well plate 145
A.3 Appendix Chapter 3 . . . 148
A.4 Appendix Chapter 4 . . . 150
A.5 Appendix Chapter 5 . . . 151

B Bibliograpy **153**

List of Tables

Table **Page**

1.1 Table of reproductive skew models 19

2.1 Table of KINSHIP tests 34

3.1 Frequency of nest sizes 52

4.1 Sources of data 76
4.2 Skew results for females 79

5.1 Table of significance values for KINSHIP tests 98
5.2 Table of group sizes and frequency 100
5.3 Standard error of average relatedness values 102
5.4 Table showing how relationships change throughout groups 104

6.1 Table of group sizes and frequency 124
6.2 Table of standard errors 126
6.3 Table of Shapiro-Wilk results 129

A.1 Table of solutions 144
A.2 Table of markers 146
A.3 Table of skew values for male and female brood 150

List of Figures

Figure		Page
1.1	Cladogram of the Stenogastrinae	6
1.2	Map of study location	6
1.3	Field site and nests	7
1.4	Food processing and male behaviour	12
1.5	Size-complexity hypothsesis	17
2.1	Field sites	26
2.2	Field techniques	27
2.3	Larvae developmental stages	30
2.4	Flow-chart of KINSHIP method	35
3.1	Average food returns	45
3.2	Experiental regime	46
3.3	Brood per-capita	50
3.4	Non-linear increases in brood number with group size	51
3.5	Brood composition spine plot	51
3.6	Total abortions per-capita	53
3.7	Transitions per-capita	55
4.1	Relatedness of nests	77
4.2	Pair-wise relatedness of nests	77
4.3	Proportion skew female brood	80
4.4	Proportion skew male and female brood	80
4.5	Effect of relatedness on proportion skew	82
4.6	Effect of group size on proportion skew	82
5.1	Relatedness estimate to the dominant	100
5.2	Worker's relatedness to the dominant	101
5.3	Graph showing worker's relatedness to the dominant for each group size	102
5.4	Relationships at each rank- spine plot	103
5.5	Graph showing worker's relatedness to the brood for the longest queues	105
6.1	Quadratic relationship between group size and effort	124
6.2	Foraging effort and rank	125
6.3	Graph of average foraging effort	126

6.4 Predicted model 127
6.5 Q-Q plots of foraging returns 128

A.1 Matrix of KINSHIP results 147
A.2 Total abortions per-capita 148
A.3 Larval transitions per-cell 148
A.4 Pupae per-capita 149
A.5 Queue dynamics of small groups 151
A.6 Queue dynamics of large groups 152

CHAPTER 1

INTRODUCTION

Sociality in animal systems has always been of particular interest to natural scientists, especially in cases where some individuals appear to incur costs from group membership, whilst others do not. Workers often behave altruistically with no direct benefits in terms of reproduction of their own. This thesis aims to investigate how some of the costs and benefits of group living, for example foraging effort and productivity, may either increase or decrease according to group size. I explore how these parameters interact with relatedness in the social insect *Liostenogaster flavolineata*, which demonstrates altruistic behaviour.

1.1 Introduction to eusociality

The defining feature of eusocial societies is a 'reproductive division of labour', in which some individuals (known as helpers, workers or subordinates) at least temporarily forfeit their own reproduction and instead rear the offspring of other individuals (known as breeders, queens or dominants). This extreme form of altruism at first appears antithetical to evolution by natural selection (Darwin 1859; Ratnieks et al. 2010). In addition to a reproductive division of labour, the criteria for eusociality include overlapping generations and cooperative care of young (Wilson 1971). Advanced eusociality also requires the presence of irreversibly distinct castes that are sterile or otherwise physiologically different, as in most ants and termites (Crespi & Yanega 1995). In contrast, a primitively eusocial species, including *Liostenogaster flavolineata*, consists of totipotent females that are theoretically free to pursue any life

history course, including mating and reproducing (Field & Foster 1999). Primitively eusocial species can furthermore be considered either facultatively or obligately eusocial, depending on whether they are found exclusively in groups or in a combination of solitary and social lifestyles. Facultatively eusocial species provide an excellent case study for investigating the evolutionary origins of eusociality, and experimenting with the full range of possible behavioural responses to group living. *L. flavolineata* is a facultatively eusocial species (Samuel 1987).

In seminal work, Hamilton (1963; 1964a; 1964b), demonstrated that these altruistic behaviours can evolve through each individual's attempts to maximize its own *inclusive fitness*, which comprises its own direct reproduction, plus any additional offspring produced via assistance to a related recipient (the latter henceforth referred to as the altruist's indirect reproduction). In an important sense, therefore, an individual that acts altruistically (in the strictly biological definition) does not act maladaptively from the gene's-eye view: i.e. in certain circumstances a greater genetic legacy will be left by those who forgo partially or entirely their direct reproduction and become social donors (Williams 1957), compared to those who reproduce directly (Dawkins 2006).

Eusociality is hypothesized to have arisen at least 24 times independently in taxa as diverse as insects, crustaceans, and mammals (Bourke 2011). Lineages that have innovated advanced eusociality have come to dominate their respective niches, and sociality in general is ubiquitous as a successful mode of living. However, in order for sociality, eusociality and increasingly large group sizes to evolve and be stable there must be non-linear increases in productivity from the solitary state upwards (Naug & Wenzel 2006). Group size can reach gigantic proportions with the largest super-colony of Argentinian ants, *Linepithema humile*, consisting of billions of individuals forming the largest cooperative unit ever recorded (Giraud et al. 2002). Non-linear increases in productivity are crucial since in primitively eusocial species a relative's offspring rarely carry as large a proportion of the helper's genes as would the helper's own offspring. Therefore, natural selection should favour altruistic behaviour only if helpers are compensated by being much more productive than they would be by reproducing directly, and larger group sizes should evolve only if individuals within them gain proportionally more through inclusive fitness benefits than when in a smaller

group (Queller 1996). Phrased mathematically, for workers the benefits in Hamilton's rule (see below) must exceed the costs of the altruistic behaviour, for larger groups to evolve and be stable.

Hamilton's Rule:

Hamilton (1964) was the first to define the central role that relatedness plays in the evolution of social systems. Hamilton's Rule (1964) specifies the conditions under which the altruistic behaviour observed in social insects could be selected for:

r b > c

Where **c** is the cost of the altruist act to the actor, **b** is the benefit of the act to the recipient and **r** is the relatedness between recipient and actor. A subordinate will help when **r > cost/benefit**.

1.2 Research questions

This thesis has four main themes: i) how per-capita productivity changes with group size, ii) how reproduction is partitioned according to relatedness and group size, iii) how relatedness is structured throughout groups and according to group size, and iv) how foraging effort changes with group size in the context of productivity, relatedness, and inheritance benefits. Here I introduce the main themes.

1.2.1 Productivity

Success equates to gene frequency, and a useful measure of gene frequency is the number of offspring produced by a group as a whole or per-individual. Therefore, comparisons between solitary living and group living, and between groups of different sizes, have often focused on per-capita productivity (Shakarad & Gadagkar 1995; Stevens et al. 2007a; Kojima 1989). However, Michener (1964) paradoxically observed that smaller groups of some social insects produced higher per-capita numbers of brood (immature offspring) than larger groups. If all these offspring were raised successfully to maturity, this would be contrary to both the evolution of sociality and of larger groups.

In order to explain this paradox, costs and benefits of group vs. solitary living must be measured to test for non-linear differences in either productivity or efficiency between groups of different sizes. These can be viewed from both the perspective of rational individuals

making decisions to maximize genetic benefits, and also from the perspective of nest or population productivity and survival. These viewpoints should not have any inherent conflict (Bourke 2011).

1.2.2 Reproductive partitioning

Reproductive skew theory is an attempt to understand what is essentially an economic issue: how do members of a group share the profits of a cooperative association? (Field & Cant 2009). Whilst reproduction is the most important of these parameters, it is a unifying theme of this thesis that all effort and resources (costs and benefits) must be partitioned amongst the group in such a way that all members still agree to the social contract. Whilst many of the models introduced here (Johnstone 2000), are phrased in terms of reproductive skew theory in cooperatively breeding societies, the concepts are applicable to many kinds of group in which the benefits of association are unequally shared among members. Partitioning of reproduction, resources and effort may change at different stages of group development, including at group initiation (Zanette & Field 2008).

1.2.3 Systematic patterns throughout inheritance queues

Groups in many species contain subordinate adults who queue to inherit the nest or territory, and the right to reproduce (Field et al. 2006). Queues can also be governed by conventions rather than the result of aggression or fighting ability, for example age or order in which individuals join the group (Bridge & Field 2007). Inclusive fitness benefits to each actor per-unit effort will vary according to the relatedness to the primary egg-layer. Previous studies that have modeled the dynamics of queuing for dominance in eusocial Stenogastrinae have assumed that genetic benefits are equal for all individuals (Toyoizumi & Field 2014a; 2014b). However, uniform relatedness between group members and inclusive fitness benefits equal to those of the reproductive dominant are unlikely to occur in many eusocial species. Any systematic patterns of relatedness within groups, according to inheritance rank, are unknown in *L. flavolineata*.

1.2.4 Differential foraging effort and group size

Foraging as an act of altruism is predicted to increase with increasing relatedness to the recipient (Hamilton 1963). Within queue forming social insects foraging effort is usually

dependent on inheritance rank, and there has been little evidence that relatedness affects effort or defense either in general, or within ranks (Cronin & Field 2006; Leadbeater & Carruthers 2010). Instead, individuals closer to inheriting the breeding position reduce foraging work to conserve future fitness in *L. flavolineata*. As such, group size (and therefore value of the nest at inheritance), has been the only variable that has affected foraging effort within ranks, with high-ranking subordinates on larger nests working less hard (Field et al. 2006). However, reduced foraging work may confer other advantages, for example group persistence (Toyoizumi & Field 2012). Group size may also provide information on average relatedness that individuals could use when making decisions about the level of effort they are willing to invest or tolerate in the group.

In order to more clearly understand costs and benefits for individuals throughout non-uniform groups, and the effect of relatedness profile on group-level traits (especially in a species with varying foraging effort), it is important to understand any systematic patterns in nest genetic structure. The subject of this thesis is how the parameters of relatedness, per-capita productivity, group size, and foraging effort interact in a species where all individuals retain the ability to reproduce. *L. flavolineata*, a facultatively eusocial tropical hover wasp with a well-defined dominance hierarchy and perennial lifecycle is an ideal species to address these questions, the natural history of which I summarize below.

1.3 Classification and life history

1.3.1 Classification

Within Vespidae, eusocial behaviour is found in only three of the six subfamilies: the Stenogastrinae, Polistinae and Vespinae. Hover wasps are located in the subfamily Stenogastrinae, which comprises of six social subfamilies (see Figure 1.1) (Carpenter 1988).

1.3.2 Distribution

Like many Stenogastrinae, *L. flavolineata* occurs across a wide distribution that includes Peninsular Malaysia. Due to the constancy of the climate (aside from a wet and dry season), nests are functional throughout the year, without a period of hibernation or dramatic decrease in number, making them very amenable to practical study and manipulation (Samuel

1987). Aggregations are particularly found in the higher altitude regions of Malaysia (Field 2008), with the following experiments undertaken at Fraser's Hill (Figure 1.2), 116 km from Kuala Lumpar.

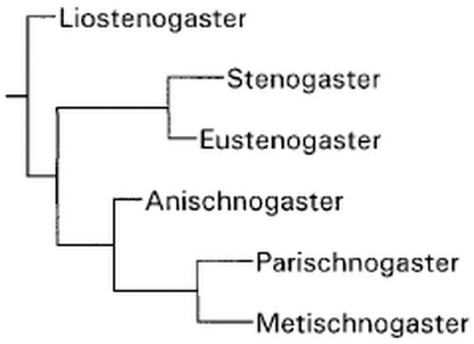

FIGURE 1.1. Cladogram of the Stenogastrinae (Carpenter 1988)

FIGURE 1.2. Map representing penninsular Malaysisa and the study site.

1.3.3 Nest architecture

Nests are robustly built from mud, and often occur in large aggregations (up to 100 nests in certain locations), typically over bodies of running water on the underside of artificial

bridges, and on the walls of concrete culverts that run under the roads for drainage (see Figure 1.3 b and 1.3 c). New nests consist of shallow cups of mud attached to the chosen substrate (see Figure 1.3 a), but established nests are durable, large (up to a maximum of 100 cells) and as such represent a large investment and a valuable material resource. Particularly when built on man-made structures, these mud nests can have a potential life of a decade or more (L. Holt pers. obs. - nests of *L. flavolineata* labeled with identifying numbers in 1997 field season by J. Field and others were still inhabited by wasps and in good condition in 2013).

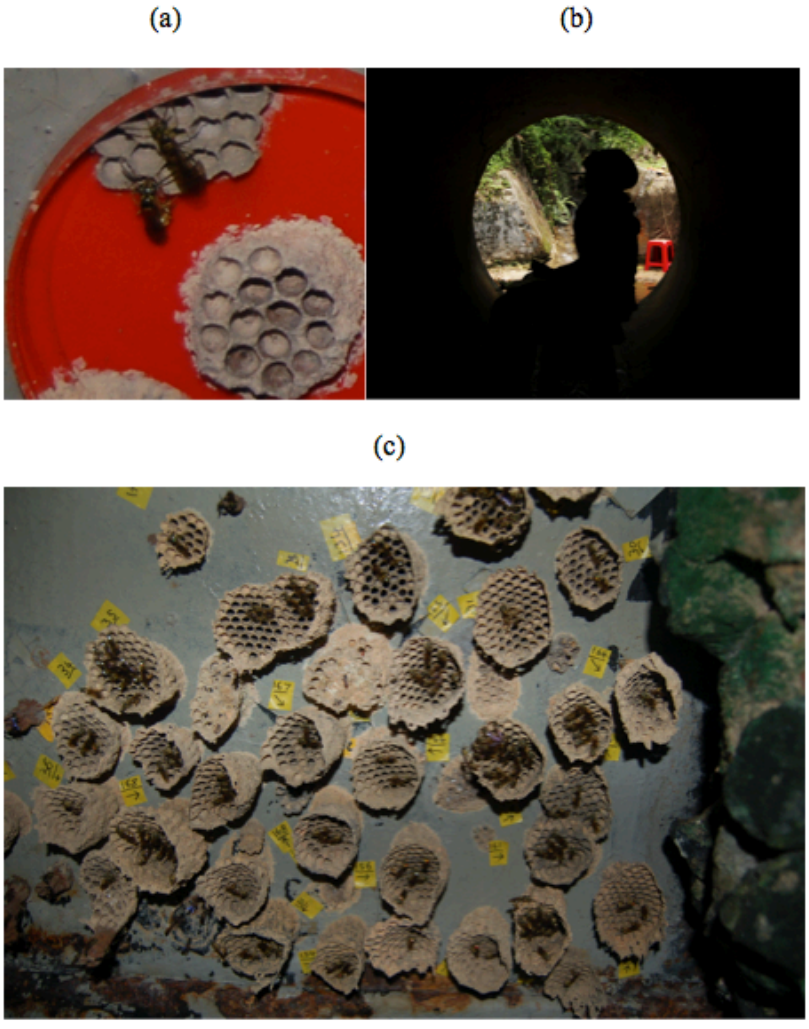

FIGURE 1.3. (a) Two *L. flavolineata* females constructing a new nest on the rim of a container from a previous field season. (b) A culvert with *L. flavolineata* nests. (c) Closely packed *L. flavolineata* nests at Site 1.

1.3.4 Ovipositon and abdominal substance

The Stenogastrinae have a unique method of oviposition and production of abdominal secretions (Keegans & Morgan 1993). In aculeate Hymenoptera, adult females possess a ducted gland (the Dufour's gland), which produces a gelatinous secretion (Turillazzi 1985). This is used in the Stenogastrinae for larval anchoring, oviposition, provisioning and sometimes as a slippery, sticky ant guard on the attachment point of nests (Turillazzi 1991). Eggs are initially expelled into a wad of this 'abdominal substance', and these are adhered to the base of a nest cell. Later, the larva emerges from the egg chorion and coils around the blob of abdominal substance. This then acts as a substrate for the young larva to anchor to, and as a surface for the deposition of liquid and solid food (Turillazzi 1985). The abdominal substance itself has no nutritional value until mixed with other substances (Keegans & Morgan 1993; Sledge & Fortunato 2000).

Subordinates are often observed producing abdominal substance, even when not producing eggs themselves (L. Holt pers. obs.). Turillazzi (1989) suggested that the energetic cost of the abdominal substance, rather than the costs of the eggs themselves, could constrain brood numbers since it seems to be a limited resource which is expensive to produce.

1.3.5 Larval development and offspring provisioning

On new or empty nests of *L. flavolineata* it is usual for the first few offspring laid to be female, since female wasps will assist in brood care whilst males play no role in nest building or food acquisition (Samuel 1987). Approximately one week after being laid, the egg hatches into the first of four larval stages. Larvae are then progressively provisioned with processed insect protein until they reach the pupal stage, when adults seal the nest cell openings in order for the larvae to pupate (Carpenter 1988). Full development from egg to imago can take up to 100 days in *L. flavolineata* (Hansell et al. 1982; Turillazzi 1985; 1991). Foraging can begin from as early as four days after adult emergence (Field et al. 1999). Foraging in hover wasps is thought to be independent, in contrast to the complex communication of food location information in honey bees. Males play no part in nest provisioning or defence, although they may sometimes remain on the nest to receive food from returning females (Samuel 1987).

Of central importance to this thesis is the observation that egg and small brood cannibalism occurs frequently in Stenogastrinae (Hansell et al. 1982; Turillazzi & Pardi 1982). This is considered as an indicator of nutritional stress and a strategic response to environmental conditions (Stevens 1997; Kozlowski & Stearns 1989). Brood are at risk from the egg layer herself, with many instances of consumption immediately after oviposition, and also by usurpers, adult nest-mates, and other predators (Turillazzi et al. 1997).

1.3.6 Nest initiation and seasonal variation

New nests (see Figure 1.3 a) of *L. flavolineata* are initiated by a solitary female (Samuel 1987; Turillazzi 1991). After the first few cells are constructed, the foundress usually attracts other female joiners (Samuel 1987; Field & Foster 1998; L. Holt pers. obs.). However, single female (solitary) nests do persist, particularly in locations with few nest-less (floating) individuals. Previous work in the same area as the following studies suggest that despite a lack of a winter and associated diapause, there remains seasonal variation in parameters such as the frequency of nest-founding, mean group size, and the mean number of immature offspring being reared (Samuel 1987; Shreeves & Field 2002; J. Field and G. Shreeves, unpublished data). Colonies typically exhibit two distinct periods of increased brood output, the first in late May and the second in August. These peaks occur during the warmest part of the year and between two wetter periods (Cronin et al. 2011).

1.3.7 Social organization and behaviour

L. flavolineata have a gerontocratic (age-based) queuing convention, where the youngest females born on the nest join the end of the inheritance queue at the most junior position and wait to inherit (Bridge & Field 2007). Alternatively, a female may leave to join another group, become a 'floating' individual, fight to move up in the hierarchy, or establish a nest of her own. Samuel (1987) found that 29% of 230 newly emerged females stayed on their natal nest, whilst the remaining 71% left, and of those that left only 22% were seen again. Group sizes rarely grow beyond 10 adult females and the rate of disappearance is not dependent on group size (Field et al. 1999). Females that have been marked on nests can disappear for long periods and then re-join up to a month later (L. Holt pers. obs.). If a female remains on her nest beyond the initial orientation stage after eclosion, she will assist significantly in the

acquisition of food for developing larvae, other females and any resident males. Unlike some other species (for example eusocial thrips such as *Oncothrips tepperi*), it is the subordinates who work the hardest. The dominant (Rank 1) female almost never leaves the nest apart from very brief excursions, while the most junior females forage the most (Field & Cant 2006; Bridge & Field 2007). Wasps that are on the nest usually participate in nest defense, first threatening conspecifics and potential predators in a C-shaped posture, and then engaging in direct contact. However, the typical response of *L. flavolineata* to any serious threat is to drop from the nest and flee (L. Holt pers. obs). Most *L. flavolineata* females leave the nest periodically to 'prospect' other nests, and are usually met with threatening gestures from wasps already in residence. The upper limit to *L. flavolineata* lifespan is 200 days, but the average is much less, usually around 100 days (Turillazzi 1989). Fights do occur, of some severity, and nests are sometimes taken over by usurping females. Bridge and Field (2007) have found evidence of limited queue jumping.

1.3.8 Colony genetic structure

Nests are typically small groups (1-10 females), with sometimes as many as five resident males on larger nests. Initially, *L. flavolineata* were judged to be only incipiently eusocial due to low relatedness among female nest-mates (r =0.22 $\pm$ 0.1]) (Strassmann et al. 1994). However, re-examination of genetic structure by Sumner et al (2002) using 3-4 hyper-variable microsatellite loci resulted in an average relatedness estimate of (r=0.56 $\pm$ 0.05). An estimate of (r= 0.46 $\pm$ 0.08) was later obtained from Fraser's Hill (Bridge 2005; Field et al. 2006). Overall, these results suggest that mean relatedness is normally (r= 0.4- 0.5), within the range typical for other primitively eusocial wasps (Strassmann et al. 1989). Relationship analysis showed that that normally only one reproductive adult lays female eggs, subordinates occasionally lay male eggs, and that the dominant reproductive female is singly mated (Sumner et al. 2002).

1.3.9 Forging and larval provisioning

Prey is sometimes processed away from the nest, but typically in *L. flavolineata* insect prey is amalgamated into balls to be distributed to adult males and females through stomodeal trophallaxis (transfer of food or fluids through month-to-mouth feeding) upon return from foraging (see Figure 1.4 a). Analysis of solid food returns in *L. flavolineata* shows that they

contain ant and sometimes termite remains (Samuel 1987), although it must be noted that at Fraser's Hill scientists have observed only ant prey when in recognisable form (L. Holt pers. obs.; J. Field pers. comm.). Liquid returns contain sugar suggesting that *L. flavolineata* are able to forage for, and digest nectar (Keegans & Morgan 1993; Sledge & Fortunato 2000). Adults are likely to require carbohydrates, and larvae primarily protein. However, consumption of protein is likely a requirement for successful egg production, and larvae of some Stenogastrinae are known to extrude carbohydrate based saliva upon stimulation in some species, potentially providing a way of processing protein into sugar (Chown & Nicolson 2004). Foraging is primarily undertaken in the morning, with a small amount of foraging in the late afternoon (J. Field pers. comm.).

1.3.10 Male behaviour and mating

Notwithstanding the first few eggs laid on new nests (which are usually female), male and female offspring are produced simultaneously (Samuel 1987). Adult males often leave during the mid-day hours to congregate in lek-like aggregations near the entrance of their culvert or bridge (see Figure 1.4 b).

1.4 An overview of group living

Group living is a complex arrangement that usually involves both costs and benefits. In totipotent species, subordinates should prefer to be in the dominant reproductive position, where the rewards of group living are most concentrated. However, subordinates may be coerced, manipulated, or have no other viable option but to accrue a cost and work in a lower position (Richards et al. 2005; Kapheim et al. 2012; Brand & Chapuisat 2012; Jaffè et al. 2007). Even for individuals waiting to inherit breeding rights there can also be several benefits to living in a group, and dominant individuals may also accrue costs. This thesis examines how some of these trade-offs of group living, for example foraging effort reproductive skew and productivity, may either decrease or increase as a function of group size. Here, I broadly introduce the costs and benefits in social systems that are important in the context of this thesis, including those specific to *L. flavolineata*.

(a)

(b)

FIGURE 1.4. (a) A female processing a ball of insect prey at the nest. (b) A hanging leaf at the entrance to a field site with males congregated into a lek.

1.5 Benefits of group living

1.5.1 Benefits of a central location

When considering sociality, a central, defendable location for brood rearing conveys many advantages and is essential for progressive provisioning of young (Hunt 2007). A central location for brood rearing can be defended and threats detected more efficiently, especially

when group numbers become larger. In fact Nowak et al. (2010) go as far as to say "the causative agent [to sociality] is the advantage of a defensible nest, especially one both expensive to make and within reach of adequate food." Apart from genetic benefits of assisting kin, Nowak et al. (2010) go on to suggest this may be a limiting factor to subordinates leaving for their own independent reproduction. This is particularly likely in species with elaborate and long-lasting nests that are used continuously, although in many species it may be the group itself rather than the nest architecture that is important (Field & Foster 1998).

1.5.2 Inheritance benefits

In species without worker sterility, altruistic behaviour need not completely exclude future direct reproduction by the social donor. When inheritance of a territory, or resources such as a nest structure or subordinate helpers are of vital importance for the future direct reproduction of the inheritor, altruistic behaviour may in fact be a complex bargain (Leadbeater et al. 2011; Bridge & Field 2007; Zanette & Field 2009). Helping others in order to qualify for inheritance of these resources is therefore a type of delayed reciprocal altruism, often augmented with inclusive fitness benefits by nesting with relatives.

1.5.3 Insurance benefits

In many species the period in which offspring are dependent on provisioning and parental care can exceed that of the reproductive phase of an adult's lifespan. Gadagkar (1990) proposed that cooperatively breeding groups benefit from Assured Fitness Returns (AFRs). If a mother dies before she is able to rear offspring through to adulthood, the surviving group members continue allo-parental care (Shreeves et al. 2003; Queller 1989; 1996; Field et al. 2000). Gadagkar (1990) demonstrated that such a mechanism could favour helping even if the relatedness between the helper and offspring is very low, since the donor in turn gains AFRs after nest inheritance.

1.6 Universal benefits to increasing group size

Above and beyond automatic advantages to group living such as a defensible central location (Crespi 1994), increased vigilance towards predators (Bertram 1980; Fernández-Juricic et al. 2004), or inheritance and insurance benefits, there may be universal advantages to

increasing group size, including from a solitary to social state. These universal benefits generally pertain to increases in stability or efficiency. Although 'Allee effects': benefits gained from the presence of conspecifics (Allee & Bowden 1932), are generally applied to populations, social groups can also be subject to Allee principles, especially when resource acquisition is shared and dependent on the number of available foragers (Gusset & Macdonald 2010).

1.6.1 The Central Limit Theorem

> "I know of scarcely anything so apt to impress the imagination as the wonderful form of cosmic order expressed by the "Law of Frequency of Error". The law would have been personified by the Greeks and deified, if they had known of it. It reigns with serenity and in complete self-effacement, amidst the wildest confusion. The larger the mob, and the greater the apparent anarchy, the more perfect is its sway. It is the supreme law of Unreason. Whenever a large sample of chaotic elements are taken in hand and marshalled in the order of their magnitude, an unsuspected and most beautiful form of regularity proves to have been latent all along." - Sir Francis Galton (1889)

A potential first principle that could help explain not only the origin and evolution of sociality, but also the findings of Michener (1964) and the apparent lower productivity of larger groups across taxa, has been posited by Wenzel and Pickering (1991). The Central Limit Theorem (hereafter the CLT) is a mathematical concept (described poetically in the box above), that when applied to social groups suggests that larger groups should receive a more stable and predictable input of food since they have a larger number of independent foragers. As group size increases, there are more foragers available to take stochastic samples of the environment. Therefore, the average food income will be more reliably met and there will be less extreme variation in food returns from day to day (Sokal & Rohlf 1994). As a consequence, larger groups of some social species might be able to better match or track brood numbers to average food availability. Conversely, smaller groups may over-produce brood either optimistically, in error, or strategically as a tropic store to use in times of food shortage (Wenzel & Pickering 1991). If aborting brood or reducing clutch sizes downward is energetically inefficient, then over time selection would favour groups with less need to do so.

Several studies have examined the predictions of the CLT in social insects (Shakarad & Gadagkar 1995; Stevens et al. 2007; Naug & Wenzel 2006; Bouwma et al. 2006) but as yet, researchers have not manipulated the variation in foraging returns directly in order to isolate this from other effects of group living.

1.6.2 Benefits of task differentiation including reproductive division of labour

A larger group size can allow for task differentiation in a way that is impossible for smaller groups (Bourke 1999; Anderson & McShea 2001), and task differentiation can potentially result in superior group functioning (Holbrook et al. 2009; Grüter et al. 2012). Toyoizumi and Field (2014a) model division of labour between queen and helpers and demonstrate that this extends nest lifespan and has a large impact on nest productivity. Poethke & Liebig (2008) also suggest that since reproducing individuals need to overcome a nutritional threshold in order to reproduce, group formation and task specialization (resulting in food returns directed primarily to one reproducing individual instead of many), can result in this threshold being crossed sooner. In extreme environments, especially those with high variance in food availability this may highly advantageous.

1.6.3 Risk management

Foraging, especially when it requires long search times and encountering live prey, is risky, involving danger of exhaustion and predation away from the relative safety of shelter. Individuals that forage more have shorter lifespans and reduced fecundity (Field & Cant 2006). Poethke & Liebig (2008) suggest gregariousness and food sharing is a way of mitigating this risk. Vampire bats are a classic example of a species with highly variable chances of foraging success which share food in an emergency (Wilkinson 1984; 1990). Without conspecifics to share food, mortality would likely be much higher, and larger aggregations in this and other species may provide some buffer to a failed foraging trip, since the chances of finding a cooperative individual who will share food increases with group size (Kerth 2008). Within species that have a more clearly defined social group, increasing group size and potential CLT-mediated benefits could also be important in this respect as a type of insurance. With more foragers, and a decreased variance of food returns to a central location,

it is more likely that each individual will receive some food even if they themselves have been unsuccessful.

1.6.4 Benefits of social complexity

Viewed from a major transitions viewpoint, the move form solitary to social behaviour is a transition to a higher state of complexity (Szathmáry & Smith 1995; Bourke 2011). All living organisms are complex adaptive systems ranging from single cells to organisms in complex social networks (Bonabeau 1998). If individuals in a group can differentiate or specialize, for example, then this is a source of additional complexity. If a larger group size enables differentiation (which in turn may enable the group as a whole to adapt better to the environment), the group then forms a higher-order complex adaptive system (Bourke 2011). Larger, more differentiated groups may be better able to adapt to the changing environment, increasing the group's survivability as a macro-structure, benefiting the constituent individuals and their genes (Grüter et al. 2012). Evolution is not actively progressive, however, a lack of an overall trend towards complexity in biology does not preclude the existence of forces driving systems towards complexity (including social complexity), in a subset of cases (Carroll 2001). Since there is likely to be variation in tolerance to stimuli, the genetic selection of aspects of social behaviour, such as number of nest-mates, is highly probable.

1.6.5 Optimization principle

The universal benefits detailed above all increase the efficiency of groups as a function of increasing group size. Group functioning can also be optimized by increased synergy. For example, communication and cooperation between group members (even as a consequence of a greater number of interactions conveying information) can in some cases allow greater exploitation of resources than by the same number of independently operating individuals (Beckers & Goss 1989; Jarau & Hrncir 2009; Naug & Wenzel 2006; Czaczkes & Grüter 2011). Optimization can also include increasing efficiency of a parameter such as foraging effort, brood production or defense.

If, as a factor of increased synergy or an effect of a principle like the CLT, larger groups are more efficient, either productivity per-unit effort will increase non-linearly or effort will be reduced. This could potentially result in a decreased mortality rate, greater group

persistence or a re-direction of this energy into reproductive output. If any of these economies increases the number of gene copies in the next generation, over evolutionary time larger group size would lead to a selective advantage, and potentially lead to a social transition (see Figure 1.5).

However, although increasing group size could decrease effort or increase productivity, there may be a point at which this relationship breaks down, especially since costs to group membership fall disproportionally on some members. It is likely that different individuals in queues face different-shaped lifespan distributions according to risky activity (Reeve & Nonacs 1997; Keller & Genoud 1997; Mesterton-Gibbons et al. 2006), whilst the queuing system of many species including *L. flavolineata* precludes any substantial direct reproduction until inheritance of the dominant position (Bridge & Field 2007). Each species will theoretically have an optimal or ideal group size up to a maximum whereby group size may begin to have negative effects. This will be dependent on parameters such as intra-specific relatedness, productivity, ecological constraints, the cost of offspring production, and rates of predation (Pulliam & Caraco 1984; Zemel & Lubin 1995; Avilés & Tufino 1998).

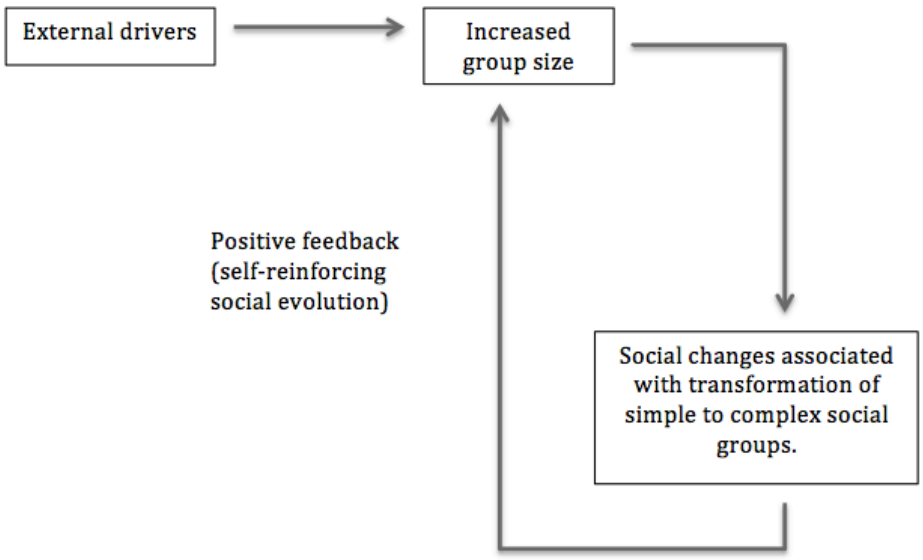

FIGURE 1.5. Schematic diagram of the extended version of the size-complexity hypothesis proposed in Bourke (2001) to underlie social group transformation in multicellular organisms and eusocial societies. Adapted from Bourke (2011).

1.7 Costs of group living

1.7.1 The costs of conflict and aggression

Aggressive interactions or 'cheating' behaviour in social groups is extremely common and can place a significant burden on productivity, nest functioning and integrity. Aggression, or lack of, may be a product of the level of relatedness between actor and participant, or of the relative status between them within a dominance hierarchy (Clutton-Brock et al. 1998; Clutton-Brock & Brotherton 2001). Cheating behaviour includes subordinates producing their own offspring, or killing the offspring of the dominant or other breeders (Sommeijer & Veen 1990; Monnin & Peeters 1997). This not only affects productivity but also can disrupt intra-nest patterns of relatedness.

Despite the established hierarchy, female *L. flavolineata* remain able to challenge the social order, moving up or down within the queue or even attempt to usurp the dominant position of a nest (Bridge & Field 2007). *L. flavolineata* can exhibit a range of aggressive behaviours including violent fights, typically involving repeated wrestling matches causing the participants to drop from the nest. Fights to the death do occur (L. Holt pers. obs.). It is hypothesized that in many social species there may be an maximum number of workers before dominance is threatened (Strohm & Bordon-Hauser 2003). For example, in annual species, too few workers and the foundress will not achieve a satisfactory number of gynes and drones in the reproductive cycle, too many and the workers may not be fully under foundress control and may lay eggs of their own. In reproductive skew models with incomplete foundress control (see below), group size is an important factor in terms of subordinate reproduction (Reeve et al. 1998).

1.7.2 The costs of task differentiation and reproductive division of labour

Although division of labour and task allocation can produce benefits in efficiency, whenever there is division of labour there is also the opportunity for cheating, exploitation and conflict. Nowhere is this more contentious than in the partitioning of reproductive privileges. In primitively eusocial species, where all females retain the ability to breed, each female

should prefer to be the reproductive dominant above any other position, especially when certain roles such as foraging are more dangerous and are associated with subordinate ranks (Richards et al. 2005).

Reproductive skew is a quantitative measure of how reproduction is partitioned among members of a society. Depending on the skew index used (Nonacs 2000), high values typically indicate that reproduction is monopolized by one or a few individuals (Faulkes & Bennett 2001), whereas low values reflect a more equitable partitioning of reproduction among group members (Cavigelli et al. 2003). There are a large number of reproductive skew models (Table 1.1), which typically fall into two broad categories. Firstly, transactional models, which assume the dominant has control over reproductive activity at low cost, and may concede some reproductive shares to subordinates in return for their assistance (with skew settling at an evolutionarily stable equilibrium). Secondly, tug-of-war (or compromise) models in contrast, assume that the dominant does not have complete control and subordinates are able to wrest reproductive shares away according to their competitive abilities and group size. This is detrimental to group productivity, with the level of skew determined by a process of attrition (Clutton-Brock 1998; Johnstone 2000; Field & Cant 2009). None of the models consistently and accurately predicts skew across all systems (Reeve & Keller 2001).

Transactional models	**Compromise models**
Reeve & Ratnieks 1993	
Reeve & Kellar 1995	Cant 1998
Reeve 1998	Reeve et al 1998
Cant & Johnstone 1999	Johnstone & Cant 1999*b*
Johnstone & Cant 1999*a*	
Johnstone et al 1999	**Combined models**
Kokko and Johnstone 1999	
Ragsdale 1999	Jonhnstone 2000
Reeve & Emlen 2000	Reeve and Shen 2006
Buston et al 2007	
Parameters:	**Parameters:**
Assumes dominant controls reproduction	Assumes incomplete reproductive control
Subordinates can choose to leave	Dominant- subordinate conflict
Value of subordinate assistance	Resource holding potential
Subordinate reproductive ability	Group size
Likelihood of subordinate nest inheritance	Effect of subordinate conflict or reproduction
Cost of offspring production or loss	on group productivity
Number of individuals considered	

TABLE 1.1. Table of reproductive skew models and their parameters.

1.7.3 Rank and foraging effort - the costs of being a worker

In many nests of social insects, subordinates work harder based on their position in the queue. Whenever a totipotent worker donates their assistance to aiding the reproduction of another individual, they suffer a cost to social living that detracts from their own future fitness. For *L. flavolineata*, being of lower inheritance rank increases the amount of effort invested in risky foraging, although in some other species the dominant forages more while subordinates guard the nest (for example eusocial thrips such as *Oncothrips tepperi*). In *L. flavolineata* this is in part due to life history traits: a higher ranked individual has greater potential direct fitness benefits, as she is more likely to inherit the egg-laying position, therefore she invests less in dangerous foraging (Field et al. 2006). However, even for the lowest ranks with highest mortality rates, remaining at the natal nest to inherit the dominant position may be a better option than dispersing from the natal nest if chances of successful independent nest founding are low (Field et al. 1998; Rehan 2011; but also see Bull & Schwarz 1996).

1.7.4 Universal energetic costs to increasing social complexity

An opposing view to the optimization principle is that an increase in social complexity, such as from a solitary to social state, primitive to advanced eusociality, or with increasing group size, requires an increase in energy requirements per-unit mass. This may be due to increased conflict, inefficiency, or even a larger amount of cognitive processing power required for social interaction (Fonck 1996; Muradian et al. 1999; Jaffé 2010). Thermodynamically, a more highly ordered system of individuals or a more complex individual animal is further from energy equilibrium, and therefore requires more 'work' to maintain (Corning & Kline 1998a; 1998b; Schneider & Sagan 2005). If social living requires more energy than being solitary, this may be a cost reflected in higher energy consumption (or food amount) per-capita, a reduction in productivity or increased foraging effort in larger groups (Jaffé & Fonck 1994).

1.8 Genetic structure of groups

In order to understand the costs and benefits of social vs. solitary living, models have been developed to test the dynamics of groups. Several have tested group or population productivity and survival, but have assumed that genetic benefits are equal for individuals (Toyoizumi & Field 2012; 2014a; 2014b). However, uniform relatedness between group

members and indirect fitness benefits equal to those of the reproductive dominant are unlikely to occur in many species. The genetic structure of a group is likely to vary with size, and may be non-uniform, particularly in species that accept unrelated joiners.

1.8.1 Genetic relatedness at nest initiation - a special case?

Studies using genetic markers suggest that nest-sharing, casteless groups often comprise of, or contain non-relatives (Queller et al. 2000). Some species go through bottlenecks in their life cycle where relatedness decreases, for example at the initiation of a new colony (Zanette & Field 2008). The establishment of a new nest by co-foundresses can be egalitarian or fraternal, that is, a union of unrelated or related entities. Those established with co-foundress peers can represent a significant deviation from a typical mature group structure and behaviour. In such situations there may be unique conflicts unmediated by kinship. Some species of social insect exhibit kin or natal nest-mate recognition (Gamboa et al. 1996; Leadbeater et al. 2013). This may increase the chances of sisters or female relatives establishing new nests together, and of subordinates gaining inclusive fitness benefits.

As yet, it is unknown whether new nests of *L. flavolineata* have a unique relatedness profile, but even if not, being required to build a new nest adds a significant burden to the foundress, particularly so in this species. Furthermore, at nest establishment, where any brood consists of very early stage eggs and larvae, there is little prospect of daughters reaching adulthood in the near future to help. Therefore, any assistance that auxiliary females can provide at nest inception is at a premium, and as such this offers a unique test of the parameters of reproductive skew models (Field & Cant 2009). This is in contrast to a dominant's situation on a mature nest, which she has likely inherited equipped with a queue of subordinates. Nest-born subordinates may also be more inclined to help without bargaining for a share of direct reproduction (Bridge & Field 2007; Abbot et al. 2011), and their decisions may potentially be dependent on cues for high or low relatedness such as nest structure and group size. By analyzing the intra-nest relatedness and reproductive skew on new nests, further light can be shed on how increased value of subordinates to a foundress, potential low relatedness, and group size can affect the level of skew.

1.9 Aims of Thesis

Since the Modern Synthesis (Wilson 1975), social behaviour has remained an active area of research as an arena to test many of the predictions of natural selection and inclusive fitness. Advances in genetics and the accessibility of genetic tools for assigning parentage have provided the opportunity to finely dissect the relationships and genetic costs and benefits for individuals participating in altruistic behaviour.

In addition the strengths of using *L. flavolineata* to test for Michener's Paradox and study potential statistical first principles of sociality, this species undergoes unique stages in group development, with potential variation in important parameters including relatedness, which has been lacking from previous studies. Genotyping the individuals and brood associated with newly formed nests with more microsatellite loci will shed better light on the conflicts and alliances during this important and dynamic stage. The females in this facultatively eusocial species face a myriad of choices of whether to act altruistically or selfishly, and unlike more advanced eusocial species, they are able to pursue the full range of social actions without physiological constraint. Since stochastic models of the effects of increasing group size have been developed specifically for species similar to Stenogastrinae, data from the following experiments can reliably test their assumptions and inform future research. The specific aims of this thesis can be summarized as follows:

1. To generate a microsatellite library for *L. flavolineata* and optimize a toolkit of primers with sufficient power to more reliably detect relationships between individuals within a nest.

2. To experimentally manipulate one of the parameters (variance in food inputs) thought to influence Michener's paradox in social insects, across *L. flavolineata* nests of varying size.

3. To identify newly established nests and collect their adults and brood in order to test reproductive skew in a context where relatedness is hypothesized to be low, in order to test current models of reproductive skew.

4. To experimentally determine the inheritance rank of wasps in queues of varying length, and investigate whether there are any systematic patterns in relatedness or

relationship to the dominant throughout.

5. To synthesize these ideas and findings in relation to foraging effort at each inheritance rank, according to relatedness and group size.

Each research chapter is presented as a largely self-contained piece of work.

1.10 Summary of Thesis

In this thesis, I use field experiments to test whether a potentially universal benefit of sociality operates in the facultatively eusocial hover wasp *Liostenogaster flavolineata* (Stenogastrinae). I also use newly developed microsatellite markers to investigate the genetic structure of colonies, and test whether genetic relatedness influences two key features, reproductive skew and foraging effort.

In Chapter 1, I introduced my study organism, *L. flavolineata*, as a model system for investigating the major questions addressed in the thesis.

In Chapter 2, I briefly outline general field and laboratory techniques referred to throughout the thesis and present methods and results from the development of new microsatellite markers for *L. flavolineata*.

Chapter 3 introduces the concept of universal benefits to sociality, and looks at the Central Limit Theorem (CLT) as a potential reason why individuals come together to provision offspring. I report the results of an experiment designed to test the central tenets of the CLT by manipulating the variance in food input to *L. flavolineata* nests.

Chapter 4. Theoretically, genetic relatedness between adult nest-mates should have an important influence on how reproduction is partitioned (reproductive skew). However, this has proved hard to test in primitively eusocial wasps because relatedness tends to be uniformly high. I use microsatellite markers to estimate reproductive skew in newly established colonies of *L. flavolineata*, where nest-mate relatedness is low.

Chapter 6. Genetic relatedness between subordinates and the dominant egg-layer could potentially vary systematically with position in the age-based queue to inherit the egg-laying position found in *L. flavolineata*. Using newly developed microsatellite markers, in combination with manipulations to determine queue position, I test for a relationship between relatedness and queue position.

In Chapter 7, I investigate how genetic relatedness and group size influences foraging effort.

Chapter 8 is a summary of the thesis with concluding comments.

CHAPTER 2

GENERAL METHODS

2.1 Field Techniques

2.1.1 Sites

The studies in this thesis were conducted in 2011 (study dates from 5 th May 2011 to 1st August 2011), and 2013 (study dates from, 19th August 2013 to 2nd October 2013). The study sites (see Figure 2.1) comprised the undersides of two bridges and one culvert along a little-used road near to Fraser's Hill, 116 km North of Kuala Lumpar, peninsular Malaysia (latitude 4°N). This road, which runs between The Gap and Raub, is the same road at which previous studies have been based since 1983 (Samuel 1987; Strassmann et al. 1994; Field & Foster 1998; Field et al. 1999; Sumner et al. 2002; Field et al. 2006). Sites shall henceforth be referred to as Site 1 ("52-nest") a bridge, Site 2 ("Jump-down site") a culvert, and Site 3 ("Scramble-site"), an aggregation of >100 *L. flavolineata* nests also under a metal bridge. A rock fall prevented access to Site 3 in 2013. Details of which sites were used for the different experiments are given in the relevant chapters.

2.1.2 Experimental preparation

Before any experiments or behavioural observations, each *L. flavolineata* nest was numbered using bright adhesive electrical tape adjacent to the nest. To identify nests that were initiated during the field season, sites were periodically scanned for new, un-numbered nests, which comprised only cells that were too shallow to house a pupae (see Figure 2.2 a).

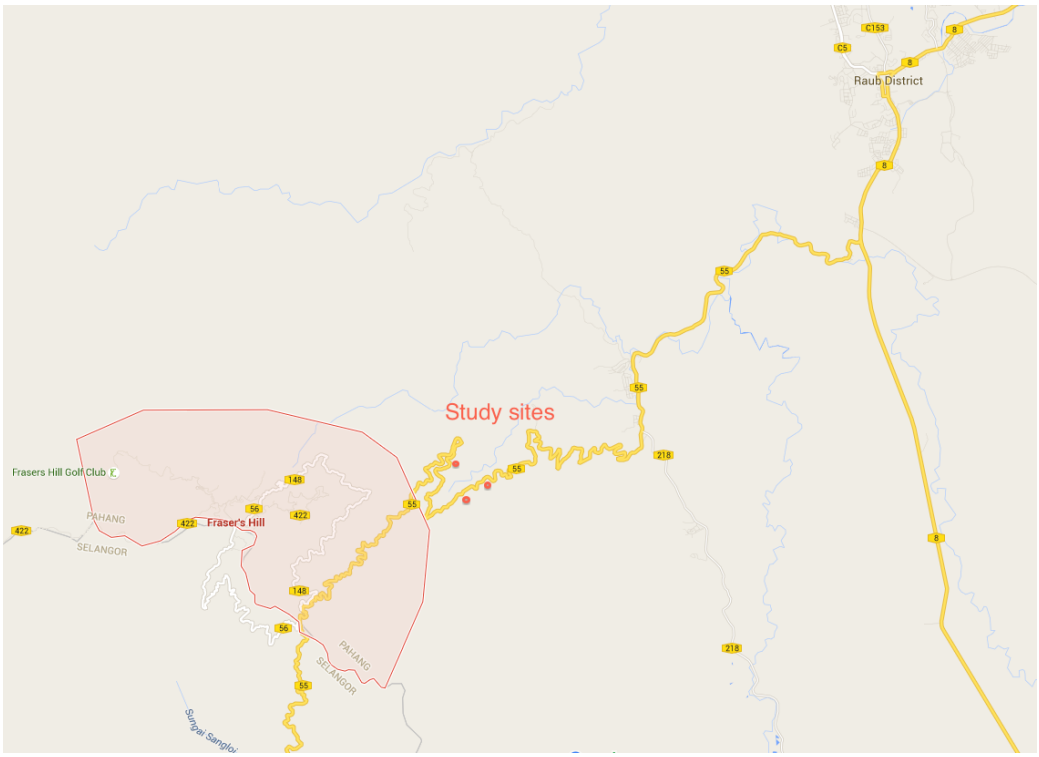

FIGURE 2.1. The study sites comprised the undersides of two bridges and one culvert along a little-used road near to Fraser's Hill, 116 km North of Kuala Lumpar, peninsular Malaysia (latitude 4°N). This road, which runs between The Gap and Raub, is the same road at which previous studies have been based since 1983.

2.1.3 Monitoring foraging returns

Positive food returns are identifiable through trophallaxsis (mouth to mouth transfer of food with a distinctive crowding movement of nest residents) and visual identification of food pellets. It was sometimes necessary to record the size of solid food pellets (graded size one to five). A size one food pellet is approximately the width of the wasp's jaws. A size five pellet is approximately as large as the wasp's head (see Figure 2.2 b).

2.1.4 Isolating nests

For the experiment in Chapter 3, nests were chosen only if they were accessible and sufficiently separate from others to allow isolation with the plastic pots (see Figure 2.2 d). The nests were isolated and the wasps prevented from foraging or returning with food to the nest by the application of a clear plastic container around the nest. The containers had ventilation windows covered with mesh (ladies hosiery), and were large enough (approximately 20 cm in

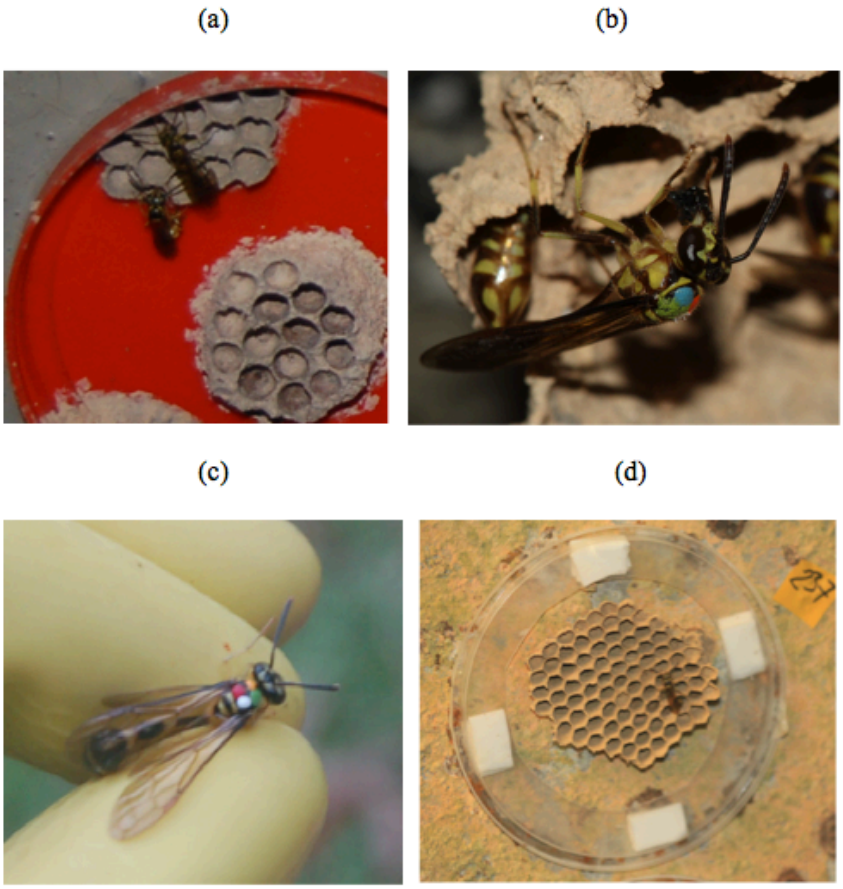

FIGURE 2.2. (a) On a plastic lid that had been added in a previous field season, *L. flavolineata* females construct a new nest (note the darker colour of the mud) (b) A marked female processing a processing a black ball of ant prey at the nest (size 3). (c) An example of the four colour matrix added to *L. flavolineata* thorax. (d) A nest prepared for the application of a pot to prevent food returns.

diameter and depth) as to not unduly constrain or alarm nest residents. In addition to its identifying number, each nest was given either a green or red identifying label, corresponding to its treatment group, in order that the pots could be quickly administered. Prior to the commencement of the experiment, the modified pot lids (with centre removed) were adhered around each nest on the underside of the bridge (see Figure 2.2 d), using foam adhesive strips, and where necessary, a small amount of superglue. Pots could then be simply snapped into place around each nest with minimal disturbance, capturing any wasps that might still be in residence, including the dominant female.

2.1.5 Marking procedure

When it was necessary to identify each individual, wasps were marked on the thorax with a unique combination of four coloured enamel paint spots from a total of nine different colours. I was careful to choose colours that were easily distinguishable and in a colour pattern sufficiently different from nest-mates. Nests were collected before dawn (approximately 06:00). A zip-lock plastic bag was placed beneath the nest and the nest comb scraped gently with a pencil so that residents were startled and dropped into the bag. The bag was then sealed and stored in a cool box with ice packs. Wasps were handled with rubber washing-up gloves. Gripping individuals securely between the forefinger and thumb by their legs, spots of enamel paint were applied to the thorax in a symmetrical pattern with a pin (see Figure 2.2 c). Wasps were held for a minute to let the paint dry and then released. Wing measurements were also taken at this time, and mark pattern, date, and size recorded for each female. Males were marked with a single large white paint identifier on the thorax. Nests were collected before dawn on consecutive days until all nest residents were marked. Unmarked wasps that joined the group or who eclosed on the nest were collected and marked as the experiment proceeded. In the case of newly emerged females a couple of days were given before collection and marking to allow the wasp to orientate to the nest.

2.1.6 Monitoring adult wasps

For each census, marked individuals were recorded as being present or absent on their nest. Day censuses allowed the role of each particular female in the nest and foraging effort to be determined by censusing at 20 min intervals (henceforth referred to as 'rapid censusing'). Night censuses were conducted allow the maximum number of nest residents to be identified, since females usually return to their respective nests at dusk and remain there until dawn (Samuel 1987). A chink of light from a Maglite® torch was used to identify wasps when it was dark.

2.1.7 Determining inheritance rank

The dominance hierarchy can be determined by comparing how long each female spends on her nest. High ranked females spend the majority of their time on the nest, and low ranked females spend most time foraging. The dominant is thus identified as the female that spends

most time on the nest (Samuel 1987; Field et al. 1998; 1999). On each day of rapid censusing, a female must have had three or more recorded observations than the individual below her in order to be ascribed higher rank. Each nest was initially observed between three to seven days to determine the first dominant, which also gave the foraging effort of all individuals before removals began. After the first removal, nests were rapid censused for a minimum of three days to determine the next dominant individual. Two days were given between each removal for the wasps to re-establish their hierarchy. Removal of the behavioural dominant continued sequentially for the whole group.

2.1.8 Behavioural observations

Behavioural observations were also used when the most dominant two individuals spent equal time on the nest. *L. flavolineata* can exhibit a range of aggressive and passive behaviour ranging form violent fights (typically involving repeated wrestling matches causing the participants to drop from the nest), to less violent signals of dominance such as antennal boxing, chasing, shoving and domination of the central area of the nest comb (with subordinates driven to the outside rim or side). Whenever a resident rested on the side of the nest or nearby during censusing, or initiated or received an aggressive act, this was noted. Nests were placed under continual behavioural observation if rapid censusing did not reveal a clear dominant. Any oviposition or production of abdominal substance was also recorded.

2.1.9 Censusing brood

The cells of *L. flavolineata* nests are hexagonal and occur (fairly) neatly in rows (see Figure 2.1). The nest can be mapped on hexagonal paper, and each cell given a coordinate. I took a high-quality digital photograph of each nest to be mapped, from directly below. An A4 print was produced, and this allowed mapping the nest to paper to be done accurately away from the site. The photos were also a good method to recognize nests, and orientate the map to its co-ordinates, as each cell was lettered and numbered on the photo as well as the brood map. Brood mapping causes disturbance, as adult nest residents need to be gently shooed away with a pencil. Therefore brood mapping was always done on the same day during an experiment, in the latter (non-foraging) part of the day. Care was taken never to brood map when it was raining, due to the risk of wasps flying outside the shelter of the

bridge or culvert. Nest contents were viewed using a Maglite® torch on narrow-beam.

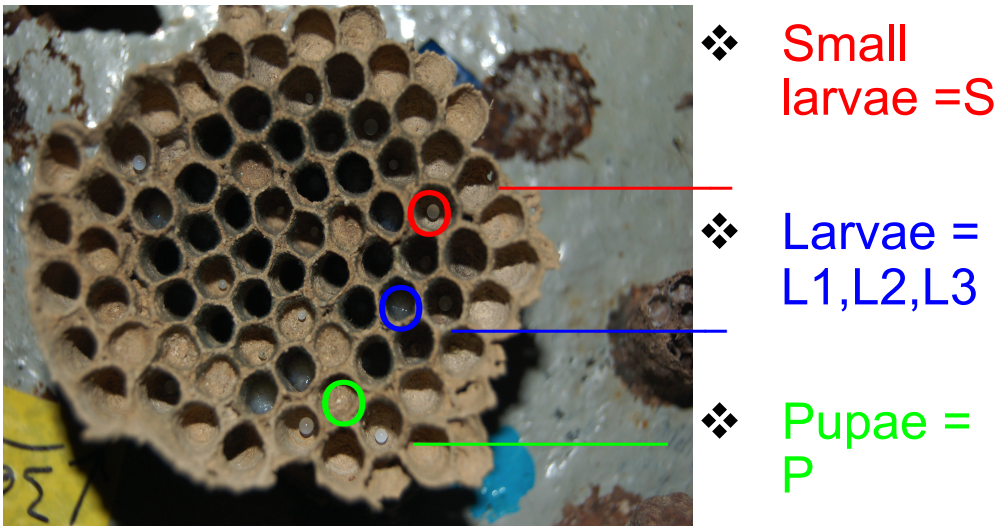

FIGURE 2.3. Typical nest of *L. flavolineata* with brood of different developmental stages highlighted.

The larval stages of brood were graded as follows: (S)= eggs and small larvae with no visible body segments, (L1)= small, comma shaped larvae with distinguishable head and body, (L2)= medium sized larvae with differentiated body segments, (L3)=large larvae, (P)= pupae in a deep brood cell capped by a mud lid (see Figure 2.1). If wasps laid a small larvae on top of a capped cell, this was designated SP. Eggs and small larvae below L1 stage were indistinguishable in the field due to the wasp's use of abdominal substance as an anchoring substrate and frequently re-applied nutritional medium (Turuillazzi 1991).

2.1.10 Collection methods

2.1.10.1 Adults

All residents were collected before dawn in zip-lock bags as described for wasp marking. Wasps required for permanent removal were stored in a cold box and euthanized at -10°C post-collection. Wasps were then stored in 100% ethanol until DNA analysis, with identifying labels.

2.1.10.2 Brood

Immediately after adult collection of new nests, all eggs and larvae were extracted from cells using fine forceps and placed directly in 100% ethanol.

2.2 Molecular genetic methods

Microsatellite analysis was used to determine colony genetic structure and relatedness of adults and brood. For microsatellite analysis, DNA from each sample was extracted, microsatellite sequences amplified, and fluorescently labelled products visualised by capillary electrophoresis. Microsatellites are short sections of DNA consisting of tandem repeated sequences of nucleotides (Nakamura et al. 1987; Queller et al. 1993). Such sequences have high mutation rate, and often occur polymorphically. As such, this allows for comparison between individuals and generations, with the difference in tandem sequence length allowing for fragments to be separated out and assigned identity due to relative size and therefore speed at which they pass through a porous polyacrylamide gel.

The four hyper-variable microsatellite loci used by Sumner et al. (2002) with *L. flavolineata* were designed at a time when genotyping was carried out manually, before autosequencers became generally available (Sumner & Field 2001). In 2013 these four primers were tested, redesigned, and tested again for use on an Applied Biosystems 3730 sequencer. Unfortunately all failed many of the tests of heterozygisity, possibly due to genetic drift in the study populations and therefore could not give sufficient power. In order to obtain more loci, a genomic library was constructed using the DNA from a single female taken from the field in 2013.

2.2.1 Extraction of DNA

Extraction of adult DNA from thoracic tissue for the genomic library using ammonium acetate extraction protocol is described in (Appendix A.2.1). For adult genotyping, the two lower tarsi of the adult wasp back leg were used, to minimize inhibitory enzymes found in other parts of the body. Adult, larval and egg extraction procedures are described in (Appendix A.2.2).

2.2.2 Development of microsatellites from a genomic library

The DNA from one individual was extracted at a concentration of minimum 20 mg/ml, and processed for the generation of genomic library by Dr. Gavin Horsborough at Sheffield Molecular Genetics Facility (SMGF), Sheffield, UK. The library was searched for potential microsatellites. Procedure and primer design was conducted according to a modification of the protocols of Armour & Neumann (1994) and Glenn & Schable (2005), details to be given in Holt & Field (in prep.).

2.2.3 Amplification using PCR

Extracted DNA combined with fluorescently labelled primers were amplified using Polymerase Chain Reaction (PCR). PCR methods and cycle sequences used are described in (Appendix A.2.3). The quantity of DNA post-PCR was measured by a plate flurometer, as polyacrylamide gel visualisation was not effective due to relatively low DNA concentration.

2.2.4 Post-PCR optimization and analysis

PCR products were analysed using an Applied Biosystems 3730 sequencer at SMGF. Post-PCR DNA obtained from using the leg tarsi was typically diluted to 1:500, and added to formamide to keep DNA strands separate for analysis. Heat shocking the samples at 95°C separated the strands, whereby the sample was then transferred to ice for three minutes (Appendix A.2.3). Plates were loaded into the sequencer for analysis and alleles were scored using the software Genemapper 3.7 (Applied Biosystems). Alleles were scored at least twice (by L. Holt and Dr. Gavin Horsborough of SMGF).

2.2.5 Optimisation, multiplexes, and fluorescent labels

Microsatellites were tested for Hardy-Weinberg equilibrium and linkage disequilibrium using GENEPOP v 3.4 (Raymond & Rousset 2004) on a subset of 24 unrelated individuals. These consisted of the Rank 1 individual from 24 separate nests collected from the 2013 field season. This minimised the chance that any of these adult female wasps were related (Samuel 1987; Bolton et al. 2006). CERVUS (Marshall & Slate 1998) was used to test whether the allele calls and bins in Genemapper were accurate.

Using the PCR protocol outlined in Appendix (A.2), it is possible to combine multiple primers into a single PCR reaction. These must be chosen so that no two primer sets in the same reaction have the same fluorescent label and overlapping range of PCR product size. In this case the potential multiplexes were restricted by only having two fluorescent dyes, although this keeps costs low with HEX and FAM being the most economical. See Holt & Field (in prep.) for temperatures, heterozygosity and EMBL numbers. All the 20 genotypes included in appendix (A.2) passed tests of Hardy-Weinberg and linkage disequilibrium. Loci not included in the analysis exhibited post-sequencing issues such as ambiguity of peaks. Full details will be given in Holt & Field (in prep.)

2.3 Analysis

2.3.1 Relatedness values

Relatedness v5.0.8 (Goodnight & Queller 1998) was used to estimate the mean inter-nest regression relatedness between various groups of different size. Colonies were weighted equally and standard errors were obtained by jack-knifing over nests. Pairwise relatedness estimates between adult individuals were determined using KINSHIP version 1.3.1 (Goodnight et al. 2005).

2.3.2 Genetic Relationships (using KINSHIP)

Relationships between individuals were determined using KINSHIP version 1.3.1 (Goodnight et al. 2005). This gives a maximum likelihood approach to compare hypothetical relationships between pairs of individuals with a set null and primary relationship based on haplodiploid inheritance patterns. The threshold likelihood ratio values are based on population allele frequencies, and therefore apply only to pairs of individuals where all loci have been successfully typed. However, KINSHIP was only used to analyze the relationships between adult females collected in 2013, extracted at Sheffield. Due to using leg tissue and obtaining very clean DNA, these samples had uniformly high coverage of typed loci. In the case of analyzing brood, sib-ships were sorted manually since brood sequence less well and KINSHIP lacks the capacity for including an error rate.

Prior to the development of a large number of loci giving sufficient power, it was only possible to detect a narrow range of relationships (e.g. mother or daughter, sister or cousin). However, with the greater power of the 19 new microsatellites, finer detail relationships can be tested, as long as it is appreciated that this type of analysis can give contradictory results, and therefore the possible relationships need to be eliminated in a systematic way. For example, when A is the null hypothesis and B the primary hypothesis, and A is rejected, it is not necessarily in favour of B. Below (see Table 2.1) is the table of relationships tested, and the type II error rates of different relationships based on the power of the loci involved. For example with Type I error rate set at ($p<0.001$, the Type II error rate of failing to detect a the effect that is present, in this case a result of sisters more likely than cousins, is 0.008. Full sisters are likely to be missed 0.8% of the time.

Sisters more likely than cousins.			
Settings for primary hypothesis: (*)			
Rm:	0.5		
Rp:	1		
Settings for null hypothesis:			
Rm:	0.38		
Rp:	0		
Results of significance simulations			
1000 simulated pairs used to calculate values			
	p<:	**Ratio**	**Type II error**
*	0.05	0.00E+00	0
**	0.01	0.00E+00	0
***	0.001	7.97E+01	0.008

Mother/ daughter more likely than aunt/ niece.			
Settings for primary hypothesis: (*)			
Rm:	1		
Rp:	0		
Settings for null hypothesis:			
Rm:	0.75		
Rp:	0		
Results of significance simulations			
1000 simulated pairs used to calculate values			
	p<:	**Ratio**	**Type II error**
*	0.05	6.91E+00	0.344
**	0.01	1.14E+01	0.796
***	0.001	1.86E+01	0.976

Aunt/ niece more likely than sisters.			
Settings for primary hypothesis: (*)			
Rm:	0.38		
Rp:	0		
Settings for null hypothesis:			
Rm:	0.5		
Rp:	1		
Results of significance simulations			
1000 simulated pairs used to calculate values			
	p<:	**Ratio**	**Type II error**
*	0.05	5.70E-04	0
**	0.01	9.95E-03	0
***	0.001	8.04E-01	0

Cousins more likely than unrelated.			
Settings for primary hypothesis: (*)			
Rm:	0.38		
Rp:	0		
Settings for null hypothesis:			
Rm:	0		
Rp:	0		
Results of significance simulations			
1000 simulated pairs used to calculate values			
	p<:	**Ratio**	**Type II error**
*	0.05	2.52E+00	0.299
**	0.01	1.09E+01	0.551
***	0.001	3.25E+01	0.723

Aunt/ niece more likely than cousins.			
Settings for primary hypothesis: (*)			
Rm:	0.75		
Rp:	0		
Settings for null hypothesis:			
Rm:	0.375		
Rp:	0		
Results of significance simulations			
1000 simulated pairs used to calculate values			
	p<:	**Ratio**	**Type II error**
*	0.05	4.00E+00	0.376
**	0.01	1.76E+01	0.719
***	0.001	5.31E+01	0.9

Mother/ daughter more likely than sisters.			
Settings for primary hypothesis: (*)			
Rm:	1		
Rp:	0		
Settings for null hypothesis:			
Rm:	0.5		
Rp:	1		
Results of significance simulations			
1000 simulated pairs used to calculate values			
	p<:	**Ratio**	**Type II error**
*	0.05	5.04E-01	0.02
**	0.01	4.30E+01	0.231
***	0.001	4.77E+02	0.52

TABLE 2.1. KINSHIP tests and Type II error rates for each of the questions asked in the analysis.

KINSHIP creates matrices of paired individuals within a given nest with a visual representation of confidence. Therefore, with successive matrices for a given nest and each analysis asking systematically whether certain relationships are more or less likely, it is possible to determine in most cases the relationship between pairs (see Figure A.1). This allows the construction of a matrix of each individual's relationship to the other in a nest.

For example, in the flow chart presented (see Figure 2.4), I start with the question, is it more likely that a given pair has a sister/sister relationship rather than a cousin/cousin relationship? This immediately sorts the nest into a group that is likely to be sisters or some other high relationship, and remaining unrelated or weak relationships. Subsequent questions narrow down the likely relationships as illustrated through a process of elimination and increase reliability. Please see Chapter 5 for further details.

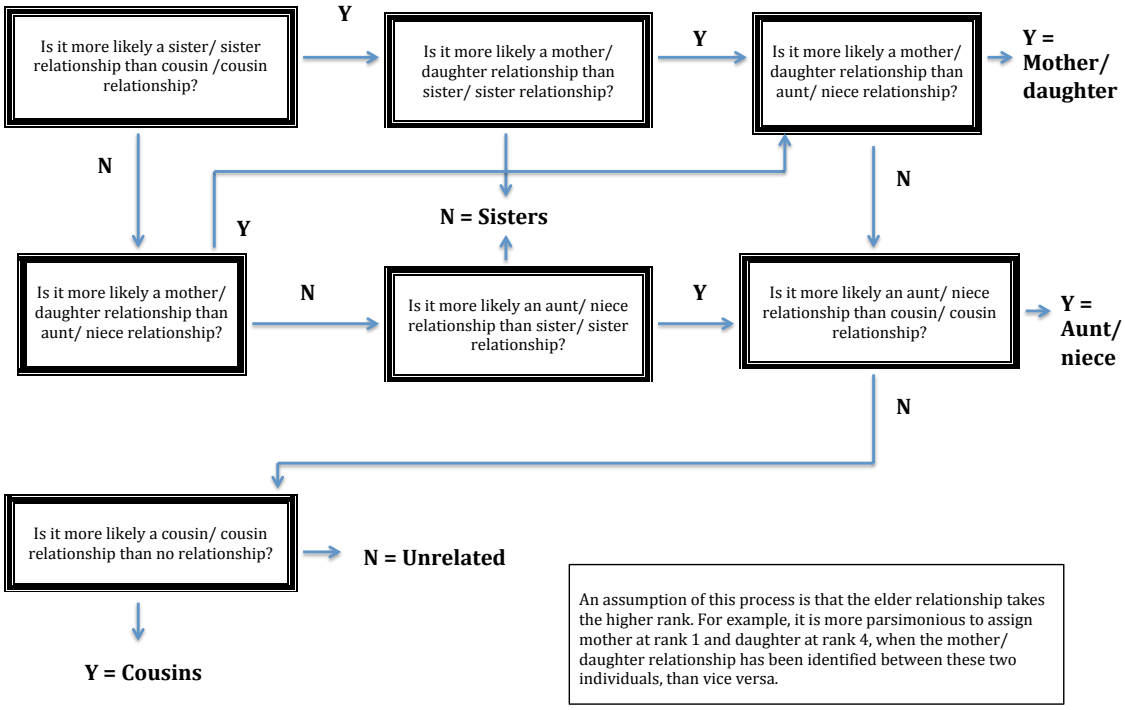

FIGURE 2.4. Flow-chart of the sequential questions asked of each pair of adult females within a nest, starting with the question whether the relationship is more likely sisters than cousins.

2.3.3 Measuring reproductive skew

Skew was calculated using Skew Calculator 2003 (Nonacs 2003b). Although there are over 20 different ways of measuring skew, a subset of eight indices are typically applied to studies of group-living: S-index (Reeve & Ratnieks 1993), Sc index (Keller & Krieger 1996), S3 index (Pamilo & Crozier 1996), Q-index (Ruzzante et al. 1996), l (or lambda) index, Id or Morisita index, Ip or standardized Morisita index (Tsuji & Tsuji 1998), and B-index (Nonacs 2000). Skew Calculator generates these eight, and for the B index calculates (by

simulation) a probability level for each group that its observed B value could represent a random distribution of offspring among nest members. The program simulates a random distribution across all groups to determine the probability level of the observed average across-group B values, and the B index value for the nest if the reproduction was monopolised by one individual. Both of these probabilities are one-tailed. The B index values and the full monopoly values are presented in Chapter 4 with accompanying p-values. The skew index value used in my analysis is the difference between each nest's B value and the complete monopoly value of that nest.

2.3.4 Identification of males and calculation of male brood skew

Individual brood that were homozygous at all loci were identified as males. As males are haploid, the power to definitively place them in a sib-group is significantly reduced. Since KINSHIP lacks the ability to assign them, males were ascribed to sib-groups manually.

2.4 General Statistics

All statistical analyses not requiring the specialist programmes mentioned above were performed in R (R Development Core team, 2011). Data that were not analysed using linear models were checked for normality using the Shapiro-Wilks test of normality, and where appropriate parametric or non-parametric tests were used. Assumptions of linear models were checked by visual inspection of model plots. A detailed description of specific statistical tests used is provided in each chapter.

CHAPTER 3

GROUP SIZE DEPENDENT FORAGING RETURNS. A TEST OF THE CENTRAL LIMIT THEOREM

3.1 Abstract:

Background: The Central Limit Theorem (CLT) is a statistical principle that states that with larger sample size, variance in sample means will decrease and means will become more normally distributed. When social insect workers forage independently, the CLT predicts that larger groups will obtain less variable daily total food rewards for their offspring. A larger group can therefore potentially match brood (immature offspring) number more closely to the more predictable food supply, a suggested 'universal benefit' to sociality. In contrast, lone provisioners and smaller groups may invest more heavily in optimistic or trophic brood to be aborted in times of dearth, and their brood may develop more slowly in response to variable nutrition. Here, I use the facultatively eusocial Malaysian hover wasp *Liostenogaster flavolineata* to experimentally test predictions of the CLT. I attempt to manipulate the variance in food input directly between matched groups of nests, with the treatment group having a more variable access to foraging.

Results: The Central Limit Theorem (CLT) is a statistical principle that states with larger sample size, variance in sample means (for example across a day) will decrease, and means will become more normally distributed. When social insect workers forage independently, the CLT predicts that larger groups will obtain less variable daily total food rewards for their

offspring. A larger group can therefore potentially match brood (immature offspring) number more closely to the more predictable food supply, a suggested 'universal benefit' to sociality. In contrast, lone provisioners and smaller groups may invest more heavily in optimistic or trophic brood to be aborted in times of dearth, and their brood may develop more slowly in response to variable nutrition. Here, I use the facultatively eusocial Malaysian hover wasp *Liostenogaster flavolineata* to experimentally test predictions of the CLT. I attempt to manipulate the variance in food input directly between matched groups of nests, with the treatment group having a more variable access to foraging.

Conclusion: The unpredictable availability of ant prey and the effect of weather patterns on opportunity to forage may result in highly variable daily foraging returns for this species. One interpretation of these results is that *L. flavolineata* adaptively adjusts patterns and frequency of foraging to manage both natural variability in food income and the experimental manipulation. Alternatively, the CLT may either be too subtle a mechanism to be detected by this experimental procedure, or nests of both treatment groups may have aborted trophic brood to the same extent in response to the food reduction inherent in the experimental design, or seasonal decrease in brood numbers.

3.2 Introduction

The evolution of sociality and group living is a fundamental problem; they can evolve only if each individual gains additional fitness at a non-linear rate when living in increasingly large groups (Naug & Wenzel 2006). Sociality is a widespread phenomenon, with many recorded benefits such as enhanced nest defence and division of labour (Wilson 1971; Bourke 2011). However, we may also want to ask whether there are any universal benefits to sociality and increasing group size that increase per-capita fitness, such as those that are based on more general mathematical principles (Wenzel & Pickering 1991). Although per-capita productivity is theoretically required to increase with group size (Naug & Wenzel 2006), the field has long been influenced by the findings of Michener (1964). He paradoxically observed that social insects can sometimes obtain higher per-capita productivity as lone individuals or in smaller groups, compared to when in larger groups (Michener 1964). Therefore, 'Michener's Paradox' would seem to act against the evolution of social living. Several subsequent studies

have failed to find evidence of his paradox (Bouwma et al. 2006; Jeanne & Nordheim 1996; Shreeves and Field 2002 but see Karsai & Wenzel 1998 for criticism).

Wenzel and Pickering (1991) evoked the Central Limit Theorem (CLT) to explain this observation, and suggested an automatic benefit to living in larger groups. The CLT is an aspect of probability theory well known to any scientist wishing to increase the reliability of their experiment by increasing the number of samples. In social insects, its application is in terms of food inputs to groups. Where foragers undertake food acquisition to progressively provision young, each trip may be considered a stochastic sample of the environment (Stevens et al. 2007). The larger the number of independently foraging group members (or the larger number of foraging trips each undertakes), the less variable the mean day to day food input should be. As the number of samples from an environment increases, total daily food returns will more closely resemble a normal distribution (Sokal & Rohlf 1994). Conversely, smaller groups will experience a more variable distribution of food returns and food income will be less predictable (Wenzel & Pickering 1991). Larger groups may therefore have an automatic advantage since more reliable foraging rewards could allow groups to ‘‘plan ahead’ and more closely match the number of offspring produced to average future food supply (Wenzel & Pickering, 1991). In contrast, lone individuals or small groups should face larger fluctuations in daily foraging rewards; they may be forced to abort offspring if foraging success is unexpectedly low, or waste excess food if foraging is unexpectedly successful (Naug & Wenzel 2006). In consequence, aborting offspring and under-exploiting food resources should reduce the reproductive efficiency of small groups compared to larger groups.

Another reason why smaller groups often have more brood per-capita, is that they may actively ‘bet-hedge’, by over-producing brood. Bet-hedging acts to lessen the impact of loss by counterbalancing it with other investments (Philippi & Seger 1989). Such bet-hedging strategies of overproducing offspring in anticipation of some loss can also be observed in other species, particularly birds, for example eagles, pelicans and boobies (Forbes 1991). To over-produce brood not only decreases the risk of under-exploiting scarce food resources (Wenzel & Pickering 1991), but the excess brood can, in carnivorous species, be relied on as a recyclable food store in times of dearth (Field et al. 2000; Karsai & Wenzel, 1998; Kozlowski & Stearns, 1989). Such optimistic or deliberate production of so-called ‘trophic brood’ could

result in the observed higher per-capita numbers of offspring in smaller groups. However, only a small fraction of the youngest brood present at any one time will ever reach adulthood, and therefore actual nest productivity (in terms of adults produced) may be substantially less than that estimated from any given snapshot (Bouwma et al. 2006). When examining the application of the CLT to all social species, insects with small eggs relative to body size may pay low costs in producing artificially high numbers of brood and may therefore be more likely to bet-hedge in this way (Kozlowski & Stearns 1989).

It also follows that *if* larger groups are more energetically efficient, they may be able to produce an average number of adults per-capita more reliably than smaller nests, and natural selection would favour those that had a lower variance in per-capita success (Gillespie 1977). Reliability would be selected for as being more rewarding on average, despite small nests occasionally being able to convert their larger per-capita number of small brood into adults in good conditions, a higher risk strategy (Philippi & Seger 1989).

Typically, previous studies investigating the CLT have focussed on i) variation in number or weight of brood, ii) the number of brood abortion events, or iii) larval development times, and generally find support for predictions of the CLT (Wenzel & Pickering 1991; Stevens et al. 2007; Shakarad & Gadagkar 1995; Kojima 1989). Shakarad and Gadagkar (1995) were able to establish an increasing likelihood of reaching the mean per-capita productivity with increasing colony size in their study of *Ropalidia marginata*. Furthermore, Stevens et al. (2007) demonstrated that in the univoltine allodapine bee *Exoneura nigrescens*, total brood weight shows less variation and becomes more normally distributed as colony size increases.

Stevens et al. (2007) also examined whether initial clutch sizes corresponded better with final brood sizes for larger colonies compared to smaller colonies of *E. nigrescens*. The mean per-capita number of brood was higher for small colonies than for larger colonies early in the season, in accordance with Michner's Paradox. However, by early summer when brood were more mature, this was no longer the case, suggesting that small colonies may indeed be initially producing more brood than they can always raise to adulthood. In contrast, in larger groups per-capita brood numbers did not change so much throughout the year, suggesting that larger colonies did not reduce the number of brood to the same extent. Abortion is likely

to be costly in *E. nigrescens* because eggs are expensive to produce (egg length is approximately 30% of total body length) and brood cannot be recycled as food; as a consequence oophagy and larval abortion in *E. nigrescens* are comparatively rare (Stevens 1997). In *E. nigrescens* at least, it might be predicted that CLT-derived advantages would give large nests a significant selective advantage through avoiding costly downwards adjustment of brood number, even though small nests may sometimes be able to achieve high fitness pay-offs when conditions are good. Importantly, Stevens et al. (2007) have not shown what causes these effects: CLT effects on foraging returns or some other factor such as better defence or division of labour in larger groups. Abortions are likely to be more common in carnivorous species where brood can be recycled; Wenzel (1989) also found a negative correlation between group size and the number of cells per-capita in *Polistes annularis*, and abortion events occurred more in smaller groups.

Studies measuring larval development times have found evidence that brood develop more slowly in smaller groups, in studies of *Polistes canadensi*s (Wenzel & Pickering 1991) and *Ropalidia fasciata* (Kojima 1989). Wenzel and Pickering (1991) also found that *P. Canadensis* demonstrated a significant bias in development times: in a nest of 69 females, 90% of the larvae pupated in 32 days or less, whereas only half the larvae on a nest of 14 females did so. The largest nests were the only ones where larval development times appeared narrowly and normally distributed about the mean value. Additionally, large colonies (six or more foundresses) of the Asian subtropical wasp *R. fasciata* demonstrated a similar, significant reduction in larval development times (Kojima 1989). The longer larval development times observed may be due either to slower development or undetected replacement of aborted eggs (or small larvae) with new eggs. Development time may therefore be a reliable barometer of the CLT's effects in species where brood removal is likely to be rare, or cannot be clearly observed.

Although Shakarad and Gadagkar (1995) were able to show larger nests were more likely to have at least the mean per-capita productivity in their study of *R. marginata*, they were also unable to show whether this was a result of the CLT or due to better protection from brood predation in large groups, an issue with several of the studies. Evidence for benefits to larger colonies are sometimes mixed. In the previous study (Shakarad & Gadagkar

1995), larger nests did not produce significantly heavier progeny and did not produce them significantly faster than smaller colonies did, at odds with the predictions of the CLT and the results of Stevens et al. (2007), and Kojima (1989).

However, Shakarad and Gadagkar (1995) were able to show empirically that deviation from expected food income decreased with increasing group size. To add weight to evidence for the CLT's effect on foraging returns, Naug and Wenzel (2006) developed a spatially explicit model which was tested using behavioural observations from five colonies of *R. marginata*. They found an increase in predictability of food supply to nests with larger adult group size, consistent with the CLT predictions. Where foragers are independent, they found advantages are gained from an increased number of foraging trips with larger group size, and that this was a product not only of adult number but also of social organization. If tasks within the nest are more efficiently organized, there is likely to be more opportunity for foraging trips, and therefore more samples from the environment (Naug 2001). They note that distribution of daily foraging success was highly skewed towards zero in smaller colonies, while approximating the normal distribution in larger colonies. This decrease in variability was most prominent in the lower range of group sizes, and they suggest that the advantages derived from the CLT are likely to be particularly important for species with small colonies and clumped resources such as invertebrate prey.

There have been both empirical and theoretical investigations into the effects of CLT on social insects, and results are broadly supportive of the concept measured in terms of increased abortions, higher food variability and slower brood development on smaller nests. Importantly, variability in food input remains to be manipulated in the field, and it is this central aspect of the CLT theory that I directly test in the following experiment.

3.2.1 Study species

Liostenogaster flavolineata is a facultatively eusocial hover wasp (Vespidae, Stenogastrinae), (Turillazzi 1991). *L. flavolineata* occur in small groups (1-10 females) across a wide distribution that includes Peninsular Malaysia. All females are capable of mating, laying eggs and independent nest founding. However they most commonly form multi-female, multi-generational groups with co-operative brood care (Field & Foster 1999) and high

reproductive skew (Sumner et al. 2002). Nests are perennial, due to the tropical climate, and their robust and long-lasting nests built from mud often occur in large aggregations (>100 nests), typically over bodies of running water, especially where artificial bridges and culverts provide an attractive location for nest building (Korb & Heinze 2008).

L. flavolineata brood are provisioned progressively, and each adult female undertakes foraging independently, with more dominant individuals spending less time foraging than subordinates (Bridge & Field 2007). *L. flavolineata* prey primarily on the alates (winged reproductives) of ants, and field observations of food returns imply temporal clumping of successful foraging events of this prey across nests in an aggregation (J. Field, pers. com; L. Holt pers. obs.). CLT effects are predicted to be particularly large in species with spatially clumped prey (Naug & Wenzel 2006), and the pattern of food returns suggests that prey items for *L. flavolineata* may also be spatially clumped, for example when the release of sexuals from ant nests is restricted to a certain location.

L. flavolineata produces relatively small eggs comparative to its body size compared to some other wasps and bees (although all aculeates have relatively large eggs compared to other insects). Loss or removal of eggs and small brood may be relatively low cost strategy, however, failing to adequately exploit food resources (which cannot be stored) may be especially disadvantageous when food supply is highly variable. The long development time of *L. flavolineata* larvae (approximately 100 days) makes loss of more mature brood highly disadvantageous, especially for single foundresses, who usually live for an average of 100 days (Field et al. 2000; Turillazzi & West-Eberhard 1996). Previous studies of brood number have found a linear relationship of brood number to group size (Shreeves & Field 2002; Shreeves et al. 2003; Field & Cant 2006), although this may vary with season.

Here I directly manipulate variance in access to food, in order to experimentally test this aspect of the CLT and measure whether increased variance has an effect on rates of brood removal or larval development.

3.3 Methods

The study was conducted in 2011 (the experiment ran from 11th June 2011 to 26th July 2011). The study sites comprised the undersides of two bridges along a little-used road near to Fraser's Hill, 116 Km North of Kuala Lumpar, peninsular Malaysia. This road, which runs between The Gap and Raub, is the same location previous studies have been based (Field et al. 2006). Initial measuring of foraging frequency was conducted at what shall henceforth be referred to as Site 1 ("52-nest"). Site 3 ("Scramble-site"), was the location of the main experiment which comprised an aggregation of >100 *L. flavolineata* nests situated under a metal bridge. Additional unpublished data of brood composition of different sized nests collected by Dr Catherine Bridge (henceforth Bridge unpubl.) between 3rd May 2001 and 30th August 2001 are also analyzed in this chapter.

3.3.1 Experimental procedure

Firstly, at the experimental site, all nests were numbered. The number of adults on each nest were counted at night (20:00 or later), when all individuals had returned to their nests. Nests suitable for the experiment were those that were accessible and sufficiently separate from other nests to permit application of the equipment (see General Methods). The number of females, males and stage of larvae in each nest was recorded, so that the nests could be ordered according to size, and randomly assigned to the control or treatment groups (treatment n= 32, control n=29). The number of resident females was also recorded at the mid-point of the experiment and at completion, to obtain the average female number used in the analysis.

3.3.2 Calculating average number of food returns throughout the day

At Site 1 (which was not suitable for the main experiment due to the tight formation of nests), 50 nests were monitored over three complete days (06:40 to 18:40) to record the number of positive food returns. These were identifiable through trophallaxis (mouth to mouth transfer of food with distinctive crowding of nest residents) and visual identification of food pellets. From this information, mean food returns were calculated (see Figure 3.1).

The bulk of the food returns occurred in the morning (07:00 to 11:30), with a second, smaller peak in the afternoon (16:30 to dusk) (see Figure 3.1). Changing the variance in potential food returns involved reducing time available to forage across all nests equally, but with the treatment group having more variable foraging opportunity. Since there was a reduction in foraging time, the morning period alone (06:45 to 11:30) was selected for manipulation in order to allow the wasps some opportunity for food and liquid acquisition. This regime also kept the reduction in food returns within the bounds of the wasp's natural experience, whereas residents might abandon the nests if conditions were too extreme.

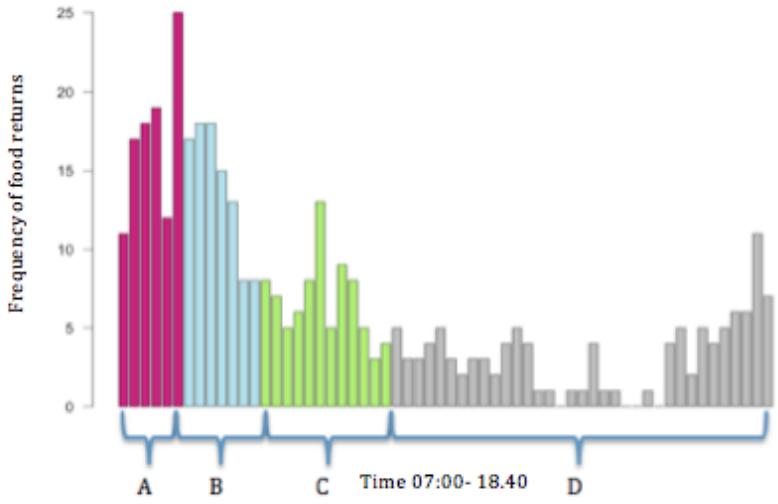

FIGURE 3.1. Bar-plot showing mean food returns (over 3 days), for n=50 nests at Site 1. The coloured bars represent first Block A (06:45 to 08:00), then Block B (08:00-09:30), and Block C (09:30-11:30). The remaining grey area D was not restricted.

3.3.3 Manipulation of variance in food returns

Both control and treatment groups were subject to an equal reduction in foraging time and therefore potential food acquisition, with this being more variable for the treatment group. The nests were isolated by fixing a clear plastic container over the nest preventing wasps either foraging or retuning with food. The containers had ventilation windows covered with mesh (ladies hosiery), and were large enough (approximately 20cm in diameter and depth) to not unduly constrain nest residents. In addition to its identifying number, each nest was given either a green or red identifying label, corresponding to its group (control

or treatment), so that the pots could be quickly administered. Prior to the commencement of the experiment, modified container lids (with centre removed) were attached around each nest on the underside of the bridge with adhesive foam tape, and a small amount of superglue if required. Pots could then be simply snapped into place around each nest with minimal disturbance, capturing any wasps that might still be on the nest comb, including the dominant female.

The main foraging period was divided into three blocks of equal food returns based on the initial observations of foraging patterns. The first block ran from 06:45 to 08:00, the second from 08:00-09:30, and the third from 09:30-11:30. The experiment consisted of 13 sets of the experimental cycle. Brood mapping was undertaken on the afternoon of the final day. The treatment group were isolated over all three blocks (06:45-11:30), on the first of these three days and was uncovered for the remaining two days. The control group received isolation each one of the three blocks sequentially, one per day, over the three days. Thus, the control had a less variable one-third reduction of food input per day (over the morning foraging period), whilst the treatment had a one entire morning out of three reduction, resulting in a more variable pattern of foraging restriction (see Figure 3.2). To control for the greater disturbance to the control nests, pots were placed over the treatment nests for one minute at the initiation of the containment of the control group, on the two days when the treatment nests were scheduled to be uncovered.

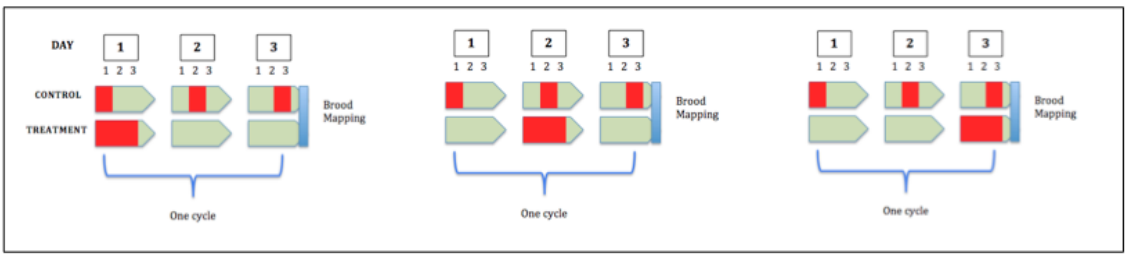

FIGURE 3.2. Experimental regime: Each red block represents a time frame with an equal amount of food returns as given by the previous observations. For the treatment group all three blocks were restricted for one whole morning of a three-day cycle. The control group had Block 1 (A) restricted on day one of the cycle, Block 2 (B) on day two, and Block 3 (C) on day three.

3.3.4 Measuring brood development and abortions

On the final day of each cycle, the contents of each cell were recorded in the afternoon (non-foraging period). On 11th June 2011 the first brood census was completed before any experimental procedure, using brood map outlines drawn from photographs of each nest's comb structure. The photographs also allowed accurate checking of the map's orientation and tracking of any newly built cells. Due to the need to remove wasps from their nests in order to clearly see cell contents, brood mapping will have caused disturbance. However this will have been equal for all nests, always done on the same day, and always done during the non-foraging part of the day. Nest contents were viewed using a powerful narrow-beam Maglite® torch. When heavy rain prevented safe driving to and access of the site in the morning, the experiment was suspended for that day and resumed the following day. Brood mapping was occasionally delayed for all nests by a day if there was heavy rain in the afternoon since the wasps were in danger of being driven outside the shelter of the bridge.

The immature brood were graded as follows: Small (S) = eggs and small larvae with no visible body segments. Larvae: L1= small, comma shaped larvae with distinguishable head and body. L2= medium sized larvae with differentiated body segments. L3= large larvae. Pupae (P)= deep brood cell capped by a mud lid. If wasps positioned a small larvae on a capped cell, this was designated SP. Whilst eggs and small larvae below L1 stage are distinguishable under a microscope, the use of abdominal fluid by the wasps as an anchoring substrate and a frequently applied nutritional medium often made it impossible to distinguish between them. Therefore all non-segmented brood were recorded as S stage. Occasionally cells were visibly parasitized, which was also recorded (n=1).When brood develop from S to L1, L1 to L2, L2 to L3 or L3 to P, these are the larval transitions referred to henceforth.

3.3.5 Abortions

Brood development, presence or absence was tracked by brood-mapping every three days (or four days if occasional heavy morning rain prevented access to the site). Whenever a brood disappeared from its cell, resulting in either recording an empty cell, or the cell containing a less developed brood (indicating it had been replaced), this was counted as an abortion event.

If large larvae (L2 or above) had clearly been moved from one cell to another, and there was no other possible explanation, an abortion event was not recorded (n=2).

3.3.6 The effect of ants

Sporadic raiding by ants was an unexpectedly large burden upon these nests. When observed, the presence of ants on a nest was recorded. However, instances of ant predation were distinctive even if the ants were not seen removing brood. Ants tended to strip the nest entirely of eggs, larvae, and occasionally even eclosing wasps. There were a total of 19 ant raids, across all nest sizes. Ants did not prefer nests in the treatment or control group (Control=10/29, Treatment=9/32). Three nests were removed from the analysis since they had been censused fewer than five times before being attacked by ants (Nest 323 (control) group size=5, Nest 355 (treatment) group size=2, Nest 356 (control) group size=2). Nests that were attacked before the end of the experiment, but with more than five censuses (n= 16/58) were included in the analysis, and number of censuses included as a random factor in the models (as it is likely nests with a longer timeframe will include more transitions and abortions). The potential difference in brood loss and transition rate as a factor of overall brood number is addressed in the analysis by calculating transition or abortion rate per active cell.

3.3.7 Data analysis

First, I wanted to investigate whether there was a linear relationship between group size and the number of brood in a nest. In order to test this, I used a generalised linear model (GLM) with negative binomial errors. Number of brood was the response variable, and 'initial group size' the explanatory variable. To test whether the relationship between initial group size and number of brood was linear, the model was run again with the inclusion of a quadratic term. Data collected in this thesis was combined with data previously collected from a nearby site (Bridge unpubl.). To test whether the two datasets could be combined, the maximal model initially included an interaction term between group size and 'dataset'. This was non-significant ($p>0.05$), and neither was 'dataset' by itself ($p>0.05$), and therefore I report results for the combined dataset for 'initial group size' only. Elsewhere, unless otherwise stated, analyses only include my data. To visualize the changing proportion of brood types

with group size, I created a spine plot.

Second I wanted to test whether manipulating the variance of food input significantly affected the rate of brood abortion or larval transitions. In this analysis I use the average number of adult females present on a nest throughout the study period, because group size fluctuated over time. Although data are presented as per-capita or per-cell rates of abortions and transitions, these variables could not be analyzed with a linear model due to difficulties transforming the data to normality. Therefore I analyzed the raw data using a Generalized Linear Model (GLM) with negative binomial errors. Since any of the given non-integer values consisted of the response variable divided by the explanatory variable in each case, all the information is retained in the models. Therefore, the number of brood abortions and larval transitions respectively were used as response variables, with 'average group size' and 'treatment' (i.e. treatment or control) included as response variables. Both models initially included and interaction term between 'treatment' and 'average group size'. These analyses were also conducted with 'average nest size' (cells) included as an explanatory variable instead of 'average group size', calculated as the total number of cells filled during the duration of the experiment, divided by number of censuses.

Data were analysed in the R statistical package (R Development Core Team 2011). Maximal models were checked for over-dispersion before proceeding with model reduction. I report results for when terms were sequentially removed from models. Starting with interactions, non-significant terms were sequentially removed until only significant terms remained (Crawley 2012).

3.4 Results

3.4.1 Do *L. flavolineata* display higher per-capita productivity in smaller groups?

Smaller groups were more productive per-capita, in agreement with Michener's paradox (see Figure 3.3). These data are represented per-capita (see data analysis). The number of adult females significantly affected the total number of brood ($z=8.423$, $df=85$, $p<0.001$). However, the model fit was significantly improved by the inclusion of a quadratic term (see

Figure 3.4), indicating that number of brood increase non-linearly with adult group size (X^2_{83}=23.239, p<0.001). The rate of increase in total brood number starts to decrease beyond group sizes of three or more. There was no interaction between group size and whether data was from the present study or from Bridge (Unpubl.) (z=1.319, df=82, p=0.187). Dataset was non significant after the interaction term was removed (z=1.341, df=83, p=0.180), and therefore both sets of data were pooled.

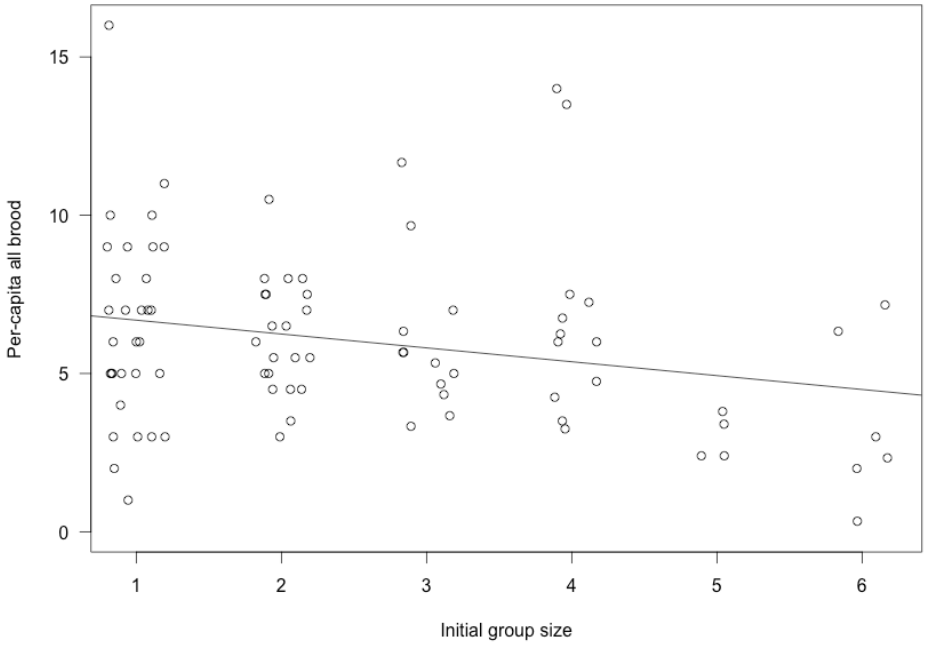

FIGURE 3.3. Graph showing that the number of brood per-capita decreases with adult female number, demonstrating Michener's paradox. Data was collected before the start of the experiment in the case of the 2011 data.

3.4.2 Brood composition

The composition of brood also changes with group size (see Figure 3.5). The proportion of larger brood (L2 and above) increases with group size, even as total brood number per-capita decreases. Crucially the proportion of pupae increases with group size, and as this represents realized brood, this is the 'true' measure of actual productivity. Notably, only the largest groups consistently achieve pupa. Most small group sizes, (one to three adult females) do not. However, small nests can occasionally raise brood to pupae, and this translates into a high per-capita payoff.

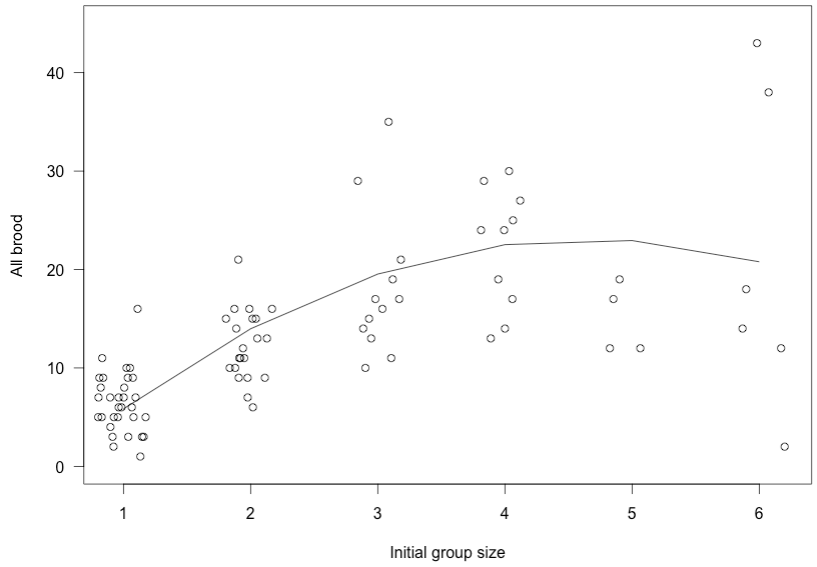

FIGURE 3.4. Graph showing that the number of brood increases non-linearly with adult female number. Data collected before the start of the experiment.

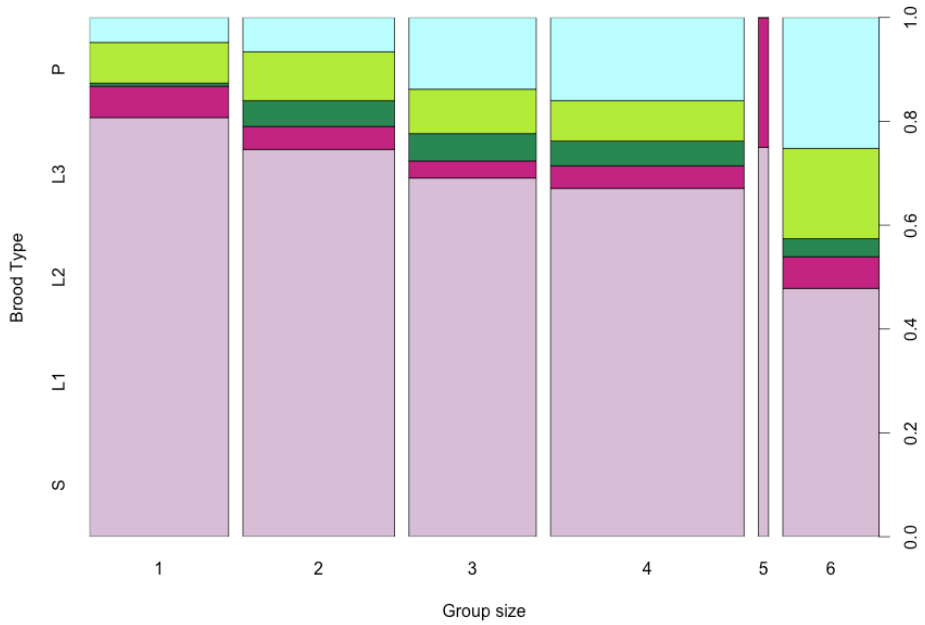

FIGURE 3.5. Spine plot representing the proportion of each developmental stage of brood in nests of varying size. The width of the bars represent the number of samples for each group size. Here only the 2011 data is presented.

3.4.3 Empirical manipulation of variance in food returns

The frequency of nests was not equally distributed among group sizes, and was highly skewed towards smaller groups Nests =58, Controls=27, Treatments= 31 (see Table 3.1 a). This was primarily due to the nests available at that site and because smaller nests are predicted to be affected more by CLT mediated effects. Due to ant attacks there was a variable number of censuses, but the majority were for the maximum 13 cycles (see Table 3.1 b).

(a)

Initial group size (females)	Frequency
1	23
2	16
3	7
4	7
5	1
6	4

(b)

Duration of experiment (no. of censuses)	Frequency
5-6	3
8	1
9	3
10	3
11	6
13 (max)	41

TABLE 3.1. (a) Table of initial group sizes. (b) Duration of the experiment. Number of censuses was controlled for in the analysis.

3.4.4 Effect of the manipulation on number of detected abortions

I manipulated the variance in foraging availability to test whether increasing the variance in food returns on treatment nests affected the number of abortions. Per-capita total abortions are represented graphically (see Figure 3.6) as this is more heuristic.

The treatment did not increase the number of abortions (and by definition abortions per-capita) relative to the controls, across all group sizes ($z= -0.725$, $df=57$, $p=0.060$). There was no interaction between group size and total abortions, indicating that treatment had no effect ($z= -0.493$, $df= 57$, $p= 0.622$) (N=58, C=27, T= 31). The number of censuses did not affect the number of abortions (or abortions per-female) ($z=1.732$ $df=57$, $p=0.083$). The overall number of total abortions increased significantly with group size, due to a greater number of brood on larger nests ($z=4.639$, $df=57$, $p<0.001$). However, the total number of abortions

did not increase linearly with group size. This is represented by (Figure 3.6), where total abortions per-capita decreases significantly with group size. I.e. there are more per-capita total abortions on smaller nests than if abortions occurred at the same rate across all group sizes.

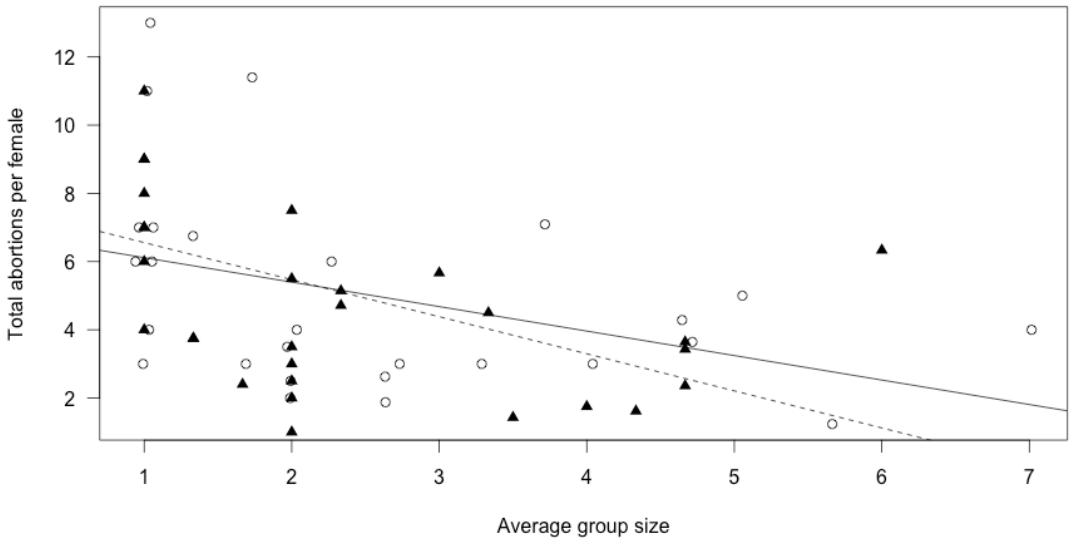

FIGURE 3.6. Graph representing the number of abortions (of all larval stages) per-capita in groups of varying size. Unfilled circles and solid line are controls, filled triangles and dashed line are treatments.

For experimental reasons, the most reliably detected abortions are of larval stage L1 and above. Lower per-capita rates of abortion in larger groups were also found when only these 'mature' (i.e. L1 stage larvae and above) abortions were analyzed (see Appendix Figure A.2). Treatment did not increase or affect the number of mature abortions (and by definition mature abortions per-capita), relative to the controls, across all group sizes (z= 0.644, df=57, p=0.520), with no interaction between group size and experimental group (z= -9.40, df= 57, p= 0.347). Number of census did not affect the number of transitions (or transitions per-female) (z= 1.223, df=57, p=0.222). The number of mature abortions increased significantly with group size (whilst mature abortions per-capita decreases significantly with group size) (z=3.206, df=57, p=0.001). Again, this relationship is non-linear, and so there are more per-capita mature abortions on smaller nests than if mature abortions occurred at the same rate per-cell across all group sizes.

Since female number fluctuated, a per-cell rate of the total and mature abortions were analyzed, and this also displayed the same relationship. There was no interaction between nest size (number of active cells) and experimental group (z= -0.323, df= 57, p= 0.747). Treatment did not reduce the number of total abortions (and by definition total abortions per-capita), relative to the controls, across all group sizes (z= -0.551, df=57, p=0.582). In this case, the number of census did significantly affect the number of total abortions (or total abortions per female (z= 2.258, df= 57, p=0.024). The number of mature abortions did increase significantly with group size (whilst total abortions per-cell decreases significantly with group size (z= 4.119, df= 57, p<0.001). And again, this did not change when 'mature' abortions only are compared per-cell. There was no interaction between nest size (cells) and experimental group (z= -0.473, df= 57, p= 0.636). Treatment did not reduce the number of mature abortions (and by definition mature abortions per-capita), relative to the controls, across all group sizes (z= 0.733, df= 57, p=0.464). Number of census did not affect the number of mature abortions (or mature abortions per-female) (z= 1.701, df= 57, p=0.089). The number of mature abortions did increase significantly with nest size (and therefore mature abortions per-cell decreases significantly with group size (z=2.760, df=57, p=0.006). Both these relationships are non-linear, and there are more total abortions and mature abortions per-cell on smaller nests than if they occurred at the same rate across all group sizes.

3.4.5 Effect of the manipulation on larval transitions

If brood are not being removed, larval development rates may instead be slower on nests with a higher variance in food input, due to larvae receiving food more variably. To determine if this is the case, I counted larval transitions as a proxy for larval growth rates; nests with faster growth rates should record more transitions. Transitions per-capita are represented graphically as this is more heuristic.

Treatment did not reduce the number of transitions (and by definition transitions per-capita), relative to the controls, across all group sizes (z= 0.956, df=57, p=0.956). There was no interaction between group size and experimental group (z= -1.593, df= 57, p= 0.111). Number of census did not affect the number of transitions (or transitions per-female) (z= 1.587, df=57,

p=0.112). The number of transitions increased significantly with group size. (z=9.005, df=57, p=<0.001). Transitions per-capita also increased significantly with group size (see Figure 3.7).

When I repeated the analysis with transition rate per-cell, the result was the same; there was no interaction between group size and experimental group (z= -0.323, df= 57, p= 0.747). Treatment did not reduce the number of transitions (and by definition transitions per-cell), relative to the controls, across all group sizes (z=0.421, df=57, p= 0.673930). In this case number of census days was significant (z=3.614, df=57, p=<0.001). The number of transitions increased significantly with nest size (cells) (z=8.558, df=57, p=<0.001). Transitions per-cell also increase significantly with group size (see appendix Figure A.3).

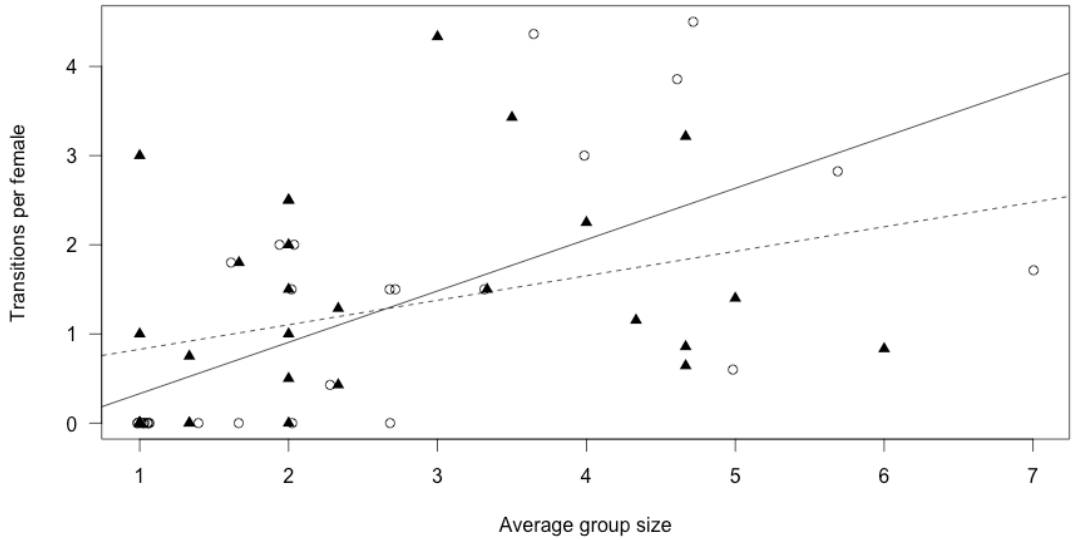

FIGURE 3.7. Graph representing the number of transitions per-capita in groups of varying size. Unfilled circles and solid line are control nests, filled triangles and dashed line are treatment nests

3.5 Discussion

According to the CLT, larger groups will be more efficient due to lower variance in foraging returns, allowing better tracking of brood numbers to available resources. Smaller groups in contrast are hypothesized to either over or under produce brood, and this is likely to be less efficient over time. If nests that experience a higher variation in food returns over-produce

brood, these excess brood are hypothesized to be adjusted downwards in times of dearth or unpredictability. To specifically test the effect of variation in food returns, nests were manipulated to have a greater variance in foraging windows on treatment nests compared to controls. Brood abortion and transition rates were then measured, with treatment nests of all sizes hypothesized to have higher rates of abortion and lower rates of transition than control nests of the same size, in response to a more unpredictable food supply.

There was no evidence of the treatment increasing abortions, or decreasing larval transition rate across all group sizes. There was evidence that smaller nests initially had more brood per-capita, and that abortions were more common, and transitions less common on smaller nests throughout the experiment. These findings support predictions of the CLT, but cannot definitively be ascribed to higher variance in food supply on smaller nests, since manipulating the variance did not cause an effect.

3.5.1 Do *L. flavolineata* display Michener's paradox?

Analysis of data from 2001 (Bridge unpubl.) and 2011 demonstrates that the total number of brood increase non-linearly with group size, with the linear relationship decelerating beyond group sizes of three or more (Figure 3.4). Represented in Figure 3.2, as per-capita data, *L. flavolineata* demonstrate 'Michener's Paradox' when all brood are counted, as in Michener's (1964) original census, and this is influenced strongly by the numbers of small brood (stage S) (Figure 3.5). Differences in productivity across group sizes appear to be species specific (Tindo et al. 2008), dependent on nest stage (Stevens et al. 2007), or season (Cronin et al. 2011). Previous studies (Shreeves & Field 2002; Shreeves et al. 2003; Field & Cant 2006) have found a positive linear relationship in *L. flavolineata* presented here were collected at seasonal peak brood times.

3.5.2 Do smaller groups of *L. flavolineata* have greater overall success?

Nests with fewer adult females predominantly contain small brood (see Figure 3.5). If these small nests fail to get brood to adulthood, for example if all the brood are cannibalized or otherwise lost, such nests could be considered to have a fitness of zero. Unobserved nest failure could be one factor contributing to studies finding the paradoxical result that smaller nests have a fitness advantage (Rehan 2011). A better measure of fitness or success, therefore, is

the number of pupae per-capita produced by nests of varying sizes (pupae are capped and relatively safe from predation).

The number per-capita pupae observed in a nest increases with adult group size (see appendix Figure A.4). Although it is probable that over time some of the brood in smaller nests will successfully grow to pupae and eclose as adults, larger groups are shown have more pupae per-capita than smaller nests at the start of experiment (before any manipulation had occurred). I suggest that although important in the context of the application of the CLT in social insects, per-capita productivity at various group sizes may be misleading, and instead, only pupae or realized productivity should be taken into account when judging the true success of nests. That Michener (1964) grouped all stages of brood together in his analysis is one of the major criticisms of his findings. These results show that larger nests will produce adults in a relatively short future time frame, whilst smaller nests will not. Next, I examine whether it is high variance in food inputs that causes smaller nests to be less successful.

3.5.3 Empirical manipulation of variance in food returns

To test for potential effects of the CLT, the variance in potential food returns were experimentally manipulated, and the number of abortions and larval transitions counted.

3.5.4 Effect of the manipulation on number of all detected abortions

There was no effect of the manipulation on the total number of abortions per-capita (when using average female number as group size), or per-cell. Although there was no effect of treatment, there were a higher number of abortions per-capita in smaller groups, consistent with CLT derived predictions of egg over-production and subsequent cannibalism. There was also a trend for smaller nests to have greater number of mature abortions per-capita and per-cell. Even though we might initially hypothesize that the higher abortion rate of brood on smaller nests is a function of smaller nests consisting mainly of small brood (which are more likely to be cannibalised as they represent a smaller investment) L1 and above larvae on smaller nests are also subject to higher rates of removal.

3.5.5 The effect of the manipulation on larval transitions/ brood development rates

If increasing the variance did not cause wasps to increase brood abortion, then the rate of larval development might have slowed if adult wasps were not able to meet the brood's nutritional needs. However, there was no effect of the treatment on the number of larval transitions achieved by different sized groups. Larval transitions are not only a reflection of larval development time but also a measure of the success rate of rearing brood from small to large larvae. As group size increases so do the number of transitions per-capita and per-cell, and this is statistically significant. Fewer transitions from small to large brood per-capita and per-cell recorded over the duration of the experiment on smaller nests are a measure of how relatively poorly they are performing, despite their initial higher per-capita brood numbers.

3.5.6 Does the CLT affect *L. flavolineata*?

I recorded higher numbers of brood per-capita in small groups of *L. flavolineata* at the start of the experiment. However, larger groups are more successful in terms of more developed brood including pupae, which I use a more accurate measure of success. This result appears to be good evidence that small groups may indeed over-produce brood compared to large groups. These smaller groups may potentially suffer a negative effect through excess brood production and later reduction of brood numbers in response to environmental conditions: failing to get as many brood per-capita to adulthood as larger nests in accordance with CLT-derived predictions. *L. flavolineata* may therefore be a good species to manipulate the variance in foraging returns in order to specifically test the CLT.

However, results from the manipulation did not find that a higher variance in food input/foraging windows affected rate of abortive events or the number of larval transitions on nests of all sizes. In doing so this work supports some of the results of Shakarad and Gadagkar (1995) where larger nests did not produce significantly heavier progeny and did not produce them significantly faster than smaller colonies. These results indicate that a higher variance in food returns itself might not be responsible for driving the higher numbers of per-capita abortions on smaller nests. If the experiment did effectively manipulate vari-

ance (but see below), then in turn it is unlikely that more variable input of food is the factor that causes small groups to over-produce brood, as observed at the outset of this experiment. Since the experimental procedure created an equal reduction in the time available to access food across all nests over each three-day cycle, we would expect treatment nests to respond to both the reduction in food, and increased variance in food inputs (compared to the controls) by decreasing their brood number. Even if wasps on treatment nests later laid more eggs to replace aborted brood or optimistically produced them in response to higher variance, those new brood should still be aborted in the next experimental cycle resulting in a higher abortion rate over the experiment. Since an effect of the experiment was not observed, the finding that small groups have more per-capita brood (and particularly small brood), may be due to small groups demonstrating behaviour typical to their stage in nest development (Stevens et al. 2007); for example, not directly a consequence of variation in food returns, but a bet-hedging strategy to protect against brood loss from predation.

A possible explanation for not observing an effect of the experiment may be that although the treatment increased the variance of foraging windows, the actual variance in food returns was not sufficiently affected. The CLT as a mathematical concept only considers the number of samples taken from the environment. Although this is likely to increase with forager number, samples from the environment could also increase with effort. Therefore, a possible reason for these results may be that although the treatment increased the variance of foraging windows, it did not affect the variance in food returns. This could happen if, for example, adult group number fluctuations during the experiment or daily variance in food availability introduced too much stochasity. Or, perhaps more likely, if wasps were compensating for the experimental procedure, by either foraging more during the remaining part of the day or over subsequent days.

If workers are able to adjust to the experimental procedure, this would buffer the effect of the adjusted variance and would explain why effects of the CLT (increased abortion on smaller nests for example) were not observed. Previous studies by Field et al. (2006) have found that wasps in smaller groups work harder than their counterparts (at the same inheritance rank), in larger groups. It is possible that wasps in small groups are already compensating for having fewer foragers by working harder. Increased or modified effort

could be in response to natural conditions e.g. weather or prey availability. Workers may also potentially adjust foraging in response to a previous deficit, the number of brood in the nest, the worker's future expected fitness payoff, or a combination of these factors (see Field et al. 2006 and Chapter 6 for an expansion on this topic). If wasps on treatment nests did forage more, or at a more hazardous (hotter) time of the day, this may have been within the bounds of their abilities, as it did not significantly affect mortality rate across the entire experiment.

The analysis did, however, show that all the response variables are correlated with group size. As groups increased in size, total abortions (and mature abortions), decreased per-capita and per-cell, and there was an increase in the number of larval transitions achieved by larger groups. This suggests that smaller nests are experiencing negative impacts that larger nests may be protected from, and this is associated with adult number. Smaller nests did abort more brood per-capita, although not in response to the treatment. If this were in response to an overall reduction in food, this would be evidence that smaller nests are more vulnerable to food restriction. This may be because they do have a higher variation in foraging returns due to fewer foragers (and especially high-ranking foragers who forage least). Despite evidence of a significant pattern consistent with predictions of the CLT, I cannot demonstrate that this is due to benefits derived specifically from the CLT as applied to food returns, as opposed to other effects of nest size. However, these results are comparable to previous studies of the CLT in social insects (Karsai & Wenzel 1998; Stevens et al. 2007; Naug & Wenzel 2006).

There remains one further reason why small larvae abortion may not respond to increased variance in food income in this species. Nests of all sizes may be optimistically producing a surplus of small brood to act as a fresh food store, to the extent they are constrained by the number of nest cells. In times of food reduction, which is a by-product of the experimental procedure and equal on all nests, these trophic brood could have been utilized equally by all groups as a fresh food source.

3.6 Conclusion

Whilst the mathematics of the CLT are sound, in practice organisms have adapted to cope with daily fluctuations in food availability, and may be able to accommodate both the

experimental procedure and natural variation to meet the energetic needs of their nest. However, higher levels of per-capita abortion on smaller nests suggest that generally, or in times of food reduction, smaller nests abort more brood per-capita than larger nests. I interpret this as an indicator of an increased ability of larger nests to maintain equilibrium without resorting to such high levels of brood reduction, although this cannot be conclusively ascribed to effects of the CLT. If higher variance in food returns is one of the reasons for higher levels of brood abortion on smaller nests, then I suggest manipulating variance in order to produce an observable effect on abortion rates or larval transitions is problematic in the field, due to stochastic factors and the potential ability of wasps to adjust to any experimental regimen. The importance of CLT needs to be further explored in other social groups to determine its wider potential facilitating role in social evolution.

CHAPTER 4

REPRODUCTIVE SKEW AND GENETIC RELATEDNESS AT NEST INITIATION

4.1 Abstract:

Background: Primitively eusocial societies with reproductive division of labour invariably experience conflicts in how reproductive rights are partitioned or allocated. Previous empirical tests of reproductive skew models within eusocial insects have been hindered by lack of natural variation in one of the key parameters: relatedness between adult females within a nest, which is often uniformly high. In order to test the role of relatedness in the context of reproductive skew theories, immature brood and adults of 26 newly initiated nests of the facultatively eusocial wasp *Liostenogaster flavolineata* are genotyped, in which relatedness is hypothesized to be low. In addition to potential indicators of low relatedness, new nests may also experience variation in other parameters (such as the value of helpers to the dominant) compared to established nests, where previous studies have found high skew.

Results: Relatedness estimates between adults within newly initiated nests were uniformly low, with the exception of two nests. These values were significantly different to relatedness estimates of established nests. Reproductive skew of brood on newly initiated nests was found to be high, and unaffected by variation in relatedness or group size. The proportion of small female brood laid by a female other than the primary egg-layer is statistically the same as in established nests. Irrespective of whether a nest is established or newly initiated,

there is a non-significant trend for less related and larger groups to have lower skew.

Conclusion: Low values of relatedness demonstrate that relatives do not preferentially nest together when initiating a nest. Despite lack of indirect fitness benefits accrued by subordinates on these nests, and despite their hypothesized high value to the dominant, a primary egg-layer dominates reproduction to the same extent as in established nests. Since neither average intra-nest relatedness of adults nor adult group size affects skew significantly, neither concession models nor incomplete control models are supported. These results suggest that peace-incentive and related models (for example Dynamic-Skew) more accurately describes the observed skew values in *L. flavolineata* nests of all stages, or some other factor may encourage high skew even in the absence of inclusive fitness benefits.

4.2 Introduction

The defining feature of eusocial societies is a 'reproductive division of labour', in which some individuals (known as helpers, workers or subordinates) at least temporarily forfeit their own reproduction and instead rear the offspring of other individuals, known as breeders, queens or dominants (Michener 1974; Wilson 1971). Primitively eusocial societies in which there is no morphological difference between dominants and subordinates, as in the study species *L. flavolineata*, represent a situation where there is choice in co-operation or conflict. Levels of assistance to the dominant may be structured by an inheritance system or queuing convention based on factors such as age or size, with differential effort between ranks (Field & Cant 2009).

Reproductive skew is a quantitative measure of how reproduction is partitioned among members of a society. Depending on the skew index used (Nonacs 2000), high values typically indicate that reproduction is monopolized by one or a few individuals (Faulkes & Bennett 2001), whereas low values reflect a more equitable partitioning of reproduction among group members (Cavigelli et al. 2003). Beginning with the seminal work of Vehrencamp (1983) a large number of theoretical models have been developed to understand variation in reproductive skew (reviewed in Clutton-Brock 1998; Johnstone 2000; Field & Cant 2009).

There are two main frameworks for understanding reproductive skew: Incentive and Compromise. Within the context of 'incentive' models, the Concessions model and the closely related Peace-Incentives model have typically been applied to primitively eusocial wasps (Reeve & Ratnieks 1993; Field & Cant 2009). In the Incentives framework, dominants are seen as making a reproductive 'concession' to subordinates when necessary to retain subordinates in the group. In the Peace-Incentives and related models, subordinates receive an incentive so that they do not gain more by fighting for reproductive dominance than by peacefully accepting the queuing convention (Reeve & Ratnieks 1993). This could involve future inheritance of the nest and reproduction if the subordinate observes the status quo, rather than a reproductive share concurrent with the dominant (Kokko & Johnstone 1999).

The concessions framework makes several well-known predictions about how social and ecological factors should affect the size of reproductive concessions, hence affecting how equitably reproduction is partitioned in the group (Vehrencamp 1983; Reeve & Ratnieks 1993). Concession models predict that the subordinate's share will be smaller (i.e. reproductive skew will be higher) when relatedness is high, because the subordinate then receives compensation in the form of greater indirect benefits from helping to rear the dominant's offspring. Concessions are also predicted to be small when group productivity and constraints on independent breeding are high, as in both cases subordinates obtain greater benefits by remaining in the group rather than departing. Finally, concessions should be smaller when subordinate resource holding potential (the ability to control the nest) is low, since then the threat to or chances of a successful challenge to the dominant is low (Reeve & Ratnieks 1993). Conversely, when the presence of subordinate females bringing food and building supplies is highly beneficial, either because of long brood-rearing time (which will be longest at an earlier stage of brood development), high energetic costs of foraging, or the need for nest defense, skew may be lower as a reflection of the subordinate's value to the dominant (Nonacs 2006). Additionally, market forces may operate; were helpers are in short supply, or there is a relatively small number associated with a nest, each individual subordinate's value may be higher to the dominant and as such helpers may be able to bargain for a greater reproductive share. Conversely, subordinates may themselves accept higher skew in the presence of high ecological constraints or when help is plentiful (Noë & Hammerstein 1995).

A key feature of these and related models is their assumption that the dominant has complete control of the partitioning of reproduction, and that such control is cost-free. Because primitively eusocial wasps have physically small nests (maximum 100 cells in *L. flavolineata*), it seems feasible that the dominant could indeed control reproduction at low cost: a dominant female rarely leaves her nest, and the brood-comb surface is often only a few times larger than she is, especially in the case of new nests (Field & Cant 2009). It therefore seems possible that the dominant could detect and prevent any attempt at egg-laying by subordinates: in *L. flavolineata*, it takes several minutes for an egg to be laid (Turillazzi 1996). Alternatively skew models under the Compromise framework assume that neither the dominant nor the subordinates have complete control. Group members engage in a tug-of-war over reproduction, with a cost to nest productivity and efficiency equivalent to the degree of conflict (Nonacs 2007; Buston & Zink 2009; Shen & Reeve 2010). Incomplete-control models predict that reproductive skew will decrease with increasing group size, as the dominant spends more time and effort maintaining reproductive monopoly. Therefore, there may be an ideal number or workers from the dominant's perspective balancing assistance with conflict (Strohm & Bordon-Hauser 2003).

Two recent reviews both found that there was little empirical evidence to support predictions of the concessions framework in primitively eusocial wasps (Nonacs et al. 2006; Field & Cant 2009; but see Lucas et al. 2011). Of the eight empirical studies reviewed, five involving paper wasps (*Polistes*) and three involving hover wasps, only one study provides clear support: Reeve et al. (2000) found that higher adult relatedness and higher group productivity were both associated with higher reproductive skew among nests of *Polistes fuscatus*. None of the other seven studies, however, found any relationship between skew and relatedness, productivity, or body size differences between dominants and subordinates (the latter assumed to reflect relative resource holding potential of the two parties). Nonacs et al. (2006) concluded from their analysis that transactional and related models simply do not predict patterns of skew in these wasps. Field & Cant (2009) on the other hand, were more circumspect, drawing attention to several aspects of the data available, and several aspects of the basic ecology of primitively eusocial wasps that could potentially explain the apparent lack of fit between models and data. For example, Field and Cant (2009) pointed out that if subordinates have a good chance of eventually inheriting the dominant position, which

seems likely with the small group sizes found in primitively eusocial wasps, subordinates may be prepared to accept a high skew while they wait to inherit (Kokko & Johnstone 1999). Indeed, Leadbeater et al. (2011) found that if inheritance was taken into account, direct fitness alone was enough to explain subordinate behaviour in *Polistes dominulus*, one of the key species in Nonacs et al.'s (2006) analysis.

Another problem with testing the predictions of skew models in primitively eusocial wasps is that there is typically little variation in skew, especially in studies of hover wasps (Field & Cant 2009). In *L. flavolineata*, *Parischnogaster mellyi* and *Parischnogaster alternate*, only dominants had reproduced on 80-85% of nests, with the remaining 15-20% that had any subordinate reproduction represented by only 2-4 nests in each study: skew was rather uniformly high (Sumner et al. 2002). This lack of variation provides little opportunity to test for correlations between skew and the variables predicted to affect it, although there was considerable variation in the explanatory variables themselves (i.e. adult relatedness and group size). The latter observation might, however, suggest that explanatory variables do not affect skew in the predicted way, or that some other unmeasured variable(s) consistently contribute an effect that leads to a uniformly high skew at any one time (Field & Cant 2009). Plausible examples of such variables could be a uniformly good chance of inheritance in small hover wasp groups, at least up to a certain inheritance rank (see above and Field et al. 1999; Shreeves & Field 2002), or uniformly strong ecological constraints against independent nesting (Field & Foster 1998).

Furthermore, genetic relatedness among adult female nest-mates, one of the key variables predicted to affect skew, has also been consistently high in several previous studies (Seppä et al. 2002; Field et al. 1998). In the only published previous study of skew in *L. flavolineata*, using three microsatellite loci, estimated average adult nest-mate relatedness was (r= 0.56 ± 0.05) (Sumner et al. 2002). This previous study was hindered by a small sample size of only 13 nests, where skew (using skew S index) was found to be consistently high (0.95± 0.033, n=13 nests, with 1 representing complete skew), a problem in many of the studies. The lower range (r= 0.296± 0.046) of adult relatedness were represented by only six nests, making any correlation between relatedness and subordinate reproduction hard to detect. Without a visible cue, it is difficult to identify and collect sufficient nests

with low relatedness to reliably test variation in skew, and the established nests previously collected with low relatedness by Sumner et al. (2002) do not represent completely unrelated associations.

In the present study, I aim to measure adult relatedness and reproductive skew in a situation where adult relatedness might be particularly low; newly founded nests (Zanette & Field 2008). Most nests are founded by a single female (Samuel 1987), and these lone foundresses soon receive joiners (Samuel 1987; Field & Foster 1998; L. Holt Pers. obs). It is not obvious how joiners could locate or identify close relatives, unless a nest was being re-established on the site of an earlier, destroyed nest, so new nests may typically comprise random individuals drawn from the pool of nest-less floaters associated with large *L. flavolineata* nest aggregations. Beyond its relevance to testing the skew models, adult female relatedness on newly founded nests is itself potentially interesting: perhaps joiners do have a way of locating relatives among the nests in a cluster, as has been found in some other social taxa which exhibit 'budding' off of groups of relatives to start new groups, e.g. *Apis dorsata* (Paar & Oldroyd 2004). *L. flavolineata* displays an age-based gerontocracy on mature nests, however, it is unknown how members of new nests arrange the dominance hierarchy, although date of arrival is hypothesized to be the equivalent of age as the deciding factor in inheritance, as in co-foundress aggregations of *P. dominulus* (Jeanne 1980; Seppä et al. 2002).

If adult relatedness is indeed lower in these newly initiated nests, the concessions model predicts that skew should also be lower, since subordinates are not profiting from indirect fitness benefits. This prediction can potentially be tested by comparing skew in newly founded nests with the high skew found by Sumner et al. (2002) in established nests, where average relatedness may be higher. The fact that a nest is newly founded (i.e. structurally different to established nests), and contains only young brood, potentially provides a cue associated with low relatedness that adult females could use. Conversely, females born into nests with even naturally low relatedness values may not be 'aware' they could bargain for a reproductive share, which might explain the high skew on established nests (Keller 1997).

4.2.1 Study species

Liostenogaster flavolineata (Cameron) (Hymenoptera: Stenogastrinae), the Hairy-Faced Hover Wasp is a facultatively eusocial wasp that lives in small groups (1-10 females) across a wide distribution in South-East Asia, including Peninsular Malaysia. *L. flavolineata* is the best-studied hover wasp; it's biology is summarized by Field (2008). Groups are perennial and usually consist of between two and five (up to 12) adult females with co-operative brood care (Turillazzi 1991). One female is behaviourally and reproductively dominant (Samuel 1987; Field et al. 1999) although all females are capable of mating, laying eggs and independent nest founding. Helpers have a chance of eventually inheriting the dominant, egg-laying position on their nests: an age-based dominance hierarchy determines the sequence of successive behavioural dominants (Samuel 1987; Field et al. 1999). Seniority can be measured in terms of time on the nest, with the most subordinate wasps undertaking the majority of the foraging (Field & Foster 1999). Since there are no morphological differences between egg-layers and helpers, females may choose to become helpers on their natal nests, or they may leave and found a nest alone, join another nest, or take over an existing one. On established nests, adult females are normally relatives (mainly sisters or cousins), and reproductive skew is uniformly high (Sumner et al. 2002).

L. flavolineata construct robust and long-lasting nests made from mud, which often occur in large aggregations (>100 nests), typically over bodies of running water, especially where artificial bridges and culverts provide an attractive location for nest building (Field 2008, and references therein). New nests of *L. flavolineata* are initiated by solitary females (Samuel 1987; Turillazzi 1991), and consist of shallow cups of mud attached to the chosen substrate. Previous work in the same area as my study suggested that despite the lack of a winter or diapause there remains seasonal variation in parameters such as the frequency of nest-founding, mean group size, and the mean number of immature offspring being reared (Samuel 1987; Shreeves & Field 2002; J. Field and G. Shreeves, unpublished data). Colonies typically exhibit two distinct periods of increased brood output, the first in late May and the second in August. These peaks occur during the warmest part of the year and between two wetter periods (Samuel 1987; Shreeves & Field 2002; Cronin et al. 2011). Although single female nests can persist, it is usual that after building the first few cells the nest initiator

attracts other female joiners, at least at sites of high density (Samuel 1987; J. Field pers. comm, L. Holt, pers. obs).

Several features of *L. flavolineata* may make subordinates, especially at nest initiation, particularly valuable to the dominant. *L. flavolineata* obtains protein primarily by preying on the variably distributed alates (winged reproductives) of ants. Long development time of *L. flavolineata* larvae (approximately 100 days), and risky foraging makes death of the egg-layer before eclosion of a first (female) offspring highly likely: only 10-30% of lone foundresses can expect to survive that long (Field et al. 2000). Cooperation between females and subsequent Assured Fitness Returns (hereafter AFRs; where subordinates continue to raise the dominant's immature offspring upon her death), may be almost essential for successful reproduction in this species, especially at the establishment of new nests (Field et al. 2000; Shreeves et al. 2003). The assistance of subordinate helpers at this stage may be so valuable that the dominant may be more likely to cede reproductive shares than on mature nests (Turillazzi 1996).

Here I mark, collect and genotype the brood and adults of newly established nests, in order to determine whether helpers are related or unrelated to each other, and if subordinates on these unique nests are able obtain a significant share of reproductive rights.

4.3 Methods

This chapter contains data on adults and brood collected from newly founded nests in 2011, (study dates from 5th May 2011 to 1st August 2011), and 2013 (study dates from, 19th August 2013 to 2nd October 2013) (total nests n=26). Relatedness data for adults and brood on established nests are taken from Sumner et al. (2002), where adults and brood were collected from sites in the general location in 1997 (total nests n=13). In both datasets the sites surveyed comprised the undersides of bridges and culverts along a little-used road near to Fraser's Hill, 116 km North of Kuala Lumpar, peninsular Malaysia. This road, which runs between The Gap and Raub, is the same location where previous studies have been based (Field et al. 2006). The majority of nests were collected in 2011 from what shall henceforth be referred to as Site 1 ("52-nest"), with a first collection of nests with marked wasps on

24th June 2011, and a second, unmarked set of wasps and brood which had colonised these abandoned nests collected on 29th July 2011. Four unmarked nests were also collected from Site 1 in 2013. In 2011 Site 3 ("Scramble-site"), also contributed four unmarked nests to the sample set. A rock fall prevented access to Site 3 in 2013.

4.3.1 Experimental procedure

On first arrival at the study sites in each year, all nests with cells deep enough to contain pupae were numbered using pieces of yellow insulation tape placed close to each nest. Frequent re-censusing identified nests that had not previously been indexed, and which lacked cells deep enough to house a pupae (this ensures no daughters could have been produced in the nest to join the group). These are the newly founded nests referred to in the following experiment (see General Methods for more details). Newly founded nests were easily identifiable, and usually comprised just a few shallow cells attached directly to the bridge or culvert surface, lacking the depth or flanges of older nests. The number of adults on each nest was counted at night (20:00 or later), when all individuals had returned to their nests. Daily observations using rapid censusing of the first set of nests at Site 1 were conducted after the wasps had been marked. Harvesting consisted of collecting the brood and adult wasps, but left the nest structure intact. Several weeks after the initial nest collection in 2011, it was observed that several of these small nests (4/7) had been re-colonized at Site 1, and contained brood. None of the wasps that re-colonized the nests were marked, but it is possible that the nests were taken over by one or more unmarked wasps associated with the previous group if they had avoided capture. However, due to the quantity of nest-less 'floating' females at this site, it is also highly probable that these secondary groups represent completely new associations of wasps. Newly founded nests from Site 3, and those from Site 1 collected in 2013 (n=5), were not re-colonized after harvesting during the (much shorter) fieldwork period.

4.3.2 Nest collections

On 24th June 2011, the adult wasps and brood present on these new nests were collected. Females were collected at night using zip-lock bags, as for marking. Once sealed in the bags, they were euthanized by placing in an -8°C freezer and stored in 100% ethanol. Immediately

after adult collection all eggs and larvae were extracted from cells using fine forceps and placed directly in 100% ethanol. Since some of the smallest nests (4-6 cells) did not provide a large enough location for more than one or two wasps to reside, subordinate females sometimes rested within a radius of 5-10 cm from the nest comb during the day, tending to cluster together more closely in the evenings and overnight. Females caught for the genotype analysis of this experiment were those in an approximately 5cm radius around the nest at night collection, whether or not they were marked. Particular care was taken to get the mouth of the bag around the wasps located in this area. Since newly founded nests were typically far away from the main aggregation, and from each other, it is very unlikely that females from another nest were accidentally caught in the collection. Typically, *L. flavolineata* do not tolerate out-group females or males on the nest overnight, and nest-less floating females tend to spend the night far from other nests (L. Holt pers. obs.). Although collecting adults was not as easy as on established nests, the collection had to be achieved in one attempt, since any unmarked females could not be identified afterwards. If a marked female was not caught during the collection, scans were conducted of the floating population and other nests to search for these individuals during the remainder of the field season.

Nests were chosen for collection if they had been observed with more than one female simultaneously resting on the nest comb. However, new nests had a more transient female adult population than more established nests. Group size fluctuated and not all marked wasps were resident at night censuses. Nests where only one female was caught were excluded from the analysis. When nest SCR4 was collected, only one female was caught, but upon analysis there were two brood sib-groups, and so skew in that instance was calculated assuming a group size of two. Maximum group size was used for the analysis opposed to the number of females finally caught, as this is more representative of the true number of females. Unmarked females were only counted as comprising more than one individual if two or more unmarked females were simultaneously observed on the nest.

4.3.3 Molecular genetic methods

The four microsatellite loci used by Sumner et al (2002) with *L. flavolineata* were designed at a time when genotyping was carried out manually, before autosequencers became generally available (Sumner & Field 2001). All four primers were tested, redesigned, and tested again

for use on an Applied Biosystems 3730 sequencer. All failed tests of heterozygosity and could not give sufficient power (see General Methods for details). In order to obtain more loci, a genomic library was constructed at Sheffield Molecular and Genomic Facility (SMGF) using the DNA from a single female taken from the field in 2013.

Adults were genotyped at 21 new polymorphic microsatellite loci, with 17 being used for the final analysis. DNA extraction, PCR and genotyping were performed as described in General Methods and Appendix (A.2.1. to A.2.4). For sample genotyping, DNA was extracted from the last two segments of one hind leg of each adult female (n=73). In the case of the brood (n=174), entire eggs or small larvae were used, or part of each large larva (n=1). Two people scored alleles independently. Genotypes were further checked if loci were inconsistent with putative sib-group membership (see below).

Adults from 46 established nests, varying from three to seven adult females, were collected for a separate experiment and analyzed with the same microsatellite markers (unfortunately it was not possible to collect brood in that experimental protocol, see Chapter 5). It is more relevant to compare adult relatedness between the two types of nests whose members were analyzed using the same markers, rather than the previous genetic analysis by Sumner et al. (2001).

4.3.4 Assignment of sex

Although the sex of adult *L. flavolineata* can be distinguished visually by facial patterning, eggs and larvae are impossible to sex visually. Eggs and larvae can be sexed using genetic data, as hymenopteran males are haploid and females are diploid. Brood that recorded mainly homozygous genotypes were re-checked and in cases where the individual was conclusively homozygous at every locus it was considered male, otherwise it was considered female. Excluding the possibility of typing error, the probability of a male being wrongly assigned as female is zero.

4.3.5 Identifying sib-groups

Previous data indicate that *L. flavolineata* mates only once (Sumner et al. 2002). Therefore a female sib-group within the brood should exhibit a maximum of three alleles at each locus,

and all individuals should share the same paternally derived allele. Likely maternal and paternal alleles could therefore be identified for the main female sib-group of brood in each nest. This was done manually, since although KINSHIP (the only available software for analyzing haplodiploid relationships) can identify sib-ships, it has no capacity for including an error rate. As brood genotyping is much less efficient than for adults due to small size of eggs, extracellular enzyme products, and inability to separate out gut and food products in larvae, there are likely to be some errors across the 17 loci. After manually sorting into putative sib-groups, all members were re-checked at loci that did not fit in with the consensus. Some nests did not have a dominant sib-group due to low number of brood and potential genotyping error, and so all loci were re-checked. When only two loci did not concur with the null hypothesis of sisters after checking across the (maximum) 17 microsatellite loci for an individual those two loci were removed (26 loci, 13 individuals). Five individuals that had very poor genotyping and potential contamination of master stock were removed from the analysis. Any female that potentially fitted into either of the (maximum two) sib-groups in the nest was ascribed to the largest.

4.3.6 Relatedness values

Relatedness v5.0.8 (Goodnight & Queller 1998) was used to estimate the intra-nest mean regression relatedness between adults and brood in groups of different size. Colonies were weighted equally and standard errors were obtained by jack-knifing over nests.

4.3.7 Measuring skew

Skew was calculated using Skew Calculator 2003 v1.2 (Nonacs 2003b). There are over 20 different ways of measuring skew, but a subset of eight indices are typically applied to studies of group-living (Nonacs 2000; Nonacs 2003a). Skew Calculator generates these eight, and for the B index calculates (by simulation) a probability level for each group that its observed B value could represent a random distribution of offspring among nest members. The program simulates a random distribution across all groups to determine the probability level of the observed average across-group B values, and the B index value for the nest if the reproduction was monopolised by one individual. Both of these probabilities are one-tailed. The B index values and the full monopoly values are presented in the results with accompanying p-values. Skew for both males and females together can be calculated, as they are

ascribed to sib-groups before entering into Skew Calculator.

Although the B Index is useful in that it produces significance values, it is difficult to compare B index results, as each nest has a unique (nest-specific) value. Therefore, proportion data (the proportion of brood not laid by the primary egg-layer) are used for the analysis. Deviation away from complete monopoly was calculated and modeled in the same way as proportion data, giving identical results. However, proportion is more heuristic and is presented here, and in order to compare results between new and established nests since the S Index was used by Sumner et al. (2002).

4.3.8 Calculation of male brood skew

As males are haploid, the power to place them definitively in a sib-group is significantly reduced. Since KINSHIP lacks the ability to assign them based on haplodiploidy, males were also ascribed to sib-groups manually. For the purposes of my analysis, a male was assumed to be a brother of the females in the largest female sib-group on his nest, if his single allele at each locus matched one of the maternally derived alleles found in the primary sib-group at that locus. When a male did not match the main sib-group at only one locus, this was accepted, and the male placed in the alternative out-group. When such a male was ascribed as out-group, it was impossible to be sure whether it was the brother of any other out-group female brood, as there was never more than one such female per-nest. Therefore, for the skew calculations, out-group male brood were always assigned to the secondary sib-group as opposed to a tertiary sib-group.

4.3.9 Data analysis

All statistical analyses not requiring the specialist programmes mentioned above were performed in R (R Core Development Team 2015). Data were checked for assumptions of normality and homogeneity of variance. Potential associations between skew and possible correlates were analyzed using general linearized modeling with binomial errors. In order to determine whether new and established nests had significantly different average intra-nest relatedness values I first performed a one-way ANOVA to test for significant differences between established nests from two datasets and newly founded nests. I subsequently con-

ducted a post-hoc Tukey's HSD test to obtain pairwise significance values.

For the subsequent analyses maximum female number observed is used as group size instead of the number of females caught at collection, as this is more biologically relevant when considering the effects of group size on potential subordinate reproduction or concessions.

I examined the effect of average adult relatedness within nests and group size on the proportion of female brood not laid by the primary egg layer, and the proportion of male and female brood not laid by the primary egg-layer, using a generalized linear model with binominal errors. In both cases adult relatedness and group size were included as explanatory variables, with site and year of collection included as covariates. In the maximal models an interaction term was initially included between average adult relatedness and group size.

Next I examined my data together with data from Sumner et al (2002), who had genotyped adult wasps and brood from established nests. Therefore, I was able to additionally test whether the type of nest (new or established) also affected the proportion of small female brood not laid by the primary egg-layer. Unlike the previous analysis I was only able to compare female brood of the same developmental stage (eggs and small larvae) across datasets. This is because established nests are more likely to have brood from a previous dominant that are well developed, whereas the new nests had no brood larger than L1 (small larvae) stage. This also gives a more accurate comparison. Explanatory variables and co-variants were included as above; additionally I included nest type (new or established), as well as an interaction term between group size and average adult relatedness. I also included an interaction term between nest type and group size, and nest type and average adult relatedness.

In all models, starting with the interaction terms, non-significant terms were removed following step-wise model reduction until only significant terms remained. Values reported in the results are Chi-squared and associated p-values for when terms were sequentially removed from the model.

4.4 Results

4.4.1 Relatedness profiles of new and established nests

Average intra-nest relatedness estimates for adults on new compared to established nests (data from 2013) are significantly different (Figure 4.1), with established nests having higher average relatedness (W= 176, p <0.01) (26 new nests n=73 adult wasps, 46 established nests n= 207 adult wasps). Pairwise intra-nest relatedness values for the two types of nest (Figure 4.2) show that established nests have a bimodal distribution, whereas adults in new nests are most commonly unrelated or almost unrelated, with only a few examples of highly related individuals. ANOVA demonstrated there were significant differences in adult relatedness between nest types (F=15.05, p<0.05) see (Table 4.1).

Year	Adults or Brood Collected	Data	No. of Nests	No. of Adults	No. of Brood	Adult Relatedness
1997	Adults and Brood	Skew and Adult Relatedness	13	80	134	0.56± 0.056
2013	Adults	Adult Relatedness	46	207	N/A	0.40± 0.067
2011, 2013	Adults and Brood	Skew and Adult Relatedness	26	73	153	0.14± 0.049

TABLE 4.1. Table of sources of data used in this analysis.

4.4.2 Developmental stage and sex of brood not belonging to the primary egg layer on new nests

With the exception of one large larvae (L3- large brood with differentiated segments) from nest SCR4, none of the brood were larger than the L1 developmental stage (small brood with differentiated segments), on new nests. This is in contrast to 22% (30/134) of brood from established nests (data from 1997), being of stage L2 (medium sage brood with differentiated segments) or higher. In established nests, any brood of L2 stage or above are likely to have been laid by a previous dominant, and so only the maternity of brood of equivalent developmental stage (up to L1 stage) are compared between new and established nests (see General Methods for further details on classification of brood developmental stages).

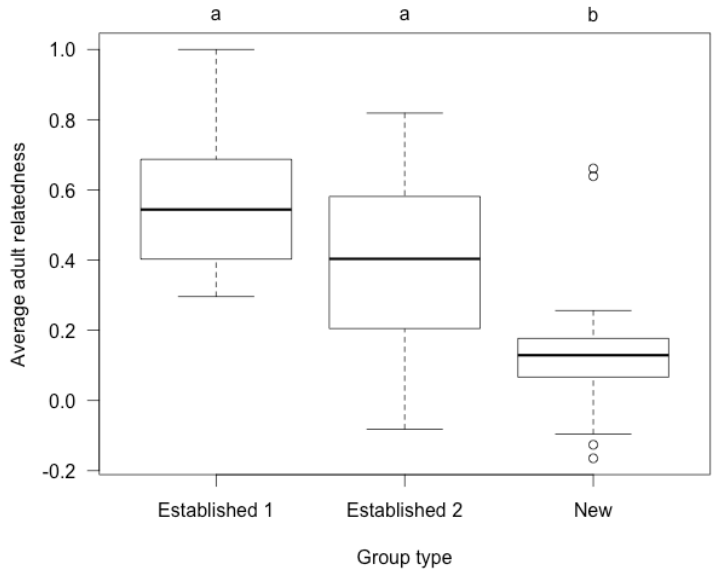

FIGURE 4.1. Box and whisker plot of the adult average relatedness values for established and new nests. Letters denote significant differences after Tukey's HSD test. Established 1 (Sumner et al. 2002) and Established 2 (Chapter 5) are statistically equivalent, but significantly different from newly founded (New) nests.

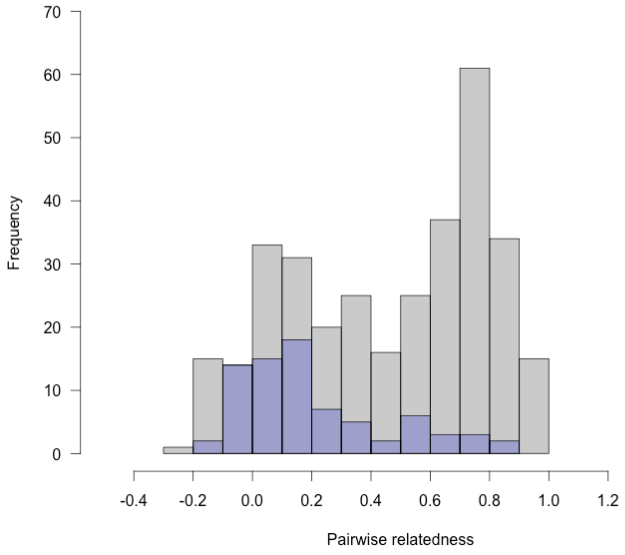

FIGURE 4.2. Distribution of raw within-group relatedness estimates for established (grey) and new (purple) nests.

4.4.3 Reproductive skew in newly established nests

Skew was high across all nests (Table 4.2). In 73% (19/26) nests, reproduction of female offspring was completely dominated by a single egg-layer (see Table 4.2). There were seven female brood, and an additional six male brood identified as not belonging to the primary egg-layer. When male and female brood are combined, this gives lower values of B index skew (see A.6 in Appendix). When male and female brood are combined in terms of skew it is still high with 58% (15/26) nests having reproduction completely dominated by one female.

4.4.4 Factors affecting the proportion of brood laid by the primary egg-layer on new nests

Considering the proportion of female brood not laid by the primary egg-layer (Figure 4.3) (n=7 brood), there was no interaction between maximum adult group size and adult relatedness on new nests (X^2= 0.0356, p= 0.8503) (n=26 nests). Site did not affect the proportion of female brood not laid by the primary egg-layer (X^2= 0.442, p= 0.506), nor did year (X^2= 0.013, p= 0.909). The proportion of female brood not laid by the primary egg-layer did not change with either adult relatedness (X^2 = 0.705, p= 0.401), or maximum number of adults (X^2= 0.831, p= 0.362).

Considering the proportion of male and female brood not laid by the primary egg-layer (Figure 4.4) (n=13 brood), there was no interaction between maximum adult group size and adult relatedness in these new nests (X^2= 0.523, p= 0.469) (n=19 nests). Site did not affect the proportion of male and female brood not laid by the primary egg-layer (X^2= 1.536, p= 0.2152), nor did year (X^2= 0.039, p= 0.843). The proportion of female brood not laid by the primary egg-layer did not change with either maximum number of adults (X^2= 0.109, p= 0.7417), or adult relatedness (X^2= 0.343, p= 0.558).

The proportion of brood not in the main sib-group is represented graphically as this is more heuristic (Figure 4.3 to 4.6). Using as a response variable the difference between the B index value for a nest and it's B Monopoly value (instead of proportion of brood not laid by the primary egg-layer), produced identical results, therefore only proportional data is presented here. Data points are nests.

Nest	Max Adults	A Relatedness	B Relatedness	Total Brood	No. Female	Sibgroups	% Sibgroup	B Index	B Monopoly	Difference	p
100	4	0.166	0.756	7	7	1	100	0.6429	0.6429	0	0
101	4	0.180	0.652	8	8	2	88	0.4375	0.6563	0.219	0.001
102	3	0.163	0.732	11	10	1	100	0.6	0.6	0	0
103	2	0.070	0.718	7	7	2	86	0.1837	0.4286	0.245	0.130
104	3	0.065	0.749	10	7	1	100	0.5714	0.5714	0	0.001
170	3	-0.095	0.320	5	4	2	75	0.125	0.4924	0.367	0.26
171	7	0.169	0.756	5	5	1	100	0.6857	0.6857	0	0.001
401	5	0.174	0.687	6	6	1	100	0.6667	0.6667	0	0
402	5	0.129	0.640	7	4	1	100	0.6	0.6	0	0.002
405	1	NA	0.606	3	3	1	100	0.3333	0.3333	0	0.263
410	3	0.090	0.770	5	4	1	100	0.5	0.5	0	0.350
411	1	NA	0.773	3	3	1	100	0.4	0.4	0	0.073
412	2	0.068	0.828	7	6	1	100	0.4167	0.4167	0	0.042
430	5	-0.126	0.650	3	3	2	67	0.0889	0.5262	0.437	0.254
171B	6	0.129	0.424	6	4	2	75	0.25	0.6176	0.368	0.073
405B	1	NA	0.736	2	2	1	100	0.25	0.25	0	0.476
411B	2	-0.165	0.869	3	3	1	100	0.3333	0.3333	0	0.255
430B	3	-0.076	0.722	5	5	1	100	0.5333	0.5333	0	0.010
NR62	2	NA	0.764	3	3	1	100	0.3333	0.3333	0	0.269
NR82	1	NA	0.737	6	4	1	100	0.5	0.5	0	0.032
NR82B	1	NA	0.580	3	3	1	100	0.3333	0.3333	0	0.226
NR85(B)	3	0.256	0.764	5	4	1	100	0.5	0.5	0	0.036
NR87B	5	0.661	0.663	22	13	2	92	0.5964	0.7385	0.142	0
SCR2	3	0.219	0.729	4	4	1	100	0.5	0.5	0	0.032
SCR3	3	0.639	0.640	3	3	1	100	0.4444	0.4444	0	0.106
SCR4	2	NA	0.084	3	3	2	67	-0.1111	0.3276	0.439	1

TABLE 4.2. Table showing data for skew calculations. Represented here are the skew values for females. Columns represent: The B index value (B Index), the value if only one female dominated skew (B Monopoly), the difference from full skew (Difference), and the significance value of that result (P). See Appendix for expanded table (A.3). The two nests with high relatedness values are highlighted.

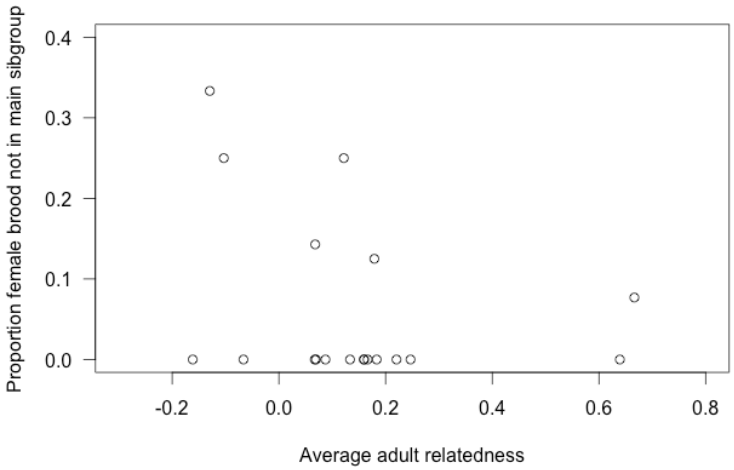

FIGURE 4.3. Graph representing the effect of average intra-nest adult relatedness on proportion of female brood not laid by the primary reproductive

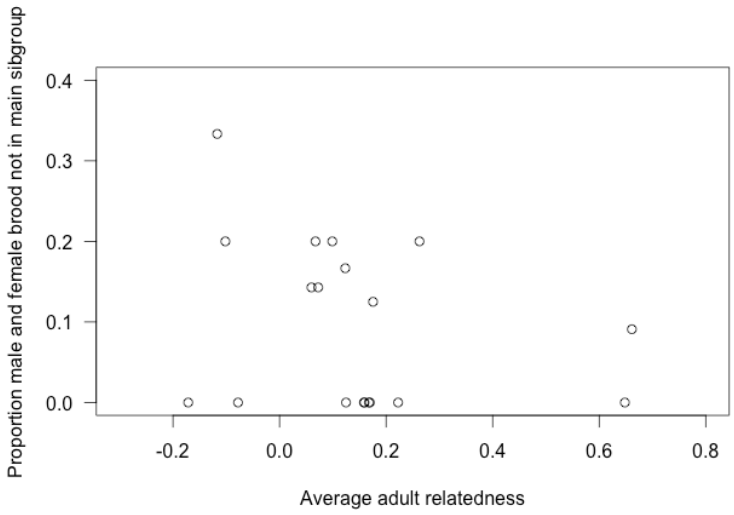

FIGURE 4.4. Graph representing the effect of average intra-nest adult relatedness on proportion of all (male and female) brood not laid by the primary reproductive

4.4.5 Proportion of males from the primary or secondary egg-layer

The proportion of males in brood laid by the primary egg-layer across nests is 12% (17/140). However, the proportion of male brood not laid by the primary egg layer is 46% (6/13). This is higher than the 90% of all male brood being laid by the primary-egg layer in the study of

Sumner et al. (2002). This suggests that on new nests unfertilized female subordinates may be more common than on established nests. Neither site, adult relatedness, adult group size, or year that the nests were collected, affects the proportion of male brood laid by a female other than the primary egg-layer.

4.4.6 Comparing between new and established nests

Here, I compare the proportion of small female brood laid by the primary egg-layer between new and established nests, and test factors which may affect it. Considering both types of nest together (new or established), and the proportion of small female brood not laid by the primary egg-layer as the response variable, there was no interaction between the type of nest and adult relatedness on the proportion of small female brood not laid by the primary egg-layer (X^2= 0.003, p= 0.961). There was no interaction between adult group size and adult relatedness on the proportion of small female brood not laid by the primary egg-layer (X^2= 2.285, p= 0.131). There was no interaction between the type of nest and adult group size on the proportion of small female brood not laid by the primary egg-layer (X^2= 1.560, p= 0.212). Site did not significantly affect the proportion of small female brood not laid by the primary egg-layer (X^2= 0.688, p= 0.407) nor did type of nest (X^2= 0.021, p= 0.884), adult group size (X^2= 0.019, p= 0.889)(Figure 4.4), or year (X^2= 0.706, p= 0.401). The proportion of small female brood not laid by the primary egg-layer also did not significantly change with adult relatedness (X^2= 0.26151, p= 0.6091)(Figure 4.5).

4.5 Discussion

Genotyping the adults of newly founded nests revealed low adult intra-nest relatedness compared to established nests. On newly started nests, a single primary egg-layer dominates reproductive rights even in the absence of relatedness between adults, and irrespective of group size. Subordinates on nests at initiation have not been able to bargain for a significantly more egalitarian arrangement as predicted by a concessions framework of reproductive skew, compared to established nests. Since group size does not affect the level of skew either in general or on new nests specifically, this suggests that the incomplete control framework is also inappropriate to describe the reproductive patterns found in this species.

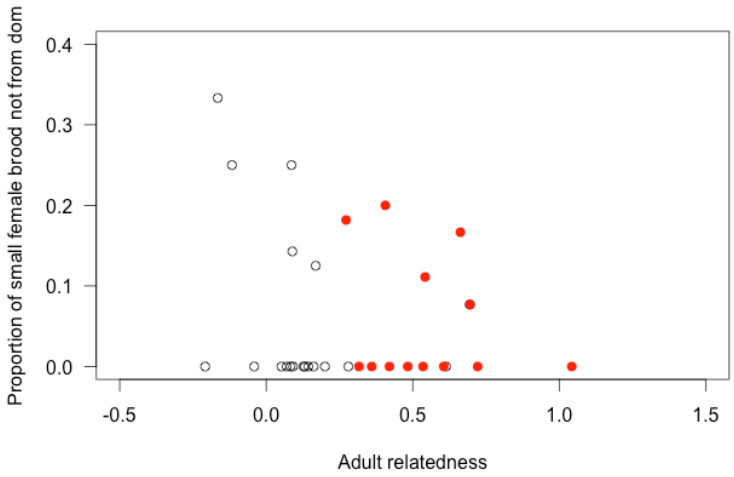

FIGURE 4.5. Graph representing the effect of of relatedness on proportion reproductive skew. Data from Sumner et al. (2002) represented by red, filled points.

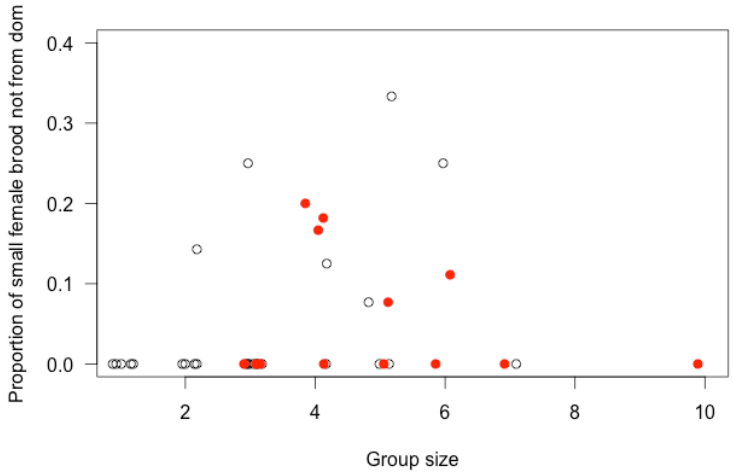

FIGURE 4.6. Graph representing the effect of group size on proportion reproductive skew. Data from Sumner et al. (2002) represented by red, filled points.

4.5.1 Relatedness profiles of new and established nests

Previous studies using genetic markers across a range of species have shown that nest-sharing females are not always relatives (West-Eberhard 1978), and that relatedness profiles may vary according to the stage of nest development (Queller et al. 2000; Zanette & Field 2008). Here, genetic analysis of adult nest-mates on newly founded nests in *L. flavolineata*

show typically low relatedness (r= 0.14± 0.049) (with the exception of two nests highlighted in Table 4.2). Furthermore, newly established nests have significantly lower relatedness than adult wasps taken from established nests in 2013 (r= 0.40± 0.067). Previous genetic analysis by Sumner et al. (2002), of established nests using three microsatellites also gave higher relatedness values than new nests (r= 0.56± 0.054).

Beyond its relevance to testing the skew models, adult female relatedness on newly founded nests is itself interesting. The most common pairwise intra-nest relatedness values are around zero (Fig. 4.2) indicating the majority of females are unrelated. There is a small secondary peak around 0.75, the value of sisters, contributed to by two highly related nests (NR87(B), and SCR4). Wasps that join sisters who have established new nests and kin re-starting nests that have been destroyed must sometimes happen, perhaps since in such cases nest-specific cuticular hydrocarbons are likely to be fresh. Samuel (1987) conducted a removal experiment on *L. flavolineata* where she found that all females on 20 nests at one site immediately re-nested in their original groups (but this was highly variable, with only two wasps out of 29 returning at another site). Joining a sister either after disruption or dispersal would be preferable in terms of inclusive fitness benefits compared to joining non-kin, especially considering the effort involved in constructing a nest from inception. While it is possible that two highly related females could come together to start a nest by chance, I suggest nest NR87(B) is likely a wholly re-established nest of kin due to its large group size (n= 5) and high intra-nest relatedness (r= 0.661).

4.5.2 Reproductive skew on new nests

Collecting newly started nests contributed 19 nests to the analysis (where more than two females were collected). Of these, 17 had very low relatedness values. Since new nests have shallow cup-like cells (far too shallow to hold a pupae), it is impossible for any daughters to have been produced on the nest. Providing the nest is not a re-establishment of an previous, mature nest after an event such as the nest dropping off the substrate (which occasionally happens), each wasp in a new group should be a joiner, and therefore 'aware' they are unlikely to be related to the dominant and vice versa. Assistance to these new foundresses is also likely to be at a premium, since extensive nest building needs to be undertaken to raise a significant number brood to adulthood. Despite these unusual parameters, none of

these nests had any more than two sib-groups among the brood, even when male brood were included, with high skew both in terms of female brood, and male and female brood combined.

4.5.3 Factors affecting the proportion of brood laid by the primary egg-layer on new nests

On new nests, neither proportion of female or proportion of male and female brood not laid by the primary egg-layer, is affected by either adult relatedness or group size. The significance values from the analyses are consistently non-significant, but become less so when the male brood are considered. The analysis remains non-significant because the average adult relatedness was uniformly low with the exception of two nests. However, there is a general trend displayed by Figures 4.3 and 4.4 which represent relatedness against the proportion of female (Figure 4.3), and male and female brood (Figure 4.4) not from the primary egg-layer. The lower values of relatedness are generally associated with higher proportions of brood outside the main sib-group. The two nests with the lowest proportion of brood outside the main sib-group (equivalent to the highest skew) consist of at least one nest which is very likely to be a re-started nest (NR87B). The other highly related nest (SCR4) represents the only other example of two highly related individuals nesting together with high skew.

According to incomplete control models of reproductive skew, larger group size might decrease reproductive skew, as a greater number of subordinates may be harder for a dominant to control. However, the maximum observed number of adult females (group size) does not influence the proportion of female brood, or male and female brood not produced by the primary egg-layer. Furthermore, if the dominant does face a battle for control of reproduction, as typified by 'tug-of-war' models under the incomplete control framework, we might expect skew to be higher on new nests compared to the much larger established nests (in terms of surface area). On new nests, it is possible that the dominant female has higher resource holding potential irrespective of variation in body size, because of the comparatively smaller size of the nest comb (up to 10 cells in new nests compared with up to 90 cells in established nests). Even if there is opportunity for surreptitious oviposition by subordinates, the dominant may be able to more effectively police and remove these eggs on smaller, new nests. However, there is no significant evidence for this. Using group size as the explanatory

variable did not reveal any trends within new nests (see supplementary).

4.5.4 Proportion of male brood

For established nests, a likelihood analysis suggested that on average, 90% of all the male eggs had been laid by the dominant (Sumner et al. 2002) compared with with 54% (7/13) of all the male eggs laid by the dominant on new nests. This suggests that female brood are prioritized by the primary egg-layer more on new nests as previously suggested (Truillazzi 1991). Early developmental stage of out-group brood on new nests makes it unlikely that these males have been laid by a previous dominant. For new nests neither site, adult relatedness, adult group size, nor year that the nests were collected, affects the proportion of male brood laid by a female other than the primary egg-layer. This is consistent with previous findings that in studies of the same population. In one study, across 22 nests only 8% of non-dominant females had developed ovaries (Field & Foster 1999), and in another 6 out of 70 (8.5%) non-dominant females had developed ovaries (Sumner et al. 2002). Although it is possible that on new nests it may be unfertilized female subordinates contributing disproportionately to the out-group brood, Sumner et al. (2002) also found that high-ranked mated subordinates with developed ovaries occasionally laid male eggs but not female eggs. Although male offspring do not contribute to group augmentation by contributing to the workforce (Kokko et al. 2001), being smaller the male brood may develop faster and this may be a deliberate strategy by (even mated) subordinates for successful surreptitious reproduction. However, since males were ascribed as not belonging to the primary egg-layer on the strength of one genotype or more being different to the maternal and paternal genotypes of the main sib-group, compared to an error rate of two genotypes for female brood, the number of male brood not laid by the primary egg-layer could be an over-estimation.

4.5.5 Comparing the proportion of small female brood laid by the primary egg-layer between new and established nests, and factors which may affect it

As the new nests had been initiated within the time window of the field season there may not have been enough time for brood to mature past the L1 stage, especially if brood abortion on smaller nests is more common (Chapter 3). Nonetheless, since none of the out-group brood were developed beyond the L1 stage I can be reasonably confident that these are not the

preserved brood of a previous dominant (with the exception of a single L3 stage larvae on nest SCR4 which was excluded from the analysis). Instead, brood on new nests are more likely to have been laid recently by a subordinate (either through cheating behaviour, force, or though concessions), or are brood laid by a newly ascended dominant (assuming that the previous dominant had laid the majority of the brood). The level of skew is high in both this study and Sumner et al. (2002). However, the latter skew values include older brood and pupae that are likely to have been laid by a previous dominant, potentially decreasing the level of skew, and furthermore, the skew indexes (S and B index) cannot be reliably compared. Therefore, I use the proportion of small female brood (eggs to L1 larvae) not laid by the primary egg-layer in order to compare potential subordinate reproduction between studies.

Analysis demonstrates that the proportion of small female brood not laid by the primary egg-layer is unaffected by the type of nest (new or established). Although this analysis has n=2, when comparing skew of small female brood (up to L1 stage development) between new and established nests, there is no significant difference between models, and this parameter can therefore be removed. Subsequently the model can be used to test whether adult relatedness or group size affects skew, irrespective of the variance contributed by nest type. This gives a more continuous range in the parameters of relatedness and group size, (Figures 4.5 and 4.6). Neither adult relatedness nor group size affects the proportion of brood laid by the primary egg-layer (or a female other than the primary egg-layer) when data from both nest types is combined. The values from the analysis are consistently non-significant, however, there is a trend. Larger group size is associated with a higher proportion of brood not laid by the primary egg-layer (up to group size of six), and lower adult relatedness is associated with a larger proportion of brood not laid by the primary egg-layer. Most studies that have examined skew in primitively eusocial species are hindered by small sample number (Field & Cant 2009), similarly these results represent 32 nests (although with a wide range of adult relatedness values), which may be a contributory factor in detecting a trend but not a significant result.

4.5.6 Why might high skew be maintained in *L. flavolineata* even when there is low relatedness?

High skew and queuing for reproductive privilege are maintained in *L. flavolineata* despite changes in important parameters in incentive and incomplete control models, and in the presence of a visual cue for low-relatedness (nest structure, exclusively small brood and each wasp being a joiner). These results suggest that the dominant has complete or almost complete control over reproduction, as required by concession models, yet she does not share reproductive rights according to group size or relatedness to subordinates, supporting the findings of several other studies (Johnstone 2000; Nonacs et al. 2006 and references therein), but contrary to some other species (Lucas et al. 2011). The few instances of subordinate reproduction (of small female brood) do follow a trend according to the predictions of concession models with higher proportion of subordinate reproduction in nests with lower intra-nest relatedness. There was a less pronounced trend for higher proportion of subordinate reproduction in larger groups.

Firstly, it is probable that *L. flavolineata* do not possess a mechanism for individual kin recognition beyond nest-specific hydrocarbons, similar to other species (Leadbeater et al. 2010; but see Leadbeater et al. 2013). This may not be surprising since detecting relatedness apart from in or out-group membership may not be regularly required. However, the very act of actively joining a new nest may be a sufficient cue as to inform a wasp that she is not related to the current or new residents of her locale. These results indicate that the incentive to join a group is likely to still be very high, irrespective of relatedness. Even a small chance of eventual direct reproduction may represent a valid gambling strategy compared to setting up a nest alone, and may represent the only possible route to direct fitness (Field et al. 1999). Therefore, even *if* low relatedness is evident to *L. flavolineata* through the act of joining a nest or through some other mechanism, it may not make a difference to the wasps' behaviour in this social context. In the Peace-Incentives model, subordinates receive an incentive so that they do not gain more by fighting for reproductive dominance than by peacefully accepting the queuing convention (Reeve & Ratnieks 1993). In the Dynamic-Skew interpretation, (Kokko & Johnstone 1999) which is a derivative of the Peace-Incentives model and under the transactional framework with staying-incentives, the parameter values (such as direct

or indirect reproduction, probability the subordinate wins a fight, and the productivity of the group), are not instantaneous. Instead, they are cumulative expected future outputs and any staying or peace incentive is a fraction of the subordinate's present, and future total group reproduction, which is likely to change over time. This forward-looking view of the level of skew and its parameters is particularly important in species like *L. flavolineata* in which workers have the opportunity to inherit the nest and become the sole reproductive. In such a case, the subordinate's fraction of reproduction instantaneous with the current dominant may be very low even though the cumulative staying/ peace incentive is predicted to be high (Kokko & Johnstone 1999; Kokko 2003; Field et al. 2006; Leadbeater et al. 2011).

Unrelated subordinates on any nest also contribute to raising future workers. In unrelated groupings, the success of raising at least one brood to adulthood (particularly likely to be female in these newly founded nests) translates into group-augmentation effects that will in turn benefit the nest inheritor. Especially when it seems typical that nest-born brood will uphold a convention of high reproductive skew in turn (Field et al. 1999; Kokko et al. 2001; Shreeves & Field 2002). Therefore, as in the original formulation of AFRs, helping could be stable even when relatedness is low.

4.5.7 Why might high skew be a universal feature of *L. flavolineata* groups?

Based on the finding of high reproductive skew on new nests despite changes in key parameters, we may speculate on the power of other factors; i.e. high inheritance benefits, extremely high ecological constraints to nesting alone, or a uniformly high chance of nest inheritance in small groups. There may even be potential benefits to workers transiently joining a group and receiving food from returning foragers, even if other reproductive strategies are later pursued elsewhere (Poethke & Liebig 2008). The main site of this study had a large number of floating individuals (L. Holt Pers. obs), but that is not unusual even at natural locations (J. Field Pers. comms). However, low market value of workers at the time of the study (and therefore low concessions) cannot be ruled out as a potential reason for high skew.

Overall, high skew itself may be a vital arrangement in terms of risk management or social structure in this species, without which nests could not maintain integrity or achieve

realized productivity. For example, in a theoretical model Toyoizumi and Field (2014b) found that in extremely difficult environments, spreading risk and task differentiation (in terms of complete reproductive division of labour) was the only way to raise a brood to adulthood. Although the environment of *L. flavolineata* is not particularly hostile, development time is very long and requires at least 100 days of protein input per-brood, (but if larvae are regularly lost or replaced, the average development time may be much longer). Since prey is highly variable (see Chapter 3), this may qualify as a difficult environment, therefore protein resources may be best directed at mainly one or two brood, and the adult that produces them (Poethke & Liebig 2008). Any deviation away from this, in terms of subordinate reproduction, may be sufficiently detrimental to jeopardize rearing any brood to maturity (or prevent a breeding individual overcoming a high nutritional reproductive threshold). According to their models, Poethke and Liebig (2008), suggest that environments with low individual foraging success and high reproductive costs will favour the evolution of reproductive skew, while large variation in foraging success, and relatively low reproductive costs will favour the formation of egalitarian groups. *L. flavolineata* are hypothesized to have relatively low reproductive costs compared to say, a social mammal, but aculeate wasps have fairly large eggs for insects. When initial nest building and the protein/calorific costs of abdominal substance are considered, this may make reproduction more expensive.

4.6 Conclusion

Despite typically low relatedness on newly established nests, high reproductive skew (of small female brood) is maintained. Both the cue of joining these visually unique nests and the hypothesized value of workers to the dominant at this stage, are important parameters when considering the concessions model of skew in this context, but do not affect reproductive partitioning. I conclude that this supports the hypothesis that dominants have strong control over reproduction on the nest and do not concede reproduction to subordinates. Nest inheritance is likely to be a major incentive to workers providing assistance to unrelated foundresses according to Dynamic-Skew models, and could restrict any subordinate reproduction concurrent with the dominant's.

CHAPTER 5

SYSTEMATIC PATTERNS OF IN INTRA-NEST RELATIONSHIPS AND NEST-SIZE DYNAMICS IN LIOSTENOGASTER FLAVOLINEATA GROUPS.

5.1 Abstract

Background: In this study 17 new microsatellite markers are utilized to identify intra-nest relatedness values and the relationship of *Liostenogaster flavolineata* workers to the nest dominant, in 46 nests ranging in size from three to seven adult females. The 17 new markers give sufficient power to identify unrelated workers and distinguish relationships between mother-daughter, aunt-niece, sisters and cousins. I examine i) whether there are systematic patterns in relatedness or relationships corresponding to inheritance rank throughout the queue, ii) whether average worker relatedness varies across different sized groups. I discuss how the results might relate to decisions on whether to stay and help or leave to pursue other reproductive options.

Results: The average relatedness of subordinates to the dominant and the average intra-nest relatedness of adult female nest-mates both increase with group size. When categorized into relationships (to the dominant), there are systematic patterns in relatedness according to rank, when controlling for group size. With decreasing seniority the chances of being a daughter or niece of the current dominant increase, while the chances of being her sister decrease. Unrelated subordinates occur at all ranks equally, but occur more frequently in

smaller groups. Across all nests a worker's relatedness to the brood increases with decreasing seniority. However, within the longest queues (group size of six and seven), there is a trend for a decrease in relatedness to the brood towards the very end of the queue.

Conclusion: Higher incidence of unrelated individuals in smaller groups suggest that these smaller nests may either be more accepting of unrelated joiners or vulnerable to usurpers than larger nests, or some other factor heavily disrupts predicted relatedness patterns. The pattern of increasing likelihood of being a daughter to the dominant with decreasing seniority is intriguing, since foraging effort is highest at low rank and this is the optimal relationship for indirect fitness benefits. In addition to decreased chance of inheritance, a trend for a decreased relatedness of workers to the brood at the very end of the longest queues may encourage newly-eclosed *L. flavolineata* workers to leave the group. Any mechanism where subordinates are able to equate rank and/or group size with relatedness values and likelihood of eventual nest inheritance would be highly advantageous.

5.2 Introduction

In primitively eusocial societies where females retain the ability to reproduce, each individual should prefer to be a reproducing dominant rather than a non-reproducing subordinate, all other things being equal. Especially if being a worker incurs risks from foraging (Field & Cant 2009). However, queuing for the right to reproduce in a group is common in social societies, including in my study species, *Liostenogaster flavolineata* (Samuel 1987; Field et al. 1999). *L. flavolineata* demonstrates a gerontocratic (age-based) queuing system, with the eldest nest resident assuming the role of reproductive (Bridge & Field 2007). Newly eclosed females of *L. flavolineata* face a choice between staying on their natal nest in the most junior position to assist the dominant female in the collection of food for the dominant's immature offspring, and leaving to pursue other options. Females that leave their natal nests may join another group, become a nest-less 'floating' individual, or establish a nest of their own (Turillazzi 1991). If she remains a subordinate, a female's behaviour and fecundity is defined by her inheritance rank (her position relative to other females in the queue for nest inheritance). In *L. flavolineata* the most junior workers forage more, spend less time on the nest or in the relative safety of the nest centre, and receive more aggressive interactions

than those of higher rank including the dominant (Rank 1) (Samuel 1987; Bridge & Field 2007; Field et al. 2006).

Previous models of queue dynamics using quasi-birth-and-death (QBD) processes have assumed that genetic relatedness between nest-mates is identical (Toyoizumi & Field 2014a; 2014b), since there is no clear information on any systematic differences in relatedness estimates throughout primitively eusocial groups. However, intra-nest genetic structure in social animals can be highly variable depending of the life history of the reproductive female(s). Haplometric species with a singly mated, long-living queen, such as *Lasius niger* (approximately 15 years lifespan) produce very uniformly related groups, while plurimetric species, with several short-lived reproductive females and multiple mating can produce very heterogeneous groups, for example in social mammals such as black-tailed prairie dogs (*Cynomys ludovicianus*) and red howler monkeys (*Alouatta seniculus*) (Storz 1999). Nest genetic structure will be influenced by the lifespan of individuals, stochastic influences such as predation, and 'cheating' behaviour by subordinates such as surreptitious reproduction, queue jumping or preferential brood predation (Bolton et al. 2006; Bridge & Field 2007). Some species also accept (or are vulnerable to) unrelated joiners who may either assume a subordinate role or usurp the dominant breeding position (Soro et al. 2009).

In haplodiploid species, there is an optimum relatedness between a worker and the primary reproductive, assuming the subordinate cannot occupy the dominant position. Namely, if the subordinate is the daughter of the current (or most recent) dominant she will be helping to rear immature sisters with a relationship value of r = 0.75. A 50:50 sex ratio of male to female brood reduces this to an average r = 0.5 relatedness, equivalent to direct reproduction by the social donor herself (Hamilton 1972; Gadagkar 1990b). Fitness pay-offs per-unit of effort are therefore largest for daughters. Instead of costly chemical-based kin-recognition systems at the individual level (Gamboa et al. 1996; Strassmann et al. 2000), wasps may be able to work by a rule-of-thumb law, associated with typical relatedness values at certain ranks, or which potentially vary predictably with group size. Rank or group size would inform their behaviour, conveying information on ideal foraging effort, since inclusive fitness theory predicts that members of non-clonal societies will benefit by directing altruistic acts towards their closest relatives (Hamilton 1964a; 1964b).

If it is possible to infer relatedness or likely relationship to the dominant through some marker, be it a chemical or visual cue (such as group size), this could be highly advantageous. Nest-specific hydrocarbon 'bouquets' typically designate group identity (Soro et al. 2010). However, studies that have tested kinship recognition capacities show little evidence for an ability to specifically recognize relatedness at the individual level, apart from chemical nestmate hydrocarbons or green-beard genes (Strassmann et al. 2000, Nonacs 2011; although see Leadbeater et al. 2013).

In *L. flavolineata* groups, Sumner (1999) found that closely ranked females were more likely to be sisters than distantly ranked females, although since her data only used 3-4 microsatellite markers fine detail relationships could not be separated which limited the scope of her conclusions. The hypothesis that newly emerged females choosing to remain on their natal nest are more likely to be daughters of the current or previous dominant could be disrupted by a number of factors, since this pattern assumes that there have been no major losses of workers or brood, limited queue jumping and that subordinate egg-laying is restricted or non-existent. Relatedness profiles can be further disrupted by unrelated joiners or usurpers (and their offspring). Furthermore, it is unknown how much stochastic effects may breakdown this predicted pattern in *L. flavolineata*.

If subordinates can detect information on relationships, then nest-wide and rank-specific patterns may also influence intra-nest behaviour (such as levels of aggression) or the decision whether or not to remain in the group. Considering that risky foraging effort increases dramatically with decreasing rank in this species (Bridge & Field 2007), subordinates with poor indirect fitness outcomes may choose to leave rather than participate heavily in this life-shortening activity; unless groups form solely as societies of "hopeful reproductives" (West-Eberhard 1978). Conversely (providing groups have not reached saturation point) larger nests may retain more female offspring if workers are aware of likely high indirect fitness returns (Reeve & Emlen 2000). However, in previous studies of *L. flavolineata*, almost half of the emerging individuals disperse even from shorter nests (observational data from Field et al. 2006). The decision to leave or stay according to relatedness will be made in combination with variables such as the value of the nest at inheritance in terms of productivity, the number of subordinates and therefore liklihood of assured fitness returns,

the likelihood of reaching the dominant position based on queue length, and potentially increased lifespan (Field et al. 1999; Mesterton-Gibbons et al. 2006). Although Michener's paradox (whereby smaller nests produce more brood per-capita) is an assumption in many social insect societies (Shreeves & Field 2002), previous studies of *L. flavolineata* (Chapter 3) demonstrate larger nests have higher per-capita realized productivity in terms of number of pupae in this species, similar to results from *polistes domminulous* (Tibbetts & Reeve 2003).

In this experiment average relatedness values for nests of differing sizes are calculated, and relationships throughout the dominance hierarchy determined using 17 newly developed microsatellite markers. The increased power of these markers (Holt & Field in prep.), allows for greater detail and confidence in relatedness and relationship estimates. Inheritance rank is the strongest indicator of effort in this species (Field et al. 2006) and so any association of relatedness with rank is interpreted in light of the level of altruistic work subordinates typically invest at each position in the hierarchy.

5.2.1 Study Species

Liostenogaster flavolineata is a facultatively eusocial hover wasp (Vespidae, Stenogastrinae), (Turillazzi 1991). *L. flavolineata* occur in small groups (1-10 females) across a wide distribution that includes Peninsular Malaysia. All females are capable of mating, laying eggs and independent nest founding. However they most commonly form multi-female, multi-generational groups with co-operative brood care and high reproductive skew (Field & Foster 1999; Sumner et al. 2002). Nests are perennial, due to the tropical climate, and their robust and long-lasting nests built from mud often occur in large aggregations (>100 nests), typically over bodies of running water, especially where artificial bridges and culverts provide an attractive location for nest building (Korb & Heinze 2008).

L. flavolineata brood are provisioned progressively, and each adult female undertakes foraging independently, with more dominant individuals spending less time foraging than subordinates (Field et al. 2006; Bridge & Field 2007). *L. flavolineata* preys primarily on the alates (winged reproductives) of ants. Foraging in *L. flavolineata* is considered a high-risk behaviour, with the threat of predation and general wear-and-tear being high. Dominant or Rank 1 individuals almost never leave the nest, receiving food and liquid from subordinates,

and policing the nest/laying eggs. This makes rank easy to determine in this species. Long brood development time of *L. flavolineata* larvae (approx 100 days) makes loss of large brood highly disadvantageous, especially for single foundresses (Field et al. 2000), who also live on average 100 days (Turillazzi & West-Eberhard 1996). Assistance with brood rearing is therefore highly advantageous, and increasing group size is correlated with increased productivity and likelihood of rearing brood to maturity (Chapter 3).

Previous data indicate that *L. flavolineata* females mate only once (Sumner et al. 2002). Previous studies have given relationship estimates of r= 0.56 ± 0.05 and high reproductive skew (Sumner et al. 2002). Henceforth wasps will be described as increasing in (numerical) rank (i.e. becoming further from inheriting the dominant position with increasing rank number) or being of low rank in the hierarchy (i.e. at a position towards the end of the queue).

5.3 Methods

5.3.1 Field site and preparation

The study was conducted between 19th August and 2nd October 2013. The study sites comprised the undersides of two bridges and one culvert along a little-used road near to Fraser's Hill, 116 km North of Kuala Lumpar, peninsular Malaysia. This road, which runs between The Gap and Raub, is the same road at which previous studies have been based (e.g. Field et al. 2006). Details of field site, marking and censusing protocols are described in General Methods (Field et al. 1998a; Sumner et al. 2002). Nests with at least three adult females and cells deep enough to contain pupae were selected and numbered. Adult female number was counted at night. Adults were collected at dawn and this was repeated on successive days until all residents were marked.

5.3.2 Collecting adults

All residents were collected before dawn in zip-lock bags as described for wasp marking (General methods). When one adult was required for removal, all other conspecifics were released from the bag, and the focal wasp retained. Wasps were stored in a cold box and euthanized at -10°C post-collection. Wasps were then stored in 100% ethanol until DNA

analysis, with identifying labels. Unmarked wasps were collected and marked during the experiment unless they were obviously trying to usurp the nest, in which case they were captured and permanently removed.

5.3.3 Identifying rank

Experimentally, each individual's position in the hierarchy can be determined with successive removals of dominants: the individual that spends the least time off the nest in comparison to her nest-mates (following the protocols of Samuel 1987; Field et al 1998, 1999; Field & Foster 1999). Before the first removal, the nests were observed using rapid censusing for a minimum of three and a maximum of nine days (where there was ambiguity) to identify the dominant individual. The designated dominant was then removed and the wasps given two days to re-establish their hierarchy. Nests were thenceforth rapid censused for a minimum of three days (07:00 to 12:00) between each removal to identify the next dominant individual, which was then removed. This continued until the queue was fully collected. For each day of rapid censusing, a female had to have three more recorded observations on the nest than the individual below her, in order to be ascribed higher rank.

Throughout censusing any behavioural events were recorded. If there was any ambiguity between the behaviors of the two most dominant females, these could be used to help discriminate between the two. Where the number of censuses and observed behaviours were not sufficient, direct observation was carried out for these nests. *L. flavolineata* can exhibit a range of aggressive and passive behaviours ranging form violent fights, typically involving repeated wrestling matches causing the participants to drop from the nest, to less violent signals of dominance, including antennal boxing, chasing, shoving and domination of the central comb of the nest, with subordinates driven to the outside rim or side. If the number of censuses and observed behaviours was insufficient to ascribe dominance, further rapid censusing or direct observation was carried out for these nests.

Due to the duration of the experiment, not all of the adult wasps marked and observed at the start of the experiment were still alive for monitoring or collection by the time they would have inherited the dominant position, or at the end of the experiment. There were no instances of marked subordinates changing between groups during this experiment. A

missing wasp's position in the queue was calculated using a combination of foraging effort and inheritance position in the queue before their disappearance in relation to surviving members. Therefore each of the workers that were marked and observed during the initial observation period were given a rank, even if they could not later be genotyped. If a female wasp ecolosed during the experiment and joined her natal group, she was given a rank corresponding to the lowest position after the original group members.

5.3.4 Molecular work and genotyping

Previous data indicate that *L. flavolineata* females mate only once (Sumner et al. 2002), therefore a female sib-group within the adults or brood should exhibit a maximum of three alleles at each locus, and all individuals should share the same paternally derived allele. Adults were genotyped at 19 polymorphic microsatellite loci. DNA extraction, PCR and genotyping were performed as described in General Methods and Appendix (A.2.1 to A.2.4). All adults were visually and genetically female.

5.3.5 Relatedness values and relationships

Relatedness 5.0.8 (Goodnight & Queller 1998) was used to estimate the mean regression relatedness within various groups of different size. Colonies were weighted equally and standard errors were obtained by jack-knifing over nests. Relationships and relatedness values between individuals of given rank were determined using KINSHIP version 1.3.1 (Goodnight et al. 2005). These programmes can give values of less then zero and more than 0.75 for relationships based on background allelic frequency. With these 17 new loci (Holt & Field, in prep.), fine-detail relationships can be determined. Since there are a large number of potential relationships in the inheritance queues of *L. flavolineata* groups KINSHIP analysis can give contradictory results, and therefore each possible relationship needs to be eliminated in a systematic way. For example, when A is the null hypothesis and B the primary hypothesis, and A is rejected, it is not necessarily in favour of B (see General Methods for more detailed description). Figure 5.1 details the relationships tested, and the type II error rates of detecting these different relationships based on the power of the markers.

Genotypes were checked and scored twice independently at Sheffield Genomic Facility (SMGF). Individuals were first analysed in KINSHIP to determine whether it was more likely

Sisters more likely than cousins.			
	p<:	Ratio	Type II error
*	0.05	0.00E+00	0
**	0.01	0.00E+00	0
***	0.001	7.97E+01	0.008

Mother/ daughter more likely than aunt/ niece.			
	p<:	Ratio	Type II error
*	0.05	6.91E+00	0.344
**	0.01	1.14E+01	0.796
***	0.001	1.86E+01	0.976

Aunt/ niece more likely than sisters.			
	p<:	Ratio	Type II error
*	0.05	5.70E-04	0
**	0.01	9.95E-03	0
***	0.001	8.04E-01	0

Cousins more likely than unrelated.			
	p<:	Ratio	Type II error
*	0.05	2.52E+00	0.299
**	0.01	1.09E+01	0.551
***	0.001	3.25E+01	0.723

Aunt/ niece more likely than cousins.			
	p<:	Ratio	Type II error
*	0.05	4.00E+00	0.376
**	0.01	1.76E+01	0.719
***	0.001	5.31E+01	0.9

Mother/ daughter more likely than sisters.			
	p<:	Ratio	Type II error
*	0.05	5.04E-01	0.02
**	0.01	4.30E+01	0.231
***	0.001	4.77E+02	0.52

TABLE 5.1. Table of significance values for KINSHIP tests. Results of significance simulations, 1000 simulated pairs used to calculate values.

that a pair were sisters rather than cousins. Any samples that did not have a significance value of $p<0.05$ for full sisters (Type II error = 0.008), were re-checked again at all loci. Since microsatellite analysis is more likely to create deviation rather than convergence to genetic similarity between pairs, this method confidently identified any female that was not part of the main sib-group of the nest, or was a member of the main sib-group that had an erroneous score at one or more loci. After re-checking, each of the remaining relationships were systematically tested (see General Methods). Through this method intra-nest relationships could be ascribed with little conflict (n= 0 nests without an ascribed relationship to the dominant). This initial separation of individuals into sib and non-sib groups was performed blind to rank.

KINSHIP does not give directionality to, for example a mother/daughter relationship, and therefore, the final assignment is based on the assumption that according to all previous research on *L. flavolineata*, the dominance hierarchy is dependent on age, and the elder relationship is more likely to take the higher rank (Bridge & Field 2007). KINSHIP is used to here as a way of more clearly visualizing patterns throughout the dominance hierarchy, which could otherwise be drowned out by noise in relatedness values (See General Methods).

5.3.6 Data analysis

All statistical analyses not requiring the specialist programmes mentioned above were performed in R (R Development Core team, 2013). I first wanted to test whether a worker's relatedness to the dominant changed with group size or inheritance rank. I examined this question using a generalized linear mixed model (GLMM) with normal errors, with 'relatedness to dominant' as the response variable and 'group size' and 'rank' as explanatory variables. 'Nest' was included as a random factor. An interaction term was initially included

between ‘group size’ and rank. Relatedness data were continuous, but significantly non-normally distributed. Therefore, before proceeding with analyses I transformed relatedness variables using the ‘powerTransform’ function in the R package ‘car’. Since some of the values were zero I used powerTransform plus one.

Since an unrelated dominant would produce the result that all the subordinates are unrelated to it using this method, even if all the subordinates were related to each other, I additionally wanted to test whether its average relatedness to other nest-mates changed with group size. I tested this using a Pearson’s correlation between ‘group size’ and ‘intra-nest relatedness’.

I next wanted to examine whether there were any systematic patterns in relationship between individuals at a given rank and the dominant. To examine this, I used the results from KINSHIP to categorize the relationships (i.e. sister, daughter etc.), then tested each relationship in turn to determine whether the likelihood of worker being a given relationship to the dominant. I used a GLMM with binomial errors to examine each relationship in turn. In each case, whether or not a worker exhibited the given relationship to the dominant was the response variable, and ‘rank’ and ‘group size’ were included as explanatory variables. Data were from two different but nearby sites. Therefore in the maximal model I initially included interactions between site and each explanatory variable, and between the two explanatory variables. ‘Nest’ was included as a random factor.

I finally wanted to investigate whether a worker’s relatedness to the dominant’s brood was affected by rank or group size. It was not possible to transform the data to meet model assumptions, and I therefore use correlations. I used a Spearman’s rank correlation to examine both the relationship between group size and relatedness to the brood, and rank and relatedness to brood. Additionally I examined group sizes of six and seven separately for a change in a worker’s relatedness to the brood according to rank using a Spearman’s rank correlation.

In all models, starting with the interaction terms, non-significant terms were then removed following step-wise model reduction until only significant terms remained. Values

reported in the results are Chi-squared and associated p-values for when terms were sequentially removed from the model.

5.4 Results

5.4.1 Group size and relatedness

Workers in larger groups tended to be more related to the dominant than workers in smaller groups (X^2= 9.556, p= 0.002) (Figure 5.1). In line with this result, there is a positive correlation of average intra-nest relatedness and group size (cor= 0.431, df = 44, p= 0.003).

Group size	Nests	Individuals genotyped
3	13	39
4	14	56
5	10	50
6	8	48
7	2	14

TABLE 5.2. Table of group sizes and frequency

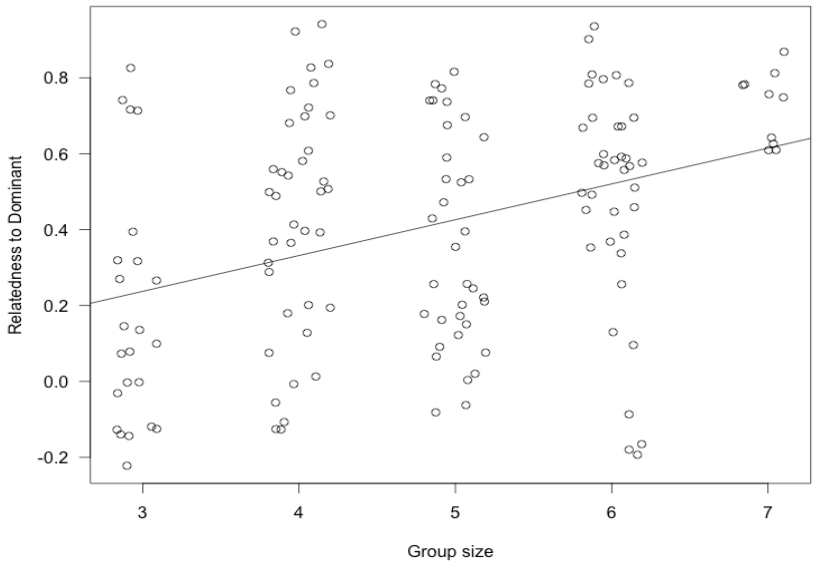

FIGURE 5.1. Each individual's relatedness estimate to the dominant according to group size.

5.4.2 Relatedness values within groups

Using relatedness values, and when all group sizes are considered together (Figure 5.2 a), there is no statistically significant difference in worker's relatedness to the dominant throughout inheritance ranks (X^2= 0.633, p= 0.426), although there is a slight trend for relatedness to the dominant to increase with rank. There was no interaction between rank and group size (X^2= 0.158, p= 0.691). However, relatedness across all ranks was significantly different between group sizes (X^2= 10.188, p= 0.001) (Figure 5.3, standard errors given in Table 5.3). When broken down by group size, rank does have a significant effect on relatedness to the dominant in groups of three (X^2= 3.9928, df=4, p=0.0457), with Rank 3 being significantly less related to the dominant than Rank 2 (Figure 5.2 b). There were no significant effects of rank in the remaining group sizes (p>0.05).

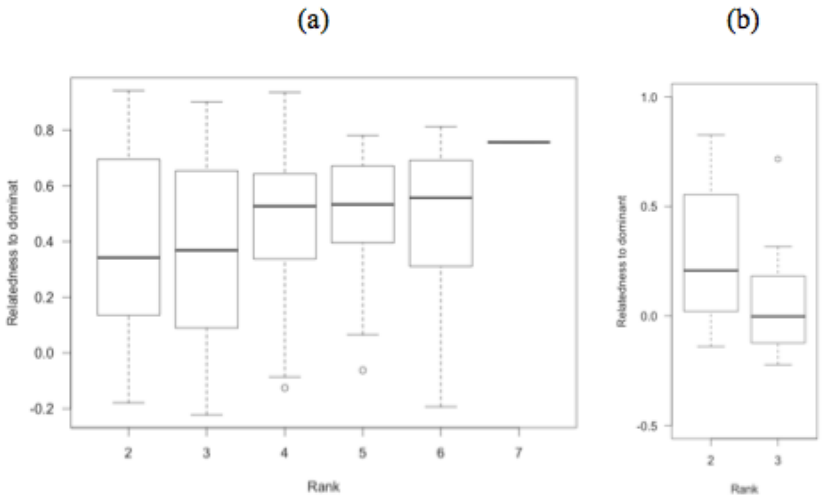

FIGURE 5.2. (a) Worker's relatedness to the dominant according to rank for all group sizes combined.(b) Graph showing the only group size (three adults) with a significant change in relatedness estimates with rank.

5.4.3 Relationships within groups

Relatedness values are sometimes an uninformative way to visualize the changing relationships through across the inheritance ranks as the spread of values can obscure patterns. By using KINSHIP relationships can be identified and the proportion of different relationships at each rank compared. Figure 5.3 represents visually, how the likelihood of each relationship to the dominant changes with rank when all groups are included. Note that only the largest

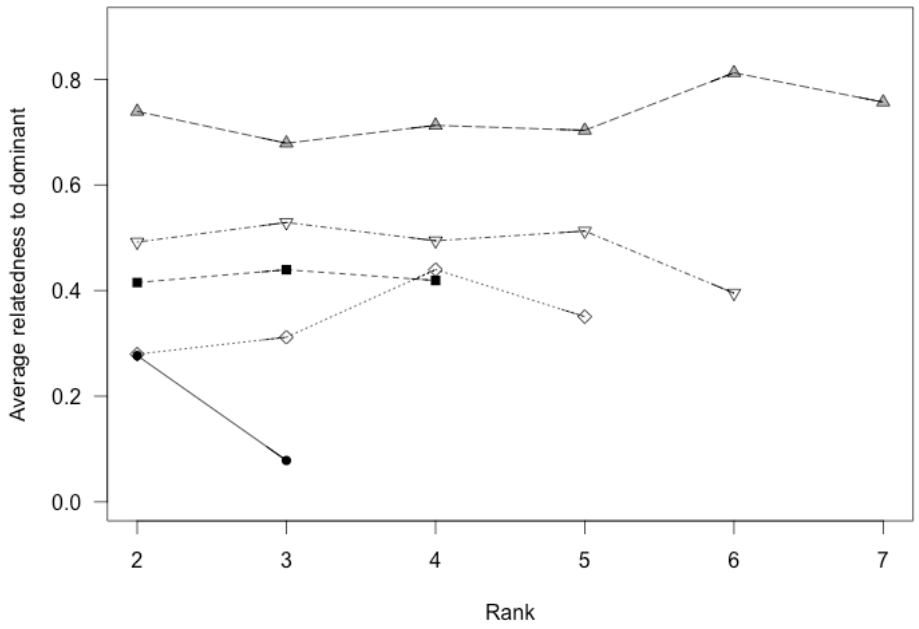

FIGURE 5.3. Graph showing the average relatedness to the dominant of nest-mates at each inheritance rank (filled circles= group size two, filled squares = group size three, unfilled diamond = group size four, unfilled inverted triangle= group size six, grey filled triangle= group size seven).

Rank	Group Size	Average r	SE
2	3	0.277	±0.0966
2	4	0.439	±0.091
2	5	0.297	±0.090
2	6	0.492	±0.126
2	7	0.739	±0.523
3	3	0.078	±0.082
3	4	0.439	±0.078
3	5	0.338	±0.095
3	6	0.529	±0.121
3	7	0.679	±0.480
4	4	0.419	±0.093
4	5	0.431	±0.087
4	6	0.494	±0.104
4	7	0.713	±0.504
5	5	0.491	±0.056
5	6	0.513	±0.053
5	7	0.703	±0.497
6	6	0.395	±0.147
6	6	0.812	N/A
7	7	0.757	N/A

TABLE 5.3. Standard error of average relatedness values for each rank and group size

nests can contribute data to the most junior ranks, and the spine plot represents the number of samples by the width of the bars. Overall there is a trend for the highest ranks to be associated with larger proportions of daughters and nieces, while the number likelihood of a

worker being the sister of the dominant decreases.

In order to analyze these trends and to control for the effect of group size on the frequency of the different relationships at each rank, I used a mixed model with binomial errors. As represented by the spine plot, the likelihood that a subordinate is a daughter of the dominant (i.e. compared to any other relationship) significantly increases with rank, the likelihood of a subordinate being a sister of the dominant significantly decreases with rank, and the likelihood of a subordinate being a niece of the dominant significantly increases with rank, when each is controlled by group size. The likelihood of being unrelated to the dominant is not affected by rank but is affected by group size, with the likelihood of being unrelated decreasing with larger group size (Table 5.4).

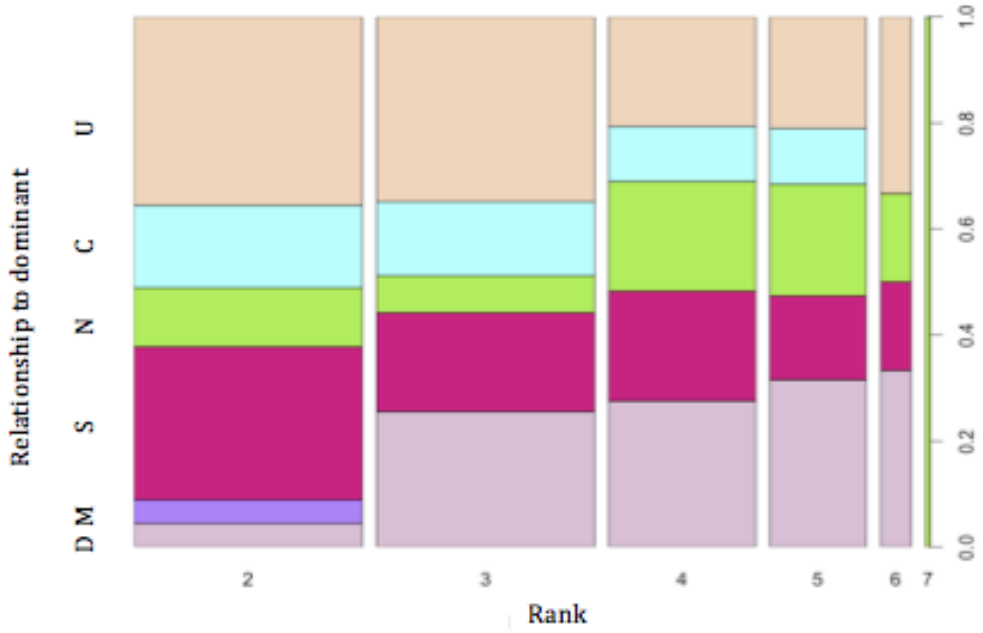

FIGURE 5.4. Spine plot representing the proportion of each rank's relationship to the dominant. The width of the bar represents the number of samples. Relationship categories, from bottom to top are, Daughter= D, Mother = M (only in rank 2), Sister= S, Niece= N, Cousin= C and Unrelated= U. There was one hypothesized granddaughter of the dominant, which is omitted for clarity (nest 209, daughter of Rank 3, who was in turn one of the daughters of the Rank 1). The spine plot weights by number of individuals at each rank, but does not separate results by group size.

Relationship	*X2* Rank	p-value Rank	*X2* Group Size	p-value Group Size	Change With Increasing Rank
Daughter	7.902	0.005**	0.004	0.948	Increase
Sister	8.787	0.003**	1.875	0.171	Decrease
Niece	4.255	0.039*	0.011	0.919	Increase
Unrelated	0.023	0.880	6.546	0.011*	N/A

TABLE 5.4. Table showing how relationships change throughout groups (stars denote significant results: *p<0.05, **p<0.01)

5.4.4 Worker relatedness to the dominant's offspring

Daughters of the dominant are more related to the brood than other relatives are, due to the haplodiploid genetic structure of *L. flavolineata*. It may therefore be useful to ask whether there are any systematic changes in subordinate's relatedness to the brood at each rank rather than to the dominant. Otherwise a sister's relatedness estimate to the dominant (r= 0.75) might be considered more important than a daughter's (r= 0.5), although the relatedness to the brood is the most important in terms of indirect fitness benefits if a subordinate is acting altruistically to raise the dominant's brood.

Using the relationship data provided by KINSHIP, the standard relatedness value for each relationship was used (e.g. r= 0.75 for sisters). This value was then converted to the standard relatedness of the worker to this dominant's brood based on a 50/50 sex ratio (e.g. 0.375 for sisters). There was a highly significant positive relationship between the group size that each wasp belonged to, and its relatedness to the dominant's brood (rho = 0.355, p<0.001). Furthermore, across all nests there is a slight positive correlation between increasing rank (decreasing seniority) and increasing relatedness to the brood, since the likelihood of being a daughter increases (rho= 0.1876181, p-value = 0.02484).

In the longest queues (group sizes 6 and 7, n=10), there is a non-significant trend for the worker's relatedness to the brood to decrease with increasing rank (rho= -0.017, p-value = 0.908). This is because there is an increasing likelihood of a worker being a niece to the dominant at the lowest ranks in the largest groups since it is very unlikely the mother of the youngest workers would still be alive. Typically, the worker's aunt has replaced the previous dominant (Figure 5.5).

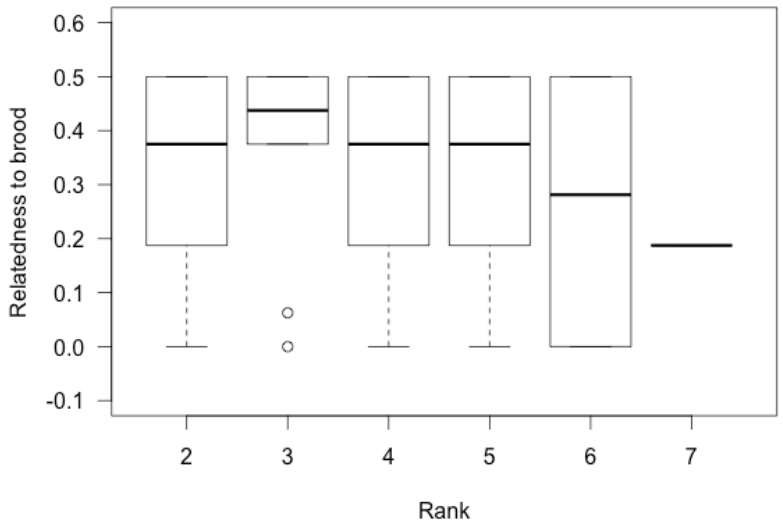

FIGURE 5.5. Graph showing worker's relatedness to the brood for the longest queues, group sizes 6 and 7 n=10

5.5 Discussion

These 17 new markers have sufficient power to elucidate relationships between mother, daughter, aunt, niece, cousin and unrelated females in *L. flavolineata* (Holt & Field in prep.), and provide better average relatedness values for groups of varying sizes then previous studies (Sumner et al. 2002). Nest-wide relatedness estimates of female nest-mates to the dominant and to each other increase with group size. There are no significant changes in the relatedness estimates of workers to the dominant according to rank, when broken down by group size. However, when categorized into relationships the results show that within any given nest there is a greater statistical likelihood of a daughter or niece of the dominant occurring at the most junior ranks, whilst the likelihood of a sister at these ranks decreases. Unrelated wasps are not more likely to occur at any given rank, but they do occur more frequently in smaller groups, which also have the lowest overall intra-nest relatedness estimates. When the relatedness of each subordinate to the brood laid by the dominant is analyzed, across all nests there is similarly a positive relationship of increasing relatedness with larger group size and numerical rank. In the longest queues, however, there was a non-significant trend of decreasing relatedness to the brood with increasing numerical rank.

5.5.1 Relatedness and group size

Both the estimates of subordinate's relatedness to the dominant (Figure 5.1) and average nest relatedness are strongly positively correlated with group size. Relatedness estimates between the workers and the dominant increase from a minimum relatedness in groups of three indicative of no kinship, up to a maximum average relatedness in group size seven consistent with relatedness of full sisters. Using average intra-nest relatedness avoids the relatedness estimates to the dominant being misleading if an unrelated wasp has usurped the nest; those results show that in the smallest groups the subordinates are not only unrelated to the dominant but also unrelated to each other.

Larger groups seem to be more successful at maintaining high relatedness, perhaps since larger groups may be better able to defend from usurpers or predators, or have less need of unrelated joiners. However, this latter point is contrary to previous research that found that group size does not affect the number of joiners (Field et al. 1999), or leavers. In previous studies, almost half of the emerging individuals disperse even from shorter nests (Field et al. 2006), although both these variables may fluctuate with season. Allee effects (a positive correlation between population size or density and the mean individual fitness) predict that smaller groups will in general be more vulnerable to outside influences (Courchamp et al. 1999). Previous studies have found the only parameter that affects foraging effort at a given rank between *L. flavolineata* nests, is group size (Field et al. 2006; Field & Cant 2006). As subordinates in smaller groups work harder than their equivalents in larger groups, it is highly probable that this translates into a higher mortality rate (Toyoizumi & Field 2012). Smaller nests may be more vulnerable to loss of adults or brood through stochastic processes, or a higher rate of brood cannibalism (Chapter 3), which could break down high relatedness values over time. Alternatively, previous research (Chapter 4) has also found that new nests consist mainly of unrelated individuals, so these smaller groups may also represent an early stage in group development. Increased aggression in groups consisting of unrelated individuals remains a possibility (Cronin & Field 2007; Tibbetts & Reeve 2000; Field & Cant 2009; Leadbeater et al. 2013; Zobel & Paxton 2007) and may disrupt smaller groups' future relatedness profiles.

5.5.2 Relatedness values within different sized groups

Smaller groups have lower relatedness values than larger groups, but there are no systematic patterns in relatedness values throughout nests of varying size, since there is no significant interaction between group size and rank. Rank 2 and Rank 3 individuals in groups of three yielded the only significant difference in relatedness between ranks. Rank 3 individuals on these nests are significantly less related, with values suggesting these are more likely to be completely unrelated to the dominant. In these cases it is possible either that the dominant is a usurper or the Rank 3 is a joiner. However, whenever the Rank 2s are more related to the dominant, it is more likely that the Rank 3s are joiners. Although groups of three had the only significant interaction between ranks this could be by chance, despite a relatively good sample size for studies of this type (n= 13 nests). As a consequence of using relatedness estimates that can have a large range outside the typical value for the relationship involved, conclusions from this data regarding any systematic patterns are somewhat unreliable.

5.5.3 Relationships within different sized groups

Analysis of data when relatedness estimates are categorized into relationships using KINSHIP suggests a significant systematic pattern. The changes in likelihood of each relationship are those hypothesized: firstly that the chances of being a daughter or niece of the dominant increases with rank (further from the dominant position), and secondly that the chances of being the sister of the dominant decreases with rank. As the relatedness estimates suggested, unrelated wasps occur more frequently in smaller nests but not in correlation with rank when controlled for group size. Lower reproductive skew might have been extended as an explanation for these lower relatedness values in smaller groups (causing a reduction of close relationships such as sisters) but previous research (Chapter 4) suggests that skew is universally high even on nests with low adult relatedness. In contrast to the results of previous studies (Field et al. 1999), these results suggest that smaller groups do potentially acquire unrelated joiners more frequently. The proportion of cousins remains consistent across group sizes but do not appear past Rank 5.

5.5.4 Subordinate relatedness to brood

Daughters and nieces of the dominant are of particular interest in the queue: daughters, since in haplodiploid species they will often be neutral about whether siblings or their own offspring are raised (and they accrue the highest indirect fitness payoffs per-unit effort out of all the possible relationships), and nieces, since the probability of a worker eclosing once an aunt has inherited the dominant position may influence her decision to stay or leave the group. It is interesting that, as effort increases with distance from the dominant position (see Chapter 6; effort is high for Ranks 4, 5 and 6), there is positive correlation with the optimum relationship to the dominant for the highest inclusive fitness benefits. If a worker's mother is still alive (and even for a significant period after her death), fitness payoffs may be largest from working in the most junior position on larger nests. This may be true for both daughters and nieces (and may explain why we observe some nieces at low rank), since previous studies have demonstrated older brood laid by the previous dominant in *L. flavolineata* are reared after her disappearance (Sumner et al. 2002). Therefore, the proportion of nieces and daughters may be considered almost equivalent in this context, providing immature brood remains in the nest. However, ants quite frequently raid *L. flavolineata* nests (L. Holt unpubl.); it is possible wasps (especially nieces) may make individual decisions on group membership based on the number and developmental stage of brood in the nest. Nests without brood would be less attractive in terms of both indirect fitness and future group augmentation (Field et al. 1998).

Additionally, the more frequent observation of daughters on larger nests (and even a grand-daughter in one group size of six) demonstrates that in larger groups the dominants have comparatively good longevity, 100 days minimum based on estimates of larval development rates (Turillazzi 1996). This may be due to improved survivability of these dominants on larger nests, or because higher rates of egg cannibalism (or loss from predation) are associated with smaller groups (Chapter 3). Mesterton-Gibbons et al. (2006) require differential survival as an important aspect of their model of group stability. High rates of brood cannibalism in small groups may also explain why there are fewer daughters detected in small nests; by the time a female worker is born on the nest, her mother has long since died.

5.5.5 Could group size and/or rank be used to indicate relatedness?

Since there are systematic patterns in relationships to the dominant and brood throughout the hierarchy, and different group sizes are on average associated with different average relatedness values, it is plausible that rank or group size could be used by this species as a cue, although further work is needed in this area. As relationships are different in different group sizes, if wasps are able to use group size or rank position (or a combination of the two), this could be highly advantageous. Previous work has ruled out any substantial reproductive concessions in this species, but relatedness and indirect fitness benefits may have implications for foraging effort.

Although it is beyond the scope of this study, I suggest that being born on a large, well-functioning nest could provide chemical, nutritional and visual cues to newly-eclosed females both pre and post-emergence. Knowledge of group productivity and inclusive fitness benefits would constitute important information for newly-eclosed wasps, potentially influencing their behaviour on whether to leave or help, independent of nest hydrocarbons. Experiments by Field et al. (2006) have implied that wasps are able to assess the number of subordinates in a group to make decisions on foraging effort based on predicted future fitness outcomes. High relatedness values on larger nests in general could potentially act as a 'catalyst' or attractor to increasing group size, since staying to help could be a more attractive when inclusive fitness benefits are predicted to be higher. Lower relatedness to nest mates or the dominant, in contrast, may discourage remaining on the nest for nest-born females in smaller groups. Previous studies in *L. flavolineata* and other social insects show that subordinates assist on a nest in part due to inheritance benefits (Kokko & Johnstone 1999, Kokko et al 2001, Shreeves & Field 2002), but unless these groups form solely as societies of "hopeful reproductives" (West-Eberhard 1978), nest-wide relatedness and indirect fitness returns are likely to have some considerable importance. I summarize these ideas on queue dynamics of small and large groups based on the results in this chapter in Appendix A.5 and A.6.

5.6 Conclusion

Workers in smaller groups are less related to each other and to the dominant, than those in larger groups. Group size is likely to be a good indicator of high relatedness to the dominant, to her brood, and to other nest members on average, providing wasps are able to use this as an visual cue. Using relationship data there are systematic patterns in larger nests similar to what we might hypothesize; namely, daughters of the dominant being at the lowest queue positions. A female eclosing on a large nest might be able to infer that she is i) likely to be in a group with high intra-nest relatedness, and ii) that she has a higher likelihood of being a daughter (with the highest possible indirect fitness returns) than if she had eclosed on a small nest. These relatedness data may be of use in contributing to models on queue dynamics in the future.

CHAPTER 6

FORAGING EFFORT AND GROUP SIZE IN LIOSTENOGASTER FLAVOLINEATA.

6.1 Abstract:

Background: Although relatedness to the primary reproductive (and by extension her offspring) in eusocial societies is hypothesized to be important in terms of how hard a subordinate should work, there has been mixed empirical evidence of the ability to individually discriminate kin (and moderate effort in response). In the facultatively eusocial queue-forming Hymenopteran *Liostenogaster flavolineata*, group size has been found to affect foraging effort at a given inheritance rank, but not relatedness to the dominant. This has previously been interpreted as workers prioritizing future direct fitness more in larger groups than smaller groups. However, new data regarding the number of brood per-capita, variance in food returns, and typical relatedness in groups of different size have not been considered in this context.

Results: Larger groups spend less time foraging than if there was a linear relationship between group size and foraging effort. There is a positive correlation between group size and the average relatedness of workers to the dominant. It is therefore difficult to disentangle effects of group size from effects of relatedness. Comparison of foraging effort across 46 nests (group size three to six adult females), demonstrates that at a given inheritance rank there is a significant decrease in effort with both increasing group size and relatedness. The

distribution of food returns to nests is not significantly affected by group size, contrary to predictions of the Central Limit Theorem (CLT).

Conclusion: That smaller groups typically have lower relatedness might provide a visual cue to inform worker's decisions on foraging effort. It is paradoxical that wasps less related to the dominant and her brood (on small nests) work harder than more highly related wasps on larger nests, since forging effort is expected to increase with higher indirect fitness benefits. This suggests that either other factors (such as group size or future fitness) outweigh relatedness in determining effort, or that the relationship between relatedness and effort is naturally negative. I discuss whether decreased levels of effort by the highest-ranking females on larger nests may be a behavioural concession bestowed or at least tolerated by the dominant. Alternatively, since there is no significant increase in variance in food returns on smaller nests, subordinates on these nests may be increasing effort to compensate for the effects of the CLT on food returns. Group-level effects such as brood number optimization and synergistic benefits from a greater number of workers could also improve the efficiency of larger groups.

6.2 Introduction

Relatedness to the current reproducing individual(s) and therefore to the immature offspring being raised in a eusocial society determines the amount of indirect fitness accrued by subordinates or helpers. Specifically, since subordinates highly related to the dominant and brood should gain more indirect fitness for every unit-effort they invest, inclusive fitness theory predicts that they should work harder, providing they have a way of detecting relatedness to the current dominant or brood (Hamilton 1963). However, if female workers retain the ability to reproduce themselves (usually only upon inheriting the breeding position), the effort they invest in dangerous activities such as foraging that lowers their own fecundity and future fitness should be balanced against their own future direct reproduction (Field et al. 2006; Field & Cant 2006).

Queuing for a dominant, reproducing position is a widespread phenomenon in social species, including social vertebrates (Johnstone & Cant 2009). *Liostenogaster flavolineata*,

a facultatively eusocial tropical hover wasp, queues according to an age-based convention (Bridge & Field 2007; Cant & Field 2001), and foraging effort is determined primarily by inheritance rank (Field et al. 2006). In facultatively eusocial insects, there has been mixed evidence that female helpers are able to detect relatedness, or if they are able to do so, they have not been observed to correspondingly modify their effort in terms of foraging or nest defense (Leadbeater & Carruthers 2010; but see Leadbeater et al. 2013). Although some studies have found the predicted positive correlation between helping effort and kinship, several others have not (see Clutton-Brock et al. 2000 and references therein). In studies of *L. flavolineata* group size is the only variable that has been found to explain variation in effort within a given rank, with subordinates in smaller groups foraging more (Field et al 2006). Their study gives evidence that this species can assess the two most important parameters that determine expected future fitness: their position in the social queue and group size (Griffin 2006). If very different average relatedness values are associated with very small and very large nests, individuals might also be selected to associate small group size or other visual cues with low indirect fitness outcomes, or a certain position in the queue in a large group with high indirect fitness benefits.

Nest value and foraging effort

Thus far, the finding that individuals at a given inheritance rank within larger groups of *L. flavolineata* work less hard than those at the same rank in smaller groups, has been understood as a pay-off between i) future direct and ii) current indirect, fitness benefits. Subordinates, informed by nest value, moderate their investment in the costly activity of foraging effort when their future direct reproduction is likely to be high. This has been modeled under the Kin-Section framework (Field et al. 2006; Field & Cant 2006; Heinsohn & Legge 1999).

For many species, and for *L. flavolineata* especially, the true value of a nest at inheritance is hypothesized to correspond to the number of subordinates or well-developed brood in a nest, rather than the nest structure itself. This is illustrated by poor uptake of vacant nests (containing only small brood) in studies of *L. flavolineata* (Field et al. 1998). Although some females do nest alone, the development time of the larvae in this species is very long

in comparison to average adult lifespan (100 days on average for both larvae and solitary foundresses (Samuel 1987; Turillazzi & West-Eberhard 1996). Brood laid by a previous dominant are usually reared by remaining group members after her death, otherwise, fewer than 50% of independent-nesting *L. flavolineata* females can expect to survive long enough to raise any offspring through to adulthood, with the remainder having zero reproductive success (Samuel 1987; Queller 1996; Field et al. 1998; Field et al. 2000b). Since there is little benefit in a worker preserving her future fitness unless she inherits subordinates along with the breeding position, a worker at the end of a queue or in a small group is hypothesized to prioritize indirect fitness benefits by investing more of her effort to raise offspring of the dominant (her putative relative), explaining the differential effort both between inheritance ranks and between group sizes (Field et al. 2006; Field & Cant 2006).

The Kin-Selection model of Field and Cant (2006) makes two major predictions: (i) subordinates will work less hard the closer they are to inheriting breeding status; and (ii) for a given inheritance rank, subordinates will work less hard in larger groups. However, relatedness (r) was not considered to vary systematically with group size in the *Polistes dominulus* co-foundress associations used to test the model, and relatedness was not found to affect effort in *L. flavolineata* (Field et al. 2006), although only a sunset of females were genotyped. Field et al. (2006) further tested predictions of the Kin-Selection model in *L. flavolineata* by manipulating group size and brood number. All subordinate wasps below Rank 2 and a proportional sub-set of brood (at all developmental stages) were removed from larger groups. The remaining Rank 2 subsequently increased her foraging effort (54 ± 10%, n = 8) to a level similar to that of control Rank 2s in un-manipulated groups of two (47 ± 9%, n = 7). Although the focal wasp was theoretically not responsible for any greater number of brood than prior to the removals, she had switched from prioritizing her future fitness (i.e. helping less), to helping more since the value of the nest in terms of the number of subordinates (and brood) had fallen (Field et al. 2006).

However, one criticism of this experiment is that by reducing group size so dramatically, all foraging duties fall upon the Rank 2 after the manipulation (dominants rarely leave the nest in any group size of two or more). Although the focal Rank 2 undoubtedly works harder after the manipulation, this could be due to aggressive prompting from the dominant,

for example, or because the Rank 2 needs to forage more for her own needs where she had previously been receiving food from returning foragers. I suggest that the understanding of results from this experiment (the finding foraging effort is increased with a manipulation downwards in group size) can be contributed to with new relatedness values and data on the per-capita number of brood for differing sized groups. Here I introduce the three concepts that I will use to interpret data on group size and foraging effort.

Optimization and the Central Limit Theorem

Greater realized productivity in larger groups is a necessary condition for the evolution of sociality and group living (Naug & Wenzel 2006). However, in addition to increased productivity at any snapshot in time, larger groups may also be able to reduce overall effort per-successfully-reared offspring if parameters (such as brood number or foraging efficiency) are optimized. In other species, greater efficiency of larger groups has been measured in a reduction in nest-wide energy requirements, or individual energy usage (Muradian et al. 1999). This 'optimisation principle' has been observed in systems as diverse as ant colonies to human cities as a product of greater synergy (synergy in biological systems refers to non-linear effects) with increasing group size (Jaffé 2010; Jaffé & Fonck 1994; Corning 1998). There are many candidate parameters for optimization, including greater synergy in terms of communication, or efficiency in division of labour, task differentiation and resource exploitation (Pacala et al. 1996; Avilés 1999; Avilés et al. 2002). However, optimization of brood number has typically been of interest in social insects (Wenzel & Pickering 1991; Naug & Wenzel 2006). If immature offspring number is sufficiently optimized through matching it to average food availability in larger groups, i.e with less waste this could allow some or all members to forage less. This may be selectively advantageous, contributing to both individual and group longevity, and in totipotent species increasing future direct fitness and fecundity (Toyoizumi & Field 2014b).

Larger groups (in terms of adult females) of many social insects are found to maintain fewer immature brood per-capita than smaller nests, although it has been shown that larger nests often rear more brood per-capita to adulthood successfully (Wenzel & Pickering 1991; Stevens et al. 2007; Shakarad & Gadagkar 1995; Kojima 1989). This includes the facul-

tatively eusocial hover wasp *L. flavolineata*. Previous work (see Chapter 3) has found the number of adult females significantly affected the total number of brood in a quadratic relationship ($X^2$83=23.239, p<0.001), indicating that number of brood increase non-linearly with adult group size. The rate of increase in total brood number starts to decrease beyond group sizes of three or more (see Chapter 3). Among other potential explanations, smaller nests may be less able to match brood number to the mean amount of food returned per-day to a central nest, according to the principles of the Central Limit Theorem (hereafter the CLT). Smaller nests will hypothetically receive more variable provisioning when there are fewer (independent) foragers, as they are taking fewer stochastic samples from the environment in total. Solitary or small groups may over-produce eggs optimistically, with the small larvae acting as a store for food when there is a glut, with their number reduced downwards in times of dearth (Alexander 1974).

Regardless of whether over-producing brood on small nests is due to the CLT (rather than, for example, a strategy typical of this stage of nest development to bet-hedge against loss), in some species at least this is hypothesized to be energetically inefficient (Gillespie 1977; Shakarad & Gadagkar 1995). Not only do excess brood need to be produced and provisioned, but the loss of a brood before adulthood represents a loss of most if not all the prior investment (depending on whether or not aborted brood can be fed to other offspring (Karsai & Wenzel 1998). Adult workers are likely to pay a survival cost to forage for the extra brood, even when this they serve as a 'nutrient storage vessel' as in the ant *Camponotus floridanus* (Nonacs 1991). In this species, workers adjust brood size downwards in response factors such as the presence of competitors, even when food remains abundant, in order to reduce the amount of foraging. The presence of competitors increases per-unit effort mortality rate of foragers, and smaller brood are likely to require adults to take fewer risks (Nonacs and Clabi unpublished data in Nonacs 1991). Larger groups of *L. flavolineata* may similarly be economizing on foraging effort with fewer brood per-capita. Furthermore, the finding of 'lazy' Harvester ants within colonies has been interpreted as an indicator of group health and robustness (Gordon 1999; Gordon 2010; Charbonneau & Dornhaus 2015).

If a species provisions progressively, then in theory, a larger number of brood per-capita to provide for may result in more effort expended in foraging per-capita, even if the brood

number is subsequently reduced downwards. Honeybees, when forced to adopt higher workloads, have a decreased lifespan such that there is a trade-off between brood biomass and adult-longevity (Schmid-Hempel & Wolf 1988; Wolf & Schmid-Hempel 1989), in part due to increased exposure to predation. If brood number is optimized, over time needing to forage less on 'good' days may give a selective advantage to individuals in larger groups (and the group as a whole), especially for workers who work the most according to rank and/or age. Reduction of foraging work below the full capacity of the group is observed in various social insects such as sweat bees (Field 1996; Field et al. 2012) and harvester ants (Gordon et al. 2013), with potential advantages (Gordon 2013). Toyoizumi and Field (2014a) found that in a harsh environment, where foraging is the main cause of mortality, a reproductive individual achieves greater productivity by reducing foraging effort below full capacity, and that may be necessary for population persistence. Population persistence itself is an important concept in Allee effects, whereby individuals in larger groups experience an increase in fitness (Pacala et al. 1996; Stephens & Sutherland 1999; Courchamp et al. 1999). Toyoizumi and Field (2014b) also demonstrate mathematically that the extended nest lifetime due to division of labour between queen and helpers has a substantial increase in nest productivity. Nest longevity is important, especially in a species with brood that take a long time to develop, and should give a selective advantage.

Even if smaller nests do not have higher nutritional requirements than larger nests, (due to their greater number of brood per-capita), small groups with few foragers may compensate for the hypothesised increased variance in food returns by working harder. The CLT as a principle is influenced only by the number of samples from the environment, therefore an increased frequency of foraging trips or increased foraging time could also reduce the variance in food returns, even if forager number remains the same. Subordinates may either undertake more frequent foraging trips or extend foraging trips until they have been successful, compared to individuals at the same rank on larger nests (see Chapter 3). Increased foraging effort on smaller nests may be the best indicator that foragers experience a higher variance in food returns, and need to work harder to meet the needs of the nest. Especially since visualizing food returns and quantifying the calorific returns from those of varying composition is difficult in *L. flavolineata* and other species.

Linear growth and increasing costs of social complexity

Optimization of overall foraging effort with larger group sizes (a non-linear decrease in effort with increasing group size, as a byproduct of brood number optimization, limitation or some other parameter) is hypothesized in this case, however, there remains two other possible distributions (Jaffé 2010). Firstly, if each additional group member can successfully rear the same extra number of brood, then there may be a linear relationship between group size and foraging effort. This might be predicted in species where there is no upper limit to brood number or group size, or in smaller groups where the limit of either has not been reached.

Secondly, it is possible for the energetic needs of a nest to increase non-linearly with group size. This may be expected in complex social groups, where the social behaviour may require a greater input of energy to maintain, for example via expensive communication, aggression and regulation. A positive association between greater social complexity and larger group sizes is well documented in a number of social systems, particularly in kin-based systems such as those in the social hymenoptera (Bourke 1999; Anderson & McShea 2001; Cassill et al. 2002). Hypothetically, the more complex a system (and the larger the group size), the more energy required to maintain it away from equilibrium (Corning & Kline 1998; Jaffé & Fonck 1994, Jaffé 2010). Various studies have attempted to test this in terms of energy expenditure/ requirements of species with varying social complexity, with mixed results. In *L. flavolineata* increased energetic expenditure or requirements due to social complexity may or may not be reflected in foraging effort; adult foraging more likely reflects the needs of the brood rather than the adults since foraging females may feed themselves outside the nest.

Behavioural concessions

Previous studies of reproductive skew (a measure of how reproduction is partitioned among group members) in this species have found high skew in groups of varying size and relatedness. This does not support so called ‘concessions’ models; where the dominant controls reproduction at low cost, and concedes a reproductive share to subordinates in return for helping (Clutton-Brock 1998; Johnstone 2000; Zanette & Field 2009). However, it is possible that although not reflected in reproductive rights being shared simultaneously with the dom-

inant, being ‘allowed’ to decrease foraging effort with increasing seniority in the inheritance queue may be considered a substantial (albeit behavioural) concession (Tibbetts & Reeve 2000). This may be particularly applicable to species where foraging duties are hazardous and associated with higher mortality.

Behavioural concessions may be tested by looking for the same patterns as those predicted for reproductive concessions (see Chapter 4); only in this case the concession involved is a resource such as food or ‘time on the nest’ which is a proxy for protection and food. Of the concession model’s predictions (see Chapter 4 for further details), two are of particular interest in this case: ii) that the subordinate’s share will be smaller (i.e. resource skew will be higher) when relatedness is high, because the subordinate receives compensation in the form of greater indirect benefits from helping to rear the dominant’s offspring, and ii) when the subordinate females are highly beneficial, skew may be lower as a reflection of the subordinate’s value to the dominant (Nonacs 2006). However, there are also other models of reproductive skew that may be more confidently applied to resource skew, such as "pay-to-stay". This has the opposite prediction that effort might be higher when relatedness is lower, as unrelated joiners may pay a premium to be accepted into a group, compared to related females that are born into one (Kokko 2002; Hamilton & Taborsky 2005; Zöttl et al. 2013; Fischer et al. 2014).

Previous kin-selection models require further testing, and experiments are required using animals of known inheritance rank in order to control for potential confounding variables. Here, I test whether larger groups expend less effort overall than smaller groups, and how group size affects foraging effort within experimentally determined inheritance ranks. I also synthesize new genetic data to examine how an individual’s relatedness to the dominant explains variation in foraging effort within ranks, and how this varies with group size. I then apply these findings in conjunction with data on the typical per-capita brood number and variance in food returns of nests of different sizes to the current interpretation of individuals moderating effort in response to nest value in *L. flavolineata*.

6.2.1 Study species

Liostenogaster flavolineata is a facultatively eusocial hover wasp (Vespidae, Stenogastrinae), (Turillazzi 1991). *L. flavolineata* occur in small groups (1-10 females) across a wide distribution that includes Peninsular Malaysia (Field 2008). All females are capable of mating, laying eggs and independent nest founding. Helpers have a chance of eventually inheriting the dominant, egg-laying position on their nests: an age-based dominance hierarchy determines the sequence of successive behavioural dominants. Seniority can be measured in terms of time on the nest, with the most subordinate wasps undertaking the majority of the foraging (Samuel 1987; Field et al. 1999). Aggressive acts occur between individuals of close rank (Cronin & Field 2007). Since there are no morphological differences between egg-layers and helpers, females may choose to become helpers on their natal nests, or they may leave and found a nest alone, join another nest or fight to take over an existing one. On established nests, adult females are normally relatives, and reproductive skew is uniformly high (Sumner et al. 2002).

L. flavolineata brood are provisioned progressively, and each adult female undertakes foraging independently, with more dominant individuals spending less time foraging than subordinates (Field et al. 2006; Bridge & Field 2007). *L. flavolineata* prey primarily on the alates (winged reproductives) of ants, and field observations of food returns across nests in an aggregation imply high spatial and temporal variability, for example when the release of sexuals from ant nests are restricted to a certain location (L. Holt pers. obs.). CLT effects are predicted to be particularly large in species with spatially clumped prey (Naug & Wenzel 2006). Foraging risk may be particularly high in *L. flavolineata*, and workers risk death and reduce their average lifetime fecundity through foraging (Field et al. 1999).

6.3 Methods

The foraging and inheritance rank data was collected between 19th August and 2nd October 2013 at Site 1 and 2. The study sites comprised the undersides of one bridge and one culvert along a little-used road near to Fraser's Hill, 116 km North of Kuala Lumpar, peninsular Malaysia. This road, which runs between The Gap and Raub, is the same road at which previous studies have been based (e.g. Field et al. 2006). The study of brood numbers and composition was conducted in 2011 (11th June), at Site 3, with the addition of unpublished

data on brood composition and food returns for different sized nests collected by Dr Catherine Bridge (henceforth Bridge unpubl.) between 3rd May 2001 and 30th August 2001. Brood data is referred to in the discussion but included in the appendix, (see Chapter 3 for methods).

6.3.1 Determining rank and foraging effort

Experimentally, each individual's position in the hierarchy can be determined with successive removals of dominants: the individual that spends the least time off the nest in comparison to her nest-mates (following the protocols of Samuel 1987; Field et al 1998, 1999; Field & Foster 1999). Details of field site, marking and censusing protocols are described in General Methods (Field et al. 1998). Nests with at least three adult females and cells deep enough to contain pupae were selected and numbered. Adult female number was counted at night. Adults were collected at dawn and this was repeated on successive days until all residents were marked. Before the first removal, the nests were observed (from 07:00 to 12:00) using rapid censusing for a minimum of three and a maximum of nine days (where there was ambiguity) to identify the dominant individual. The designated dominant was then removed and the wasps given two days to re-establish their hierarchy. Nests were thenceforth rapid censused for a minimum of three days (07:00 to 12:00) between each removal to determine the next dominant individual, which was then removed. This continued until the queue was fully ascertained. For each day of rapid censusing, a female had to have three more recorded observations on the nest than the individual below her, in order to be ascribed higher rank. See Chapter 5 for detailed methods.

6.3.2 Standardizing foraging effort

For the foraging effort analyzed in this chapter, the same three days at Site 2 and the same four days at Site 1 were used to standardize the results. Data is presented as either a proportion of time off the nest, or average time off the nest in minutes. Total wasp minutes or proportion of foraging effort only represents the main foraging period of the nests (07:00 to 12:00) identified from observations in 2011 (see Chapter 3). The initial size of groups, i.e. the group size when the foraging data was collected is used in subsequent analyses.

6.3.3 Adult removal

All residents were collected before dawn in zip-lock bags as described for wasp marking (General Methods). When one adult was required for removal, all other conspecifics were released from the bag, and the focal wasp retained. Wasps were stored in a cold box and euthanized at -10°C post-collection. Wasps were then stored in 100% ethanol until DNA analysis, with identifying labels. Unmarked wasps were collected and marked during the experiment unless they were obviously trying to usurp the nest, in which case they were captured and permanently removed.

6.3.4 Molecular genetic methods

A genomic library was constructed at Sheffield Molecular and Genomic Facility (SMGF) using the DNA from a single female taken from the field in 2013. Adults were genotyped at 17 polymorphic microsatellite loci. DNA extraction, PCR and genotyping were performed as described in General Methods and Appendix A.2.1 to A.2.4.

6.3.5 Relatedness values

Relatedness 5.07 (Goodnight & Queller 1998) was used to estimate the mean regression relatedness between various groups of different size. Colonies were weighted equally and standard errors were obtained by jack-knifing over nests. Pairwise relatedness estimates between adult individuals were determined using KINSHIP version 1.3.1 (Goodnight et al. 2005).

6.3.6 Data analysis

All statistical analyses not requiring the specialist programmes mentioned above were performed in R (R Development Core team, 2013). First I wanted to test whether average foraging effort increased with group size, and whether the relationship was linear. To test this I first ran a generalised linear model (GLM) with normal errors, with 'average foraging effort' as the response variable and 'group size' as the single explanatory variable. To test whether the relationship was linear, I ran the model again but included a quadratic term for 'group size', and compared the models with and without the quadratic term using ANOVA. Previous research has shown that nest-wide relatedness to the dominant increases

with group size and that these two parameters are significantly correlated (see Chapter 5). Therefore, for statistical integrity these two variables cannot be included in the same model since doing so will cause colinearity. I therefore separately analyze the effect of relatedness to the dominant and the effect of group size on the proportion of foraging effort within ranks. For both tests I use a GLM with binomial errors, with 'proportion effort' as the response variable in both cases, and 'group size' and 'relatedness' as explanatory variables respectively. In both cases, 'rank' and 'site' were included as covariates. In maximal models, I initially included interaction terms between 'site', and 'group size' and 'rank'. There was a significant interaction between 'group size' and 'rank' (see Results), and therefore I used the 'predict' function in R to explore this relationship.

Using data from Catherine Bridge's 2001 data collection, I tested whether there was a difference in the variance of food inputs to nests of different sizes. I follow Stevens et al. (2007) in visualizing normality of food returns using Q-Q plots, and test the distributions using Shapiro-Wilk normality tests. To correct for low sample size of larger nests, groups of six or more adult females were combined. Solid and all liquid food returns were combined, as it impossible to distinguish sugar-containing liquid returns from water returns to the nest. In all models, starting with the interaction terms, non-significant terms were then removed following step-wise model reduction until only significant terms remained. Values reported in the results are Chi-squared and associated p-values for when terms were sequentially removed from the model.

6.4 Results

6.4.1 Does total foraging effort increase linearly with group size?

Average total foraging time of groups (mins) increased with increasing adult group size ($F_{1,42}$= 98.576, p= <0.001). The model fit was significantly improved by the inclusion of a quadratic term (Figure 6.1), indicating that total foraging time of increased non-linearly with group size ($F_{1,41}$= 4.5488, p= 0.03913). Details of sample sizes are given in Table 6.1a and 6.1b.

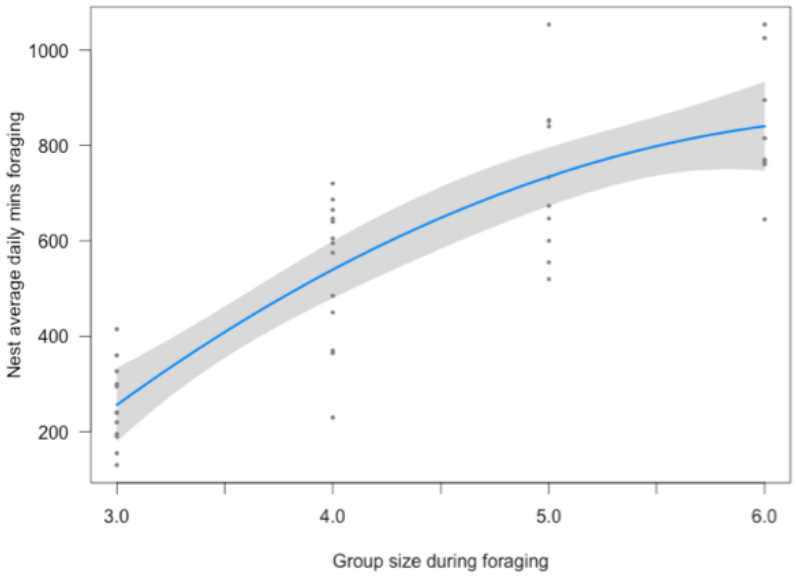

FIGURE 6.1. Graph showing quadratic relationship between group size and effort. 95% confidence intervals shown.

(a)

Year	Data on Adults or Brood	Used in This Analysis	No. of Nests
2001	Adults and Brood	Number of Brood and Food Returns	27
2013	Adults	Number of Brood and Foraging Effort	46

(b)

Group size	Nests	Individuals genotyped
3	13	39
4	14	56
5	10	50
6	8	48
7	2	14

TABLE 6.1. (a) Table of data used in analysis. (b) Table of group sizes and frequency.

6.4.2 Foraging effort in *L. flavolineata* groups

In *L. flavolineata* effort is strongly determined by rank (X^2=-1381.9, p<0.001), with the dominant (Rank 1) individual rarely leaving the nest. Figure 6.2 illustrates how effort changes with rank, showing standardized effort (average proportion effort over the census days) of 46 nests (N=207 wasps) across two sites.

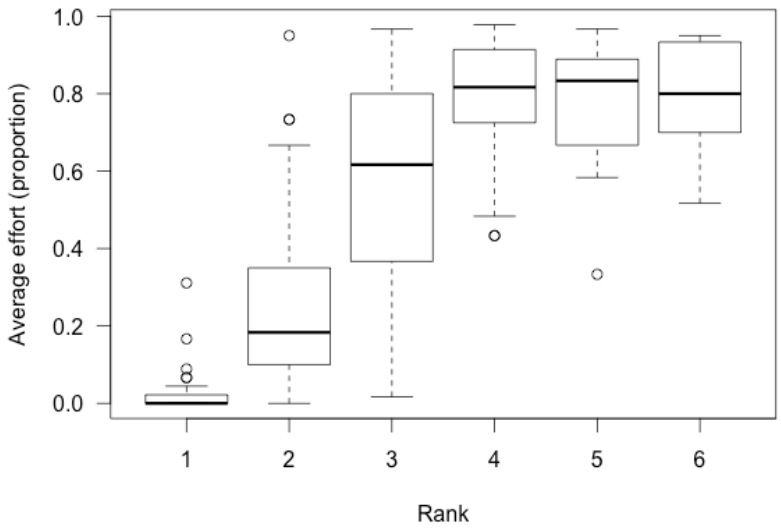

FIGURE 6.2. Graph showing relationship between foraging effort and inheritance rank.

6.4.3 Factors affect foraging effort within ranks

Group size and foraging effort within ranks

When group size was used in the model but not relatedness: the effects of rank and group size on the proportion of foraging effort could not be separated in this model due to a significant interaction between the two (X^2=82.283, p= <0.001) (see Fig. 6.4). Group size was significant (X^2=-231.43, p<0.001) as was rank (X^2=-1381.9, p<0.001). There was no interaction between the effect of site and group size, on the proportion of time spent off the nest (effort) (X^2= 0.798, p= 0.372). There was no interaction between the effect of rank and site, on the proportion of effort (X^2= 1.103, p= 0.294). Site itself (n=2) did have a significant effect on the proportion of foraging effort (X^2=75.106, p=<0.001).

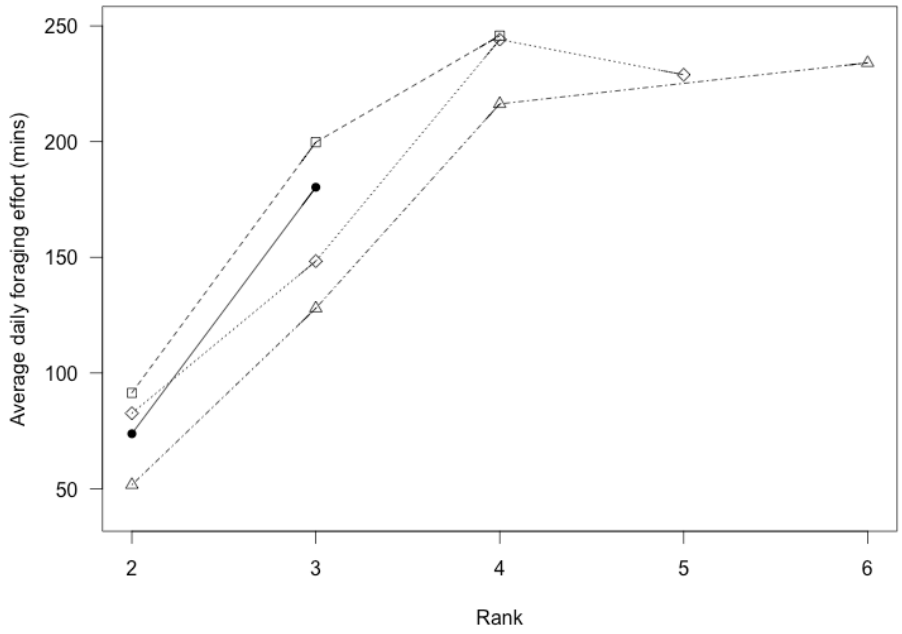

FIGURE 6.3. original data of the mean foraging effort per rank, for groups of different size. Triangles=group size six, diamonds= group size five, squares= group size four, filled circles= group size three (n=207)

Rank	Group Size	Average Effort	SE
2	3	73.750	±15.339
2	4	91.410	±14.053
2	5	82.593	±35.116
2	6	51.667	±13.617
3	3	180.278	±21.975
3	4	199.744	±20.147
3	5	148.333	±30.562
3	6	127.970	±28.653
4	4	245.769	±15.496
4	5	244.074	±4.340
4	6	216.296	±18.79
5	5	228.889	±19.446
6	6	234.020	±10.322

TABLE 6.2. Standard errors for each rank at each group size.

The interaction between group size and rank

The significant effects of rank and group size on the proportion of foraging effort could not be separated in this model due to a significant interaction between the two (X^2=82.283,

p<0.001). This is modeled in Figure 6.4 as a visual representation of how foraging effort changes with rank, according to group size. The separate lines are different sized groups. For both the analysis of relatedness and group size, effort within ranks responds differently at different sites. This is likely to be a spurious interaction due to group size, food availability or weather conditions being different between sites, which is not at all unusual for this type of study. Site 2 contributed only 11 nests to the dataset, compared to 36 nests from Site 1, and therefore this subset may contain greater variation in results (the CLT in action!). For this reason the effect of site is removed from the predicted model. According to Figure 6.4, workers in the smallest group (three adult females) forage the most. This is the only inconsistency with the raw data (Figure 6.3), where workers in groups of three forage less hard than those in a group of four.

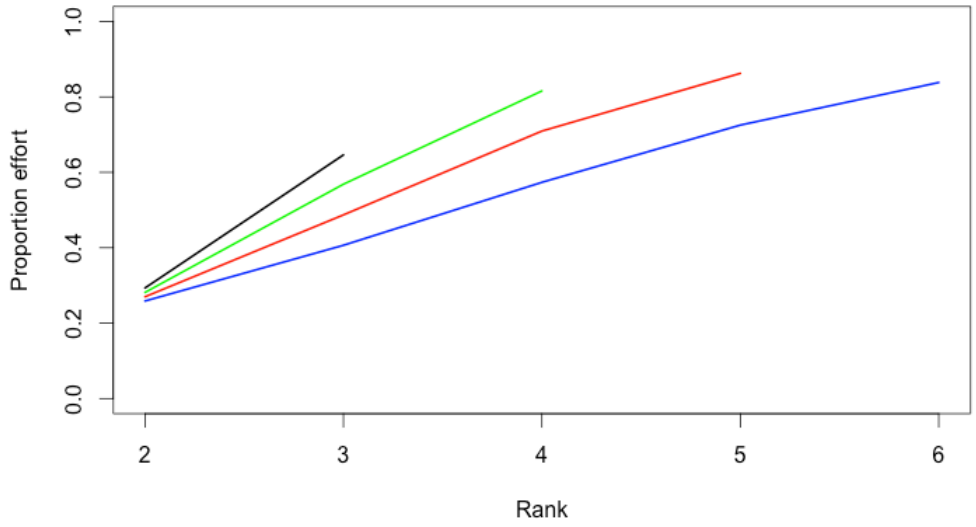

FIGURE 6.4. Distribution of proportion effort predicted by minimum model, which combines the data from the two sites, and separated by group size. Blue= group size 6, red= 5, green= 4, black= 3.

Relatedness and foraging effort within ranks

When relatedness to the dominant was used instead of group size (r= 0.40± 0.067), Relatedness to the dominant did have a significant effect on the proportion of foraging effort (X^2=33.590, p<0.001). There was no interaction between the effect of rank and the related-

ness to the dominant on the proportion of time spent off the nest (effort) (X^2= 0.3802, p= 0.537). There was no interaction between the effect of rank and site, on the proportion of effort (X^2= 1.103, p= 0.294). The effects of rank and site on the proportion of foraging effort could not be separated in this model due to a significant interaction between them (X^2= 8.018, p= 0.005).

6.4.4 Does variation in foraging returns in *L. flavolineata* meet assumptions of the CLT?

Results from Shapiro-Wilk normality analysis (Table 6.3) (Bridge Unpubl.) demonstrate that there is no significant change in normality with group size. However, it should be noted that of the two p-values greater than 0.001, both are in the category of 4+ females. Food returns are to group sizes one to 10 adult female(s), 27 nests, 12 separate days. Solid and liquid food returns were combined. Visual assessment of the normal quantile Q-Q plots (Figure 6.5) show that for a single foundress (group size 1), residuals are at maximum deviation from normality.

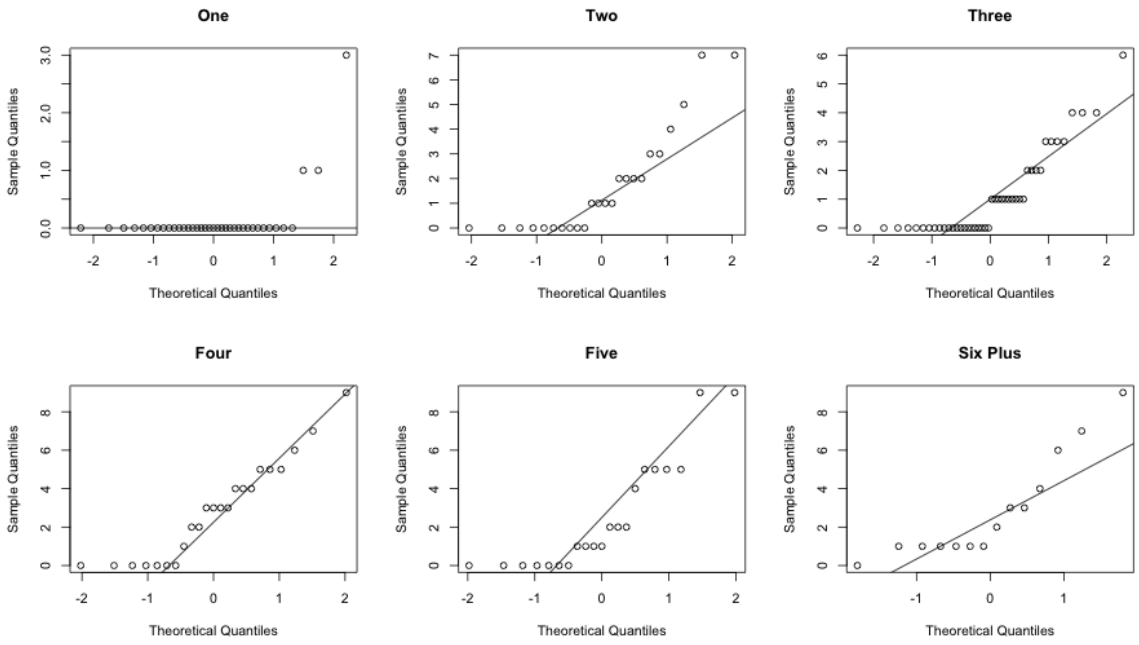

FIGURE 6.5. Normal quantile (Q-Q) plots of total food returns of differing sized groups. Groups of six or more females were combined together.

Group size	Sample size	Shapiro-Wilk statistic	Significance
1	37	0.279	<0.001
2	24	0.7845	<0.001
3	44	0.7845	<0.001
4	23	0.9087	0.038
5	21	0.8079	<0.001
6+	14	0.8283	0.011

TABLE 6.3. Table of Shapiro-Wilk results

6.5 Discussion

6.5.1 Does total foraging effort increase linearly with group size?

Average total foraging time of groups (in minutes) increased significantly with increasing adult group size. However, there was a significant improvement in fit with the inclusion of a quadratic term, indicating that total foraging time increased non-linearly with group size. This could be interpreted as either smaller nests working harder than larger nests (due to increased foraging trips or increased foraging time), or larger nests working less hard than smaller nests.

6.5.2 Factors which affect foraging effort within ranks

Since group size has been found to be correlated with relatedness values in *L. flavolineata* (Chapter 5), using both parameters in the same analysis may obscure the importance of the other. It may be that both relatedness and group size are significant when included in their respective models. Either one of these variables is important and is confounding the other, or both are important in some way. I have found that when considering group size in a model whilst excluding relatedness there is a significant effect of rank on the proportion of effort, but this interacts with group size. By producing predict plots of foraging effort of ranks when separated by group size the interaction between the two can be visualized. Foraging effort increases with rank (as shown by Figure 6.2), but larger groups forage less than smaller nests (Figure 6.3). This supports the findings of Field et al. (2006), and the predictions of the Kin-Selection model of Cant and Field (2001).

6.5.3 Does variation in foraging returns in *L. flavolineata* meet assumptions of the CLT?

Whether variance of food acquisition is higher on smaller nests of *L. flavolineata* in the field is an interesting question, since this was found to be the case in *Ropalidia marginata* and suggested by modeling (Naug & Wenzel 2006). Q-Q plots of multiple female groups appear more normal than the plot for a single foundress. This is not surprising since lone foundresses are required to protect the nest as well as forage, and may prioritize guarding over foraging. Only when at least one subordinate female is free to forage is it possible for the variance of food inputs to be decoupled from essential nest defence. The results presented here are pseudo-replicated, as some nests contribute multiple data-points to the analysis (nests = 27). However, the dates on which the food returns were recorded on the same nests were up to a month apart and in many cases the adult group size had changed, as well as brood composition. In this case it may be appropriate to consider these data points as independent samples.

Food returns are sometimes difficult to identify, especially in smaller groups where the distinctive trophallaxis movements of adults exchanging food may not be observed if the nest is empty when a forager returns. These data and previous attempts to quantify the exact calorific content of food returns to wasp nests of varying sizes have been hindered by difficulties visualizing the type of food substance. Even when the number of foraging returns is recorded, this gives little indication of their relative worth since solid food balls vary in size, and liquid food returns are indistinguishable from water (L. Holt pers. obs). Assuming that this data is reliable, I suggest that a possible reason why there is no significant change in normality of food returns between group sizes two to six-plus may be workers foraging more in smaller groups.

6.5.4 What are the potential reasons for a non-linear relationship between total foraging effort and group size?

Total foraging effort per-nest increases with group size but deviates significantly from linearity, with larger nests foraging less in total than expected from a linear relationship. This requires explanation, as with the addition of each low-ranking wasp the group gains an

increasingly hard-working individual according to rank (see Figure 6.2).

Kin-selection theory, group-augmentation and total foraging effort of nests: side effects of 'selfish' actions.

Previous interpretations of group-size dependent decrease in foraging effort of subordinates have focused on the trade-off between a worker's current indirect and future direct fitness benefits (Cant and Field 2001). Therefore, it follows that as a result of subordinates on larger nests acting 'selfishly' in order to preserve their fecundity and the chances of inheriting a comparatively more valuable nest, this should result in total reduced foraging time at the group level, because the potential foraging ability of all group members is not fully utilized (Toyoizumi and Field 2014a).

Field et al. (2006) manipulated group size and found an increase in effort of focal Rank 2 subordinates when all other workers were removed. What is less obvious is why Rank 2 wasps on small, un-manipulated nests, with typically lower relatedness to the dominant and brood (see Chapter 5), should already be working as hard as a wasp who is (now) putting all her effort into raising relatives. Among other reasons, the potential group augmentation benefits of raising a female larva to adulthood will be higher in smaller groups, irrespective of relatedness, as a new helper will be more valuable (Kokko et al. 2001). Group augmentation alone may be enough to cause high foraging effort in small groups irrespective of relatedness, explaining the increased effort of wasps on manipulated nests in Field et al.'s (2006) study. The potential benefits to group augmentation in smaller groups may have been under-appreciated in previous studies.

However, this is only one interpretation of the results of Field et al. (2006), and the empirical results from this chapter. Next, I synthesize the findings presented here into alternative explanations for decreased effort of workers in larger groups, or increased effort of workers in smaller groups. I present arguments for both views, although they are not mutually exclusive and these findings could result from a combination of hypothesises.

System-level effects

Field et al. (2006) interpret decrease in foraging effort in larger groups to individual decision-making, and I suggest that total group reduction in foraging effort is a by-product of these decisions. However, I also posit that the decrease in foraging effort in larger groups effect may be a by-product of group-level or system-wide phenomena. It is possible that larger nests work less in total due to an optimization of brood number (see Chapter 5 for the relationship between brood number and group size), or greater synergy in some other parameter. Conversely, individuals on smaller nests might work harder because of a higher number of immature brood per-capita, or each forager on smaller nests works comparatively harder to compensate for an increased variance in food returns (a predication of the CLT). Reducing the number of foragers whilst reducing the number of brood proportionally, as Field et al. (2006) did in their experiment, indirectly tests potential increased variance of food returns with decreasing group size. In fact, *it would be difficult to think of another experiment that so effectively tests a prediction of the CLT with foraging effort as the response variable*.

L. flavolineata produces relatively small eggs compared with its body size, therefore loss or removal of eggs and small brood is hypothesised to be a lower cost strategy than to failing to adequately exploit food resources that cannot be stored. Under-exploiting resources may be especially disadvantageous for *L. flavolineata* since its protein food supply is also highly variable. However, eggs in aculeates are larger compared to body size than eggs in other insect species (Turillazzi 1991). In combination with the long development time of *L. flavolineata* larvae (approximately 100 days), this may make heavy over-production of brood and any loss of mature larvae disadvantageous (Field et al. 2000b). It seems reasonable to posit that higher rates of brood loss (associated with smaller nests) could be an energetically ineffective outcome for this species (even if the brood are recycled trophically), and therefore it might be expected that wasps would work hard to avoid this outcome, or to reduce the negative relationship between group size and brood loss (Chapter 3).

Data compiled from previous observations of food returns (Bridge unpubl.), have shown that there is no significant difference in variance of food returns (solid and liquid combined) between nests of group size two to six-plus. I show here that normality of food returns also

does not significantly increase with group size. These results are contrary to models of the CLT, and data from (Naug & Wenzel 2006) which support CLT predictions that group size should affect variance in food returns. Furthermore, a previous experiment that experimentally increased variance in food availability windows (Chapter 3), definitively failed to find an increase in brood abortion levels in response. One explanation for this result is that the wasps adjusted to the experimental regime and may be highly capable of adjusting their daily foraging effort to meet the fluctuating needs of the brood or changing food availability. If so, this may explain group-size dependent variation in foraging effort as wasps attempt to compensate for the comparatively harder task of meeting brood nutritional needs with fewer foragers, explaining the increased effort of Rank 2 wasps on manipulated nests in the experiment of Field et al. (2006), and also why there is no significant difference in variance of food returns detected between nests of varying size.

The two alternative distributions for total foraging effort and group size: either a neutral relationship or an increase in effort with increasing group size, (in order to fuel a more expensive social structure or to support a greater proportion of mature brood for example), are not supported. This concurs with some studies of Fonck & Jaffé (1996) that have found mixed results for this concept.

Behavioural concessions

The dominant individual in *L. flavolineata* groups is quite capable of chasing subordinates off the nest surface, or even initiating relatively small social prompts to encourage workers to leave the nest. When this occurs, subordinates (presumably) forage or retreat to the side of the nest comb where they neither receive food or shelter (L. Holt pers. obs.). If dominants control foraging, reproduction and membership of the groups, they may also be in position to tolerate or prevent reduced foraging work. Wasps that spend more time on the nest are more likely to receive food from returning foragers, including protein which is essential for future egg-production and for passing any nutritional reproductive threshold (Poethke & Liebig 2008; Chown & Nicolson 2004). In fact, unequal protein distribution among colony members may be one of the proximate mechanisms that underlie differential reproduction of individuals in a group (summarised in Hunt 2007). Overall, behavioural concessions

may be particularly plausible in *L. flavolineata*, as there is no reason to assume that the higher-ranking wasps could linger and feed on the nest if they were not being permitted by the dominant. Furthermore, extruded carbohydrate from larvae may be an important energy source.

In the largest nests, subordinates are more likely to be either the dominant's sisters or daughters (Chapter 5), than on smaller nests (see also Sumner 1999). In smaller nests, particularly those that are new, usurped, or consist mainly of joiners, it may only be important that the dominant has information about her likely relatedness to the subordinates (which will be low). As such, it may be in the dominant's interests to exploit the assistance of unrelated subordinates more than subordinates who may be her kin. These results show the opposite patterns to those predicted by concessions models (of reproductive skew); that skew of resources should be higher i) when participants are highly related, and ii) when the subordinate females are highly beneficial to the dominant (Nonacs 2006). It is beyond the scope of this study to test whether correlates such as aggression or time on the nest are consistent with transactional or tug-of-war models (although Tibbets and Reeve (2000) have examined resource sharing and aggression in *Polistes dominulus* and found support for the former). However, as a point of interest, the association between relatedness values, foraging effort and group size found across my experiments fits predictions of pay-to-stay models more closely than concessions models. As a response to, or in addition to aggression from the dominant, unrelated subordinates on small nests may be willing to work harder in this framework. 'Rent-payment' is predicted to occur when relatedness between subordinate and dominant is low, ecological constraints are at least moderately high, and retaining non-helping subordinates might harm the dominant's fitness (Kokko 2002; Fischer et al. 2014). If joiners represent a potential dominance threat to lone foundresses or those in small groups, this may discourage dominants from accepting helpers unless they pay-to-stay with foraging effort disproportionately higher than related subordinates.

6.6 Conclusion

Decreased total foraging effort in larger groups of *L. flavolineata* could be interpreted as either a by-product of 'selfish' behaviour of high-ranking individuals, larger groups

optimizing brood number, or smaller groups working harder to compensate for increased variation in foraging returns. However, these need not be mutually exclusive. I suggest that the previous interpretation as to why subordinates at high ranks work harder on smaller nests; to prioritize current inclusive fitness over poor future direct fitness may be incomplete, since smaller nests both tend to be less related and have more brood per-capita to maintain. I also suggest that pressure from the dominant on small nests or the higher price of acceptance in small groups may result in less related subordinates working harder; while in larger, more related groups, it might be in the dominant's interests to allow closely related nest-mates to work less hard.

CHAPTER 7

SUMMARY AND CONCLUDING COMMENTS

7.1 Summary of results

In this thesis *Liostenogaster flavolineata* was used to investigate: productivity benefits to group living, the structure of genetic relationships in a facultatively eusocial society, reproductive skew, and foraging effort across different sized groups.

Firstly, I explored the relationship between group size and productivity. *L. flavolineata* was used in a large-scale experiment testing implications of the Central Limit Theorem (CLT), in the functioning of nests. Here, I conducted the first experiment that attempted to directly manipulate the variance in food input to nests, in order to test assumptions of the CLT. The experiment did not demonstrate that increased variance in food income to treatment nests significantly increased the rate of brood abortion or decreased larval development rate. However, some assumptions of the CLT were found to hold, such as smaller groups producing more brood per-capita and higher rates of brood removal.

Secondly, I developed and optimized 17 new microsatellite markers in order to test relatedness between adult nest-mates and their brood.

With these, I explored the reproductive dynamics of *L. flavolineata* nests. Females of facultatively eusocial species are capable of laying eggs and therefore have the potential to

practice direct reproductive strategies in addition to assisting related or non-related females. Newly initiated nests consisted of unrelated conspecifics, in comparison to mature or established nests where relatives of the current or previous dominant constitute the hierarchy. Reproductive skew was measured for newly initiated nests in order to test concession models in a context where relatedness of adults was low. Contrary to the predictions of concession models, one female dominated as much reproduction as was found by Sumner et al. (2002) on established nests, with the proportion of brood laid only by the primary egg layer (skew), being very high in both cases, and statistically unaffected by relatedness or group size.

Then, in a large-scale study, I investigated changes in the proportion of each relationship to the dominant at each hierarchical rank, compared between 46 nests of different size. *L. flavolineata* females queue in an age-based hierarchy to inherit the breeding position, and the inheritance rank of each female was experimentally determined. Changes in the proportion of each relationship to the dominant at each inheritance rank were compared between different sized groups. The likelihood of a subordinate being a daughter or niece of the current dominant increased with inheritance rank (distance from the breeding position) whilst controlling for group size. The average relatedness to the dominant also increased significantly with group size, an important finding, with groups of three being typically unrelated.

Finally, I investigated the relationship between foraging effort and inheritance rank of workers according to group size and relatedness to the dominant. Since group size and relatedness to the dominant are strongly significantly correlated, these variables could not be analyzed in the same model to test their effects on foraging effort. When analyzed separately both have a significant effect on the amount of effort that workers invest in foraging at each inheritance rank. Group size interacts with rank, with subordinates in smaller (and less related) groups working harder than those at an equivalent rank in larger groups. This finding supports those of Field et al. (2006), but is also paradoxical in light of the new relatedness data, since subordinates that are more closely related to the dominant and her brood might be predicted to work harder. Larger groups also forage less than predicted by a linear relationship.

7.2 Concluding comments

This thesis has investigated how some of the costs and benefits of group living, for example foraging effort, productivity, and inclusive fitness benefits, may either increase or decrease according to group size. I investigated a potential universal mechanism for increased fitness in larger groups of progressively provisioning species: less variable food returns. Here, I briefly discuss the wider implications of my finding that larger groups are ultimately more productive per-capita despite less effort overall, and how this is applicable to other social systems. Any group that shares resources, profits, and benefits while its members face conflict over the costs of association (such as businesses, economies and even research groups!), will experience different levels of synergy, resource skew and productivity according to group size, although not necessarily in the same patterns as found in *L. flavolineata* (Cook et al. 2015).

For example, although no individual is required to know all of the details about its environment, by contributing stochastic samples that are aggregated at the group level, collectively a larger group could be more attuned to the average resource availability, similar to (but not exactly the same as) 'hive mind' or wisdom of the crowd (Beckers & Goss 1989; Giraldeau & Caraco 2000; Couzin 2009). With increasing forager number extreme variation in resource returns could also be reduced (Naug & Wenzel 2006). Increasing fitness in terms of productivity or adaptive function with increasing group size may also be due to synergy or optimization of parameters, and these effects of group size are likely to be applicable to a large range of phenomena; from organs with a greater number of cells that are able to become more diversified (Reader & Laland 2002; Herculano-Houzel 2009), to larger populations of animals being more diverse and robust (Courchamp et al. 1999; Stephens & Sutherland 1999; Gusset & Macdonald 2010).

Experiments presented in this thesis (Chapter 3) have provided evidence that smaller nests of *L. flavolineata* do contain more brood per-capita. This concurs with predictions of how the CLT is thought to affect social insect behaviour, as does these smaller group's higher rates of brood removal which results in lower realized productivity (in terms of pupae per-capita). Since the experiment to increase the variation in food inputs to a treatment

group of nests did not significantly increase brood removal rates compared to a controls, I cannot conclusively prove that higher rates of brood removal on smaller nests are a result of increased variance in food returns, as opposed to some other effect of group size. However, failure to find an effect of the treatment could be explained if group members adapted to experimental changes in food inputs by increasing foraging effort. Since other experiments presented here demonstrate that individuals in smaller groups do in fact work harder, I later discuss an alternative to Field et al.'s (2006) interpretation of increased effort in smaller groups: that this could instead be a strategy of smaller groups to compensate for higher variance in food returns, supporting predictions of the CLT.

Alternatively, if Field et al.'s (2006) interpretation of reduced foraging work is correct: that each wasp makes decisions on effort based on self-interest and future fitness returns, I suggest that the observation that total foraging effort of groups decreases with increasing group size could be analogous to another general concept: that of Adam Smith's 'invisible hand', since the problems of social organization are broadly problems of economics (Field & Cant 2009). It has been argued that the same concept exists in a number of other areas under different names, including Darwinian natural selection (Nozick 1974). Smith's original application of the invisible hand was in the context of businesses choosing to use local materials in production; although not deliberately done to be helpful, this inadvertently created subsequent benefits to the business and local area. However, it more generally refers any undirected or self-interested individual action that has unplanned or unintended positive consequences. Particularly those that arise from actions not directed by a central command, and have an observable, patterned effect on the community. In *L. flavolineata*, if each individual worker makes a decision based on its own future direct fitness returns depending on nest value (according to the Kin-Selection model), then there would be an overall decrease in effort of larger groups. There may also be other 'unintended' overall group-level benefits; for example increased group stability, individual and group longevity, and overall productivity, which might be positively selected for.

These two effects need not be mutually exclusive. Further work is required to determine whether a decrease in total foraging effort on larger nests is due to an optimization of brood number (or some other parameter), whether it is orchestrated by the dominant, or

is a consequence of individual decisions about foraging effort made by the subordinates at the front of the inheritance queue. Separating these effects experimentally is likely to be challenging, as with a decrease in forager number nest value also decreases.

That subordinates are willing to wait at all at the most junior ranks in very large groups has been understood as benefits such as high indirect fitness returns, increasing lifespan at the most senior ranks, and potentially improved group survival working synergistically to compensate subordinates for their decreased chance of direct reproduction (Field et al. 1999; Field et al. 2000). These benefits are further augmented by increased productivity (in terms of number of pupae per-capita) in larger groups of *L. flavolineata*, similar to studies by (Tibbetts & Reeve 2003) of *Polistes dominulus*. This pattern was not found in previous studies of *L. flavolineata* (Shreeves & Field 2002; Shreeves et al. 2003; Field & Cant 2006), but since the data complied form Bridge (Unpubl.) also demonstrated both increased pupae per-capita and fewer brood per-capita overall on larger nests, it is likely that these parameters can fluctuate.

This finding, (that sociality and increasing group size are advantageous in terms of group productivity), is likely to be linked to higher inclusive fitness returns for *L. flavolineata* in larger groups, since relatedness to the dominant (and average intra-nest relatedness) is strongly positively correlated with group size, a new finding. I discovered there were systematic associations between a subordinate's rank and relationship to the dominant (and hence any brood laid by that dominant), with a significant trend for daughters to be associated with lower positions in the queue. Stochastic effects, including death of foragers, predation, and the possible assimilation of unrelated joiners could be factors that disrupt this relatedness pattern in smaller nests, which were much less related. Less buffering from detrimental stochastic effects, resulting in lower group-wide relatedness in smaller groups, could be a common factor of social aggregations (Gusset & Macdonald 2010).

Wasps on larger nests are more productive, work less, are more closely related, and seem to suffer no detectable negative impact of larger group size on energetic requirements (as measured by foraging effort). Therefore, I suggest that smaller groups are sub-optimal compared to larger groups. In small groups, wasps with no other option may gamble on realizing the higher end of the range of potential fitness outcomes available to them. Con-

trary to theories that suggest that there may be reproductive concessions that compensate workers to make small and newly-initiated groups attractive to joiners, experiments in this thesis demonstrate high reproductive skew is maintained even in the presence of low relatedness at nest inception. In general, if smaller groups (of any type) tend to exist in a more chaotic and less efficient state, for example with lower relatedness, more infighting, more work, and superfluous/ wasted offspring (or product), this arrangement may be more prone to breakdown, instability, loss of group members, and leave fewer decedents over time. Conversely, larger groups may reap/ provide benefits for all members in terms of rewards such as reduced work effort. Future work on these topics might use social insects and *L. flavolineata* specifically to investigate other variables sensitive to changing synergy (such as foraging success) according to group size.

Since group size is strongly correlated with relatedness to the dominant, group size may act as a signal of high or low relatedness for recently emerged wasps, and contribute to decisions on whether to say or leave. Since the experiments of Field et al. (2006) have suggested that wasps look to the future in making decisions about effort and the relative values of nests (Griffin 2006), it would be reasonable to assume that group size, rank (or both) could be cognitively associated with average relatedness values, in this and other species (Riehl et al. 2015). It would be interesting if experiments could be conducted in the future to test this further in *L. flavolineata*.

Currently, the microsatellite markers I developed in 2014 have been optimized into 11 duplexes. There remains scope to use more then the two current fluorescent dyes and streamline the current set into a toolkit with fewer multiplexes. This would be a valuable resource for future studies of *L. flavolineata*, an excellent species to study many of the principles of social evolution.

APPENDIX

A.1 Appendix Chapter 1

A.2 Appendix Chapter 2

A.2.1 Protocol for tissue preparation: procedures for eggs, larvae, adults and library tissue

1. Eggs: Examine egg through a microscope to ensure no additional material is adhered to it, then use whole egg for DNA extraction.

2. Larvae: For L1 stage use whole larva. For L2-L3 stage cut small piece 1-2mm in length from the larva's body, ensuring not to include any gut contents or tissue from the head.

3. Adults: remove the two last tarsi of the hind leg (this is preferable to keep concentrations consistent across samples, but any of the legs can be used). Cut into multiple pieces.

4: Library tissue: Using one adult female, remove the head, wings and abdomen. Chop the sample as much as possible before extracting using the procedure below. DNA concentration must be at least 20mg/ml.

A.2.2 Protocol for DNA extraction using ammonium acetate precipitation method

1. Place in a 1.5ml flip-top tube 250 μl Digsol buffer and 10 μl Proteinase K (10mg/ml).

2. Chop the tissue into small pieces on a sterile glass plate (sterilized with bleach) before adding to tube.

Vortex to mix and wrap the rack in tissue and elastic band and place in rotating oven at 55°C (3hrs or overnight) or 37°C centigrade (overnight).

3. Once digested add 300μl 4M ammonium acetate to each sample.

4. Vortex several times over a period of at least 15 minutes at room temperature to precipitate the proteins.

5. Centrifuge for 10 minutes at 13,000rpm.

6. Aspirate supernatant (clear liquid containing the DNA) into clean, labelled 1.5ml flip- top tubes (discarding the gunky protein stuff which usually pellets on the bottom although could be floating on the top).

7. Add 1ml 100% ethanol.

8. Invert tubes gently several times to precipitate DNA.

9. Centrifuge for 10 minutes at 13,000rpm.

10. Pour off ethanol taking care not to lose DNA pellet.

11. Add 500μl 70% ethanol and invert several times to rinse pellet.

12. Centrifuge for 5 minutes at 15,000rpm in case the pellet has dislodged from the bottom of the tube.

13. Pour off ethanol in a smooth movement (or using a 200μl pipette gently draw off the supernatant if there is a fear of losing the pellet). Stand tubes upside-down on clean tissue (approx. 30-60 minutes). Use heat lamp if required.

14. Once fully dry add 50μl Low TE.

15. Flick sample to dislodge pellet.

16. Place tubes in water-bath for 30 minutes (37 or 65 °C) to dissolve pellet (flicking every 10 minutes).

17. Store at -20 °C (long term) or 4°C (short term).

Solutions:

Digsol:	Low TE ($Tris_{10mM}$ $EDTA_{0.1mM}$):
To make 500ml	To make 500ml
20ml 0.5M EDTA (pH 8.0) 3.425g NaCl 25ml 1M Tris-HCl (pH 8.0) 430ml ddH_2O	5ml 1M Tris-HCl (pH 8.0) 100µl 0.5M EDTA (pH8.0) 495ml ddH_2O
Autoclave then add 25ml of 20% SDS.	Autoclave

TABLE A.1. Table of solutions.

A.2.3 Microsatellite amplification: PCR of individual or multiple loci using Quiagen PCR master mix.

1. Place extracted DNA into each well of a PCR plate and allow to dry.

2. Prepare a primer mix, in which all primers (both reverse and fluorescently labeled forward primers) for all loci to be analyzed together are present at the appropriate concentration)

3. Prepare a mastermix as follows:

Ingredient: Volume per sample: Quiagen PCR Mastermix 1μl

Primer mix 1μl (where all primers are at 0.2 μM)

4: Add 2μl of the mastermix of each well containing DNA.

5. Add a drop of mineral oil to each well.

6. Transfer plate to thermocycler and run the following programme:

PCR programme:

95°C 15 minutes

30 cycles of

94°C 30 seconds

57°C 1 minute 30 seconds

72°C 1 minute 30 seconds

followed by 2min extension at 60°C

Hold at 4°C

A.2.4 Genotyping: ethanol/EDTA/sodium acetate precipitation 96 well plate

1. After performing sequence reactions in a 96 well skirt plate do the following:

2. Prepare a mix of Hi-Di formamide and size standard as follows:

Ingredient: Volume per sample:

Hi-Di formamide 9μl

ROX size standard 0.036 μl

3. Make a 1:25-1:200 dilution of the PCR product depending on the strength of the primer (for adult legs dilute by 1:500)

4. For each well of a genotyping plate, mix 1-1.5 μl of the diluted DNA and 8.5-9 μl of the formamide and size standard mix (for a final volume of 10 μl in each well).

5. Denature for 3 minutes at 95 °C, place on ice until ready to load on ABI3730 sequencer.

Locus	Sequence	DYE	Multiplex	Used for analysis?
HFW22989	F: GCACGACGAGGAGGAAAG	FAM	1	Y
	R: CGGAAAGGAGAAGACAGAGC			
HFW00614	F: GAGATGCTGGGTATAAAGCGATAG	HEX	1	Y
	R: GACATTACTGCGCGTCTCG			
HFW05385	F: GATCCGCAATATTTCATCGAG	FAM	2	Y
	R: TTCGTTCGACACCGACAG			
HFW05748	F ATGCGACTTTGCTCGTTTC	HEX	2	N
	R: CAGTCCCGAATGCGTAGAG			
HFW05396	F: ATTAATCACCGGTGCAATCG	FAM	3	Y
	R: GGCTTGAATTTGGGTTTGG			
HFW05172	F: ATTATCGAGTTCCGCTGGTC	HEX	3	Y
	R: ATTCGAAGTATTTCCGATTAGAGC			
HFW05394	F: AAACGCGAGCAAAGATACTTCT	FAM	4	Y
	R: GCCGAAATAATTGGCGATAA			
HFW05624	F: CAAAGAAAGCTCCGTCATCC	HEX	4	N
	R: CAACATCATCTAAGAGACGAATGG			
HFW05205	F: GTCTCGCGTAGTCGATGTAGG	FAM	5	N
	R: GTCTATTTATACGTCCAGAGCAAGG			
HFW05732	F: CACTAATATGCGCCATACGC	HEX	5	Y
	R: GGCGTTAACCGTCTCAGG			
HFW05357	F: TTCCAATCGTATTCCACTCTCC	FAM	6	Y
	R: GACCTTTCGTCGTTTCGATG			
HFW05595	F: GTTATTGTCGCCAGCATGAA	HEX	6	Y
	R: AATAATCGAAGTGCATAGGCTGA			
HFW42942	F: AAGTGTAAAGAGATTCCTCCTTCG	FAM	7	N
	R: TTGTCATTCCGCCTTTCAG			
HFW05502	F: CAAATTTGCCAGCCTATTGC	HEX	7	Y
	R: CGACACCGAGTCGTTTGAC			
HFW42477	F: CGTGGAGGTGGAGGATAGTG	FAM	8	Y
	R: GCGCTTCACCCGAATTATTAC			
HFW05743	F: CCACGAAGTTTCGGATAAGG	HEX	8	Y
	R: GCACTCTGCTCCAGAGACG			
HFW00443	F: CACCCGGTATAATGAAGAAACG	FAM	9	Y
	R: GGGCAGGAGGACAAGAGG			
HFW05236	F: GAATAACCGCGTGTACGTGTC	HEX	9	Y
	R: CGTTCAGACTGCCAGAACAA			
HFW05603	F: CATTGTGGTCGATTCTGTCG	FAM	10	Y
	R: ACGTGTAATTGAGGGATAACGTG			
HFW05173	F: CGTCAATGGGTTGACGTTC	HEX	10	Y
	R: CTTGATTCCGTTTAGCTCGTG			
LF25OLD	F: GCGACTACCGTGGAAATCAG	FAM	11	N
	R: ATTCGACAGCACGAAGAACAG			
HFW05485	F: CGAGTTTAAAGGGAGCCAGAG	HEX	11	Y
	R: GAATCGGAGCAACGTACCAC			

TABLE A.2. Table of markers

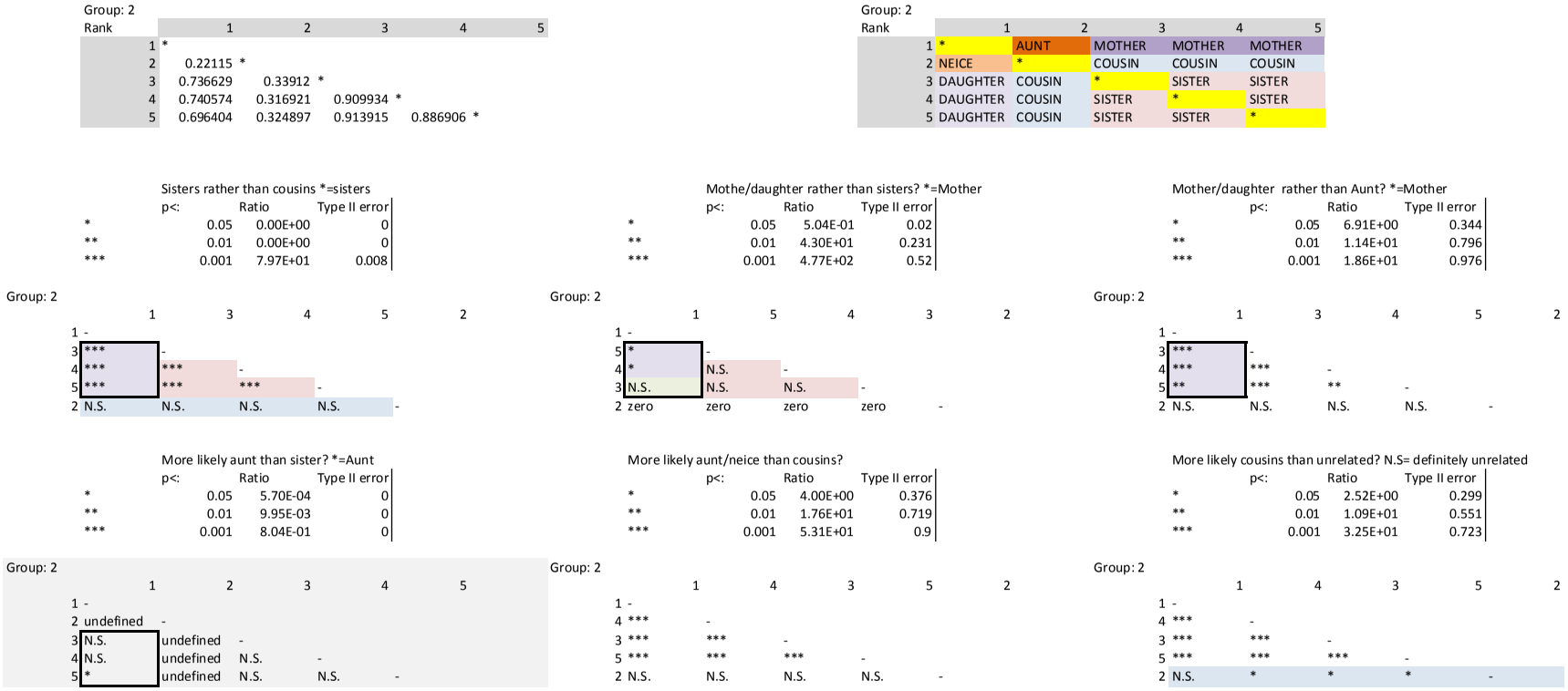

FIGURE A.1. Sequential read-outs from KINSHIP resulting an a matrix of relationships within Nest 2.

A.3 Appendix Chapter 3

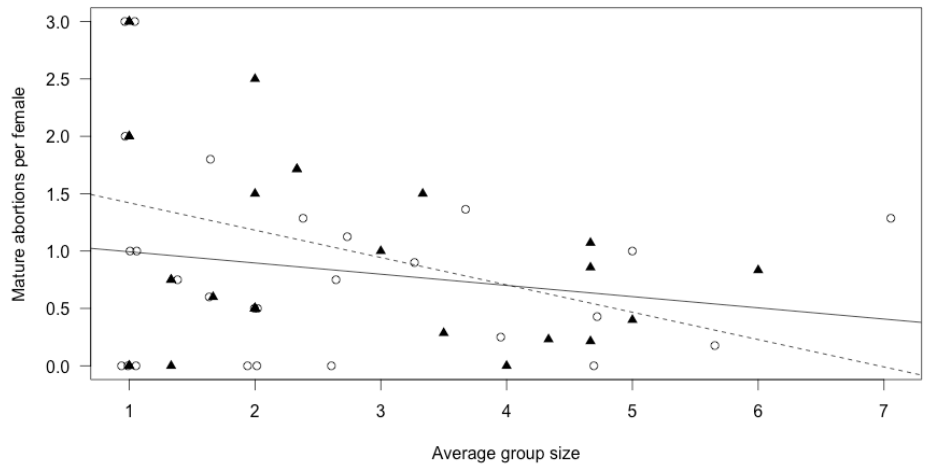

FIGURE A.2. Graph representing the number of abortions (of all larval stages) per-female in groups of varying size. Unfilled circles and solid line are controls, filled triangles and dashed line are treatments.

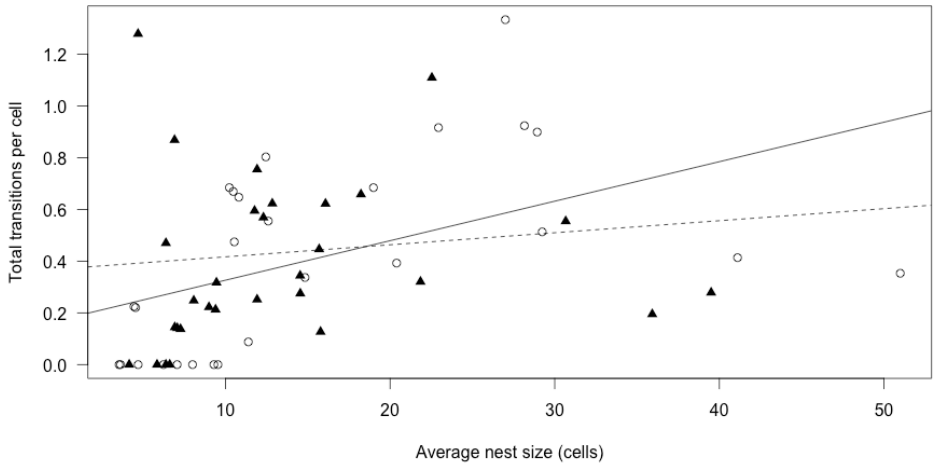

FIGURE A.3. Graph representing the number of larval transitions per-cell in groups of varying size. Unfilled circles and solid line are controls, filled triangles and dashed line are treatments.

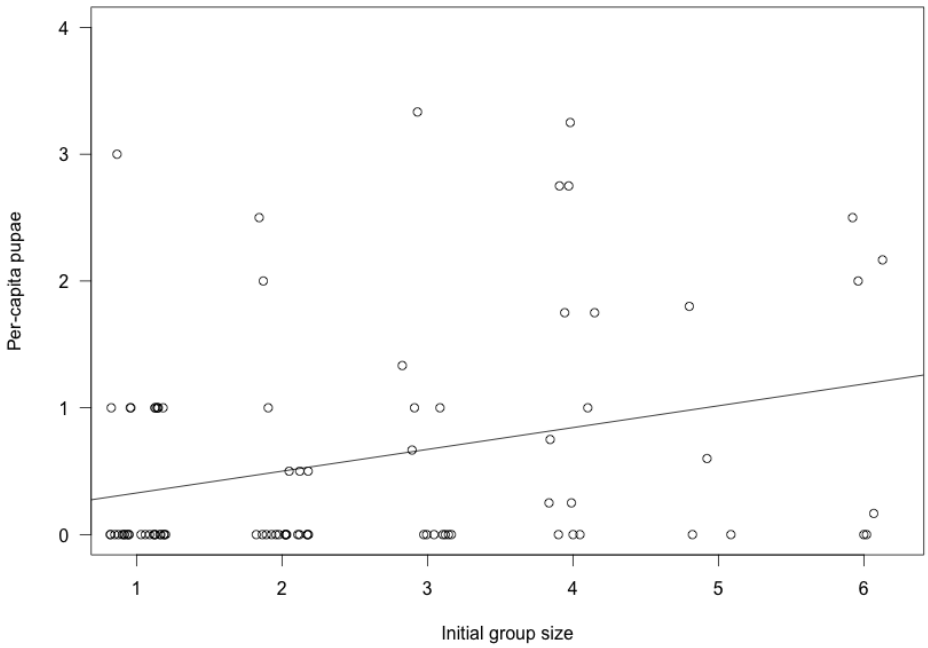

FIGURE A.4. Graph representing the number of pupae per-capita produced by groups of varying size. Both datasets combined.

A.4 Appendix Chapter 4

Nest	Max Adults	A Relatedness	B Relatedness	Brood No.	% Male	Sibgroups F	% Sibgroup F	Deviance F	P value F	Sibgroups MF	% Sibgroup MF	Deviance MF	P value MF
100	4	0.166	0.756	7	0	1	100	0	0	1	100	0	0.001
101	4	0.180	0.652	8	0	2	88	0.219	0.001	2	88	0.219	0.001
102	3	0.163	0.732	11	9	1	100	0	0	1	100	0	0
103	2	0.070	0.718	7	0	2	86	0.245	0.130	2	86	0.245	0.124
104	3	0.065	0.749	10	30	1	100	0	0.001	2	80	0.355	0.002
170	3	-0.095	0.320	5	20	2	75	0.367	0.26	2	80	0.320	0.132
171	7	0.169	0.756	5	0	1	100	0	0.001	1	100	0	0.001
401	5	0.174	0.687	6	0	1	100	0	0	1	100	0	0.001
402	5	0.129	0.640	7	43	1	100	0	0.002	1	100	0	0
405	1	NA	0.606	3	0	1	100	0	0.263	1	100	0	0.257
410	3	0.090	0.770	5	20	1	100	0	0.350	2	80	0.320	0.15
411	1	NA	0.773	3	0	1	100	0	0.073	1	100	0	0.066
412	2	0.068	0.828	7	14	1	100	0	0.042	2	86	0.245	0.149
430	5	-0.126	0.650	3	0	2	67	0.437	0.254	2	67	0.444	0.217
171B	6	0.129	0.424	6	33	2	75	0.368	0.073	2	83	0.278	0.002
405B	1	NA	0.736	2	0	1	100	0	0.476	1	100	0	0.497
411B	2	-0.165	0.869	3	0	1	100	0	0.255	1	100	0	0.274
430B	3	-0.076	0.722	5	0	1	100	0	0.010	1	100	0	0.01
NR62	2	NA	0.764	3	0	1	100	0	0.269	1	100	0	0.268
NR82	1	NA	0.737	6	33	1	100	0	0.032	1	100	0	0.001
NR82B	1	NA	0.580	3	0	1	100	0	0.226	1	100	0	0.247
NR85(B)	3	0.256	0.764	5	20	1	100	0	0.036	2	80	0.320	0.126
NR87B	5	0.661	0.663	22	41	2	92	0.142	0	2	91	0.142	0
SCR2	3	0.219	0.729	4	0	1	100	0	0.032	1	100	0	0.036
SCR3	3	0.639	0.640	3	0	1	100	0	0.106	1	100	0	0.124
SCR4	2	NA	0.084	3	0	2	67	0.439	1	2	67	0.444	1

TABLE A.3. Table of skew values for male and female brood.

A.5 Appendix Chapter 5

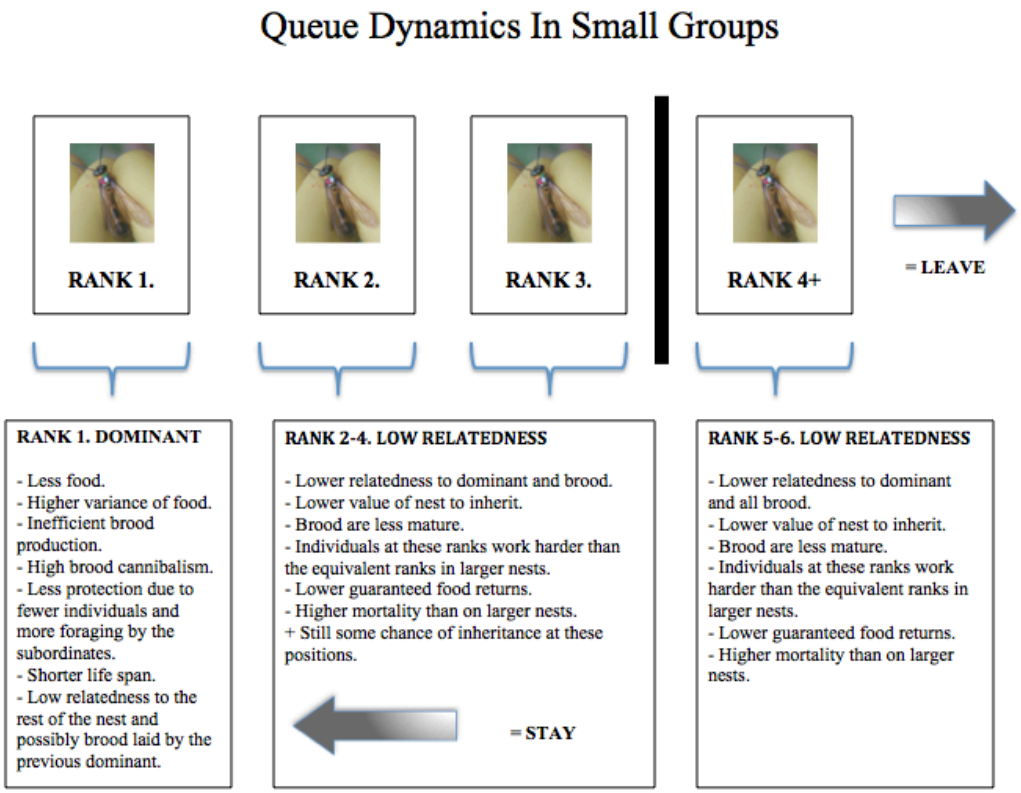

FIGURE A.5.

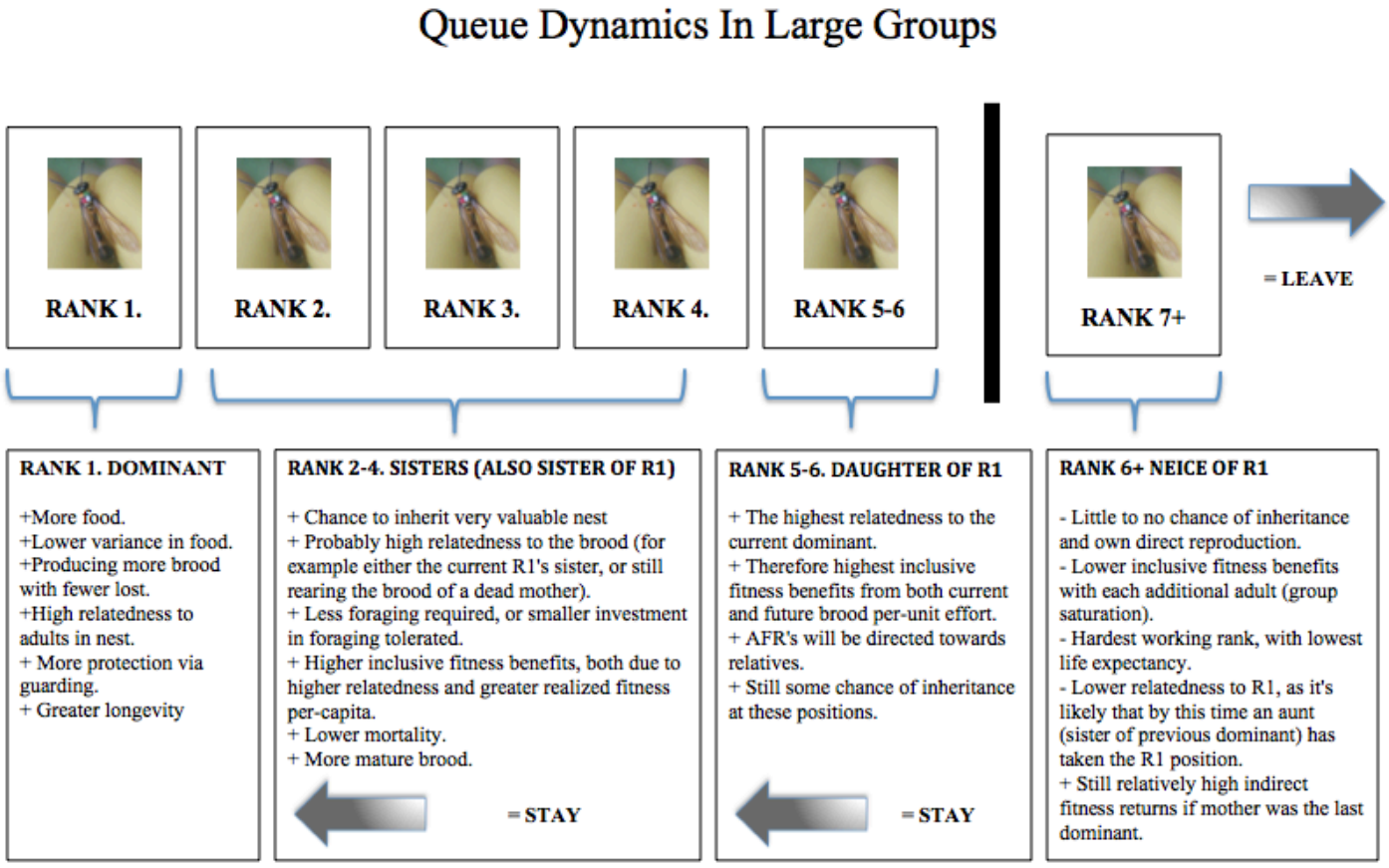
Queue Dynamics In Large Groups
RANK 1.
RANK 2.
RANK 3.
RANK 4.
RANK 5-6
RANK 7+
= LEAVE
RANK 1. DOMINANT
+More food.
+Lower variance in food.
+Producing more brood with fewer lost.
+High relatedness to adults in nest.
+ More protection via guarding.
+ Greater longevity
RANK 2-4. SISTERS (ALSO SISTER OF R1)
+ Chance to inherit very valuable nest
+ Probably high relatedness to the brood (for example either the current R1's sister, or still rearing the brood of a dead mother).
+ Less foraging required, or smaller investment in foraging tolerated.
+ Higher inclusive fitness benefits, both due to higher relatedness and greater realized fitness per-capita.
+ Lower mortality.
+ More mature brood.
= STAY
RANK 5-6. DAUGHTER OF R1
+ The highest relatedness to the current dominant.
+ Therefore highest inclusive fitness benefits from both current and future brood per-unit effort.
+ AFR's will be directed towards relatives.
+ Still some chance of inheritance at these positions.
= STAY
RANK 6+ NEICE OF R1
- Little to no chance of inheritance and own direct reproduction.
- Lower inclusive fitness benefits with each additional adult (group saturation).
- Hardest working rank, with lowest life expectancy.
- Lower relatedness to R1, as it's likely that by this time an aunt (sister of previous dominant) has taken the R1 position.
+ Still relatively high indirect fitness returns if mother was the last dominant.

FIGURE A.6.

APPENDIX B

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