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Author
Narraway, Claire Louisa

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Group Benefit, Nepotism and Intragenomic Conflict: Multiple Levels of Selection on Reproductive Behavior in Honey Bees (*Apis mellifera*).

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Biology

by

Claire Louisa Narraway

2015

ABSTRACT OF THE DISSERTATION

Group Benefit, Nepotism and Intragenomic Conflict: Multiple Levels of Selection on Reproductive Behavior in Honey Bees (*Apis mellifera*).

by

Claire Louisa Narraway

Doctor of Philosophy in Biology

University of California, Los Angeles, 2015

Professor Peter Nicholas Nonacs, Chair

There is no doubt that selection acts at the individual level but, there is heated debate over the relative importance of higher and lower levels of selection. Kin selection is the dominant paradigm explaining the evolution of cooperation. Whereby, individually detrimental traits can be selectively favored if they increase the fitness of genetic relatives. Kin selection operates at the individual level, biasing cooperation towards those sharing the most genes. However, kin selection may also act at the group level when efficiently functioning groups are more productive than dysfunctional ones, provided group success correlates to kin structure. Finally, kin selection can occur at the genome level within individuals, where paternally and maternally-inherited

genes may favor different behaviors and actions. The imprinting of genes to parent-of-origin could also have important ramifications for social evolution. Here, I exploit the conflict over male production in honey bees to examine how these three levels of selection operate. Honey bee workers could 'police' eggs laid by other workers either to maintain colony-level productivity, favor more closely related individuals or as a result of intragenomic conflict (i.e. the paternal genome favors laying, the maternal one favors policing). Firstly, I found that although African workers lay eggs more rapidly than European workers, there is no difference in their times to ovary activation. Significant effects of both the juvenile and adult social environment on ovary activation, suggest that environment has a larger effect on the propensity to activate ovaries rather than subspecies. Secondly, I mathematically simulated a typical eusocial colony where I varied the number of mates per queen, viability of worker-laid males, colony efficiency costs of reduced worker helping, and whether or not intragenomic conflict could be expressed. Genome level selection dominated over both individual and group levels, and group level selection was more significant than the individual level in determining when queens dominate male production. Thirdly, individual level selection predicts policing late into larval development whilst benefits accrued through colony efficiency predicts workers should stop policing viable larvae soon after hatching. To this end I reared queen and worker laid male larvae in a queenless colony and transferred male larvae, from both sources, of differing ages, into a queenright colony. Post transferal (4 and 24 h), I found that workers equally removed larvae regardless of age or maternal source. With the observed high efficiency of policing eggs, these results suggest no mechanism has evolved to police larvae. Alternatively, drones may have a higher level acceptance threshold than female larvae, due to the possibility that they are laid by

workers. Finally, I examined genome level selection by crossing African and European honey bees and then placing the emerging worker offspring into a queenless colony. I observed behavior from day 8 to 28 and collected marked workers on day 16 and 28. I predicted that parent-of-origin effects would occur, but instead found workers of both crosses have higher levels of ovarial development than their purebred counterparts. This suggests an imprinting mismatch such that only the paternal imprint is expressed. Together these results indicate that selection is acting at levels besides that of the individual. Continued research is needed to understand how selection, interacting over multiple levels, impacts behavior, across the animal kingdom.

The dissertation of Claire Louisa Narraway is approved.

Gregory F. Grether

Kirk Visscher

Peter Nicholas Nonacs, Committee Chair

2015

To Mum, Dad and Mark.

TABLE OF CONTENTS

LIST OF FIGURES xiii

LIST OF TABLES xvii

ACKNOWLEDGEMENTS xix

VITA xxiii

CHAPTER 1 **1**

Introduction **1**

1.1 Introduction to the Study of Social Behavior **2**

1.2 Models of Sociality **4**

1.2.1 Reciprocal Altruism 4

1.2.2 Group Selection 5

1.2.3 Inclusive Fitness 6

1.2.4 Multilevel Selection 9

1.2.5 Kin, Group Selection and Multilevel Selection Today 10

1.3 *Apis mellifera* **13**

1.3.1 European and Africanized Honey Bee Subspecies. 15

1.4 Reproductive Conflict in *Apis mellifera* **17**

1.4.1 Group and Individual Levels of Selection 18

1.4.2 Genome Level of Selection 20

1.4.3 Multilevel Selection 22

1.5 Summary **22**

1.6 Figures and Tables **23**

1.8 References **29**

CHAPTER 2 **45**

Genetic and Environmental Effects on Ovary Activation **45**

2.1 Abstract **46**

2.2 Introduction **47**

2.3 Methods **50**

2.3.1 Sample Collection 50

2.3.1.1 Experiment 1 - Rate of Ovary Activation, Prior to Egg Laying, in African and European Honey Bees. *50*

2.3.1.2 Experiment 2 - Effect of Queenless Period on Ovary Activation in African and European Honey Bees. *51*

2.3.1.3 Experiment 3 - Effect of Genetic and Social (Rearing and Adult) Environment on Ovary Activation in African and European Honey Bees. *52*

2.3.2 Ovary Assessment 54

2.3.3 Statistical Analyses 54

2.4 Results **55**

2.4.1 Experiment 1 - Rate of Ovary Activation, Prior to Egg Laying, in African and European Honey Bees. 55

2.4.2 Experiment 2 - Effect of Queenless Period on Ovary Activation in African and European Honey Bees. 56

2.4.3 Experiment 3 - Effect of Genetic and Social (Rearing and Adult) Environment on Ovary Activation in African and European Honey Bees. 57

2.5 Discussion **60**

2.6 Figures and Tables **64**

2.7 References **78**

CHAPTER 3 **84**

Multilevel Selection in Social Insect Reproduction **84**

3.1 Abstract **85**

3.2 Introduction **86**

3.3 Methods **90**

3.3.1 Model Description: Group and Individual Level Selection 90

3.3.1.1 Model Set-up and Starting Conditions *92*

3.3.2 Genomic Imprinting 94

3.3.3 Statistical Analyses 95

3.4 Results **95**

3.4.1 Group and Individual Level Selection 95

3.4.2 Group, Individual and Intragenomic Level Selection 98

3.5 Discussion **100**

3.6 Figures and Tables **105**

3.7 References **118**

CHAPTER 4 **123**

Worker Honey Bees Do Not Distinguish Between Queen and Worker-Laid Male Larvae **123**

4.1 Abstract **124**

4.2 Introduction **125**

4.3 Methods **128**

4.3.1 Statistical Analyses 130

4.4 Results **130**

4.5 Discussion **131**

4.6 Figures and Tables **135**

4.7 References **139**

CHAPTER 5 **143**

Intragenomic Conflict Over Worker Laying in the Honey Bee **143**

5.1 Abstract **144**

5.2 Introduction **145**

5.3 Methods **149**

5.3.1 Behavioral Data & Sample Collection 149

5.3.2 Ovary Dissection 150

5.3.3 Statistical Analyses 151

5.4 Results 152

5.4.1 Ovary Activation and Ovariole Number 152

5.4.2 Subcaste Determination 152

5.5 Discussion 153

5.6 Figures and Tables 159

5.7 References 165

CHAPTER 6 172

Conclusion 172

6.1 References 176

APPENDIX I 177

Unsuccessful Methodologies from Chapter 4 177

A1.1 Full Factorial Design 178

A1.1.1 Problems with Full Factorial Design 178

A1.2 *In vitro* Rearing of Male Larvae 179

A1.2.1 Problems with *in vitro* Rearing of Male Larvae Design 180

A1.3 Figures and Tables **182**

A1.4 References **185**

LIST OF FIGURES

Figure 1.1 Diploid pedigree showing the degree of relatedness of an individual to: mother, father, full sibling, half sibling, aunt and cousin. 24

Figure 1.2 Illustration of the mechanism by which group level can prevail over individual level selection, according to multilevel selection theory. 26

Figure 1.3 Haplodiploid pedigree showing the degree of relatedness of an individual to: mother, father, full sister, brother, half sister, son, full nephew and half nephew. 27

Figure 2.1 Pictures denoting different levels of ovary activation. 64

Figure 2.2 Average number of days to appearance of first worker-laid egg and promiscuous egg laying. 65

Figure 2.3 Level of ovary activation, of European and African honey bees, on the first day post dequeening and once promiscuous laying had commenced. 66

Figure 2.4 Rate of ovary activation in African and European honey bees. 67

Figure 2.5 Average level of ovary activation of African and European honey bees aged 0, 8, 16 and 24 days. 69

Figure 2.6 Average level of ovary activation of African and European honey bees aged 0, 8, 16 and 24 days, reared in **a**) a colony that had only just become queenless and **b**) a colony that had been queenless for 2 weeks and had commenced egg laying. 70

Figure 2.7 Interaction plots of ovary activation in 8 day old African and European honey bees, in colonies that had been queenless for short and long periods of time. 71

Figure 2.8 Interaction plots of ovary activation in 16 day old African and European honey bees, in colonies that had been queenless for short and long periods of time. 72

Figure 2.9 Average level of ovary activation of African and European honey bees aged 0, 4, 8, 12 and 16 days old. 75

Figure 2.10 Effect of ancestry and environment on ovary development over time. 76

Figure 3.1 Model structure. 106

Figure 3.2 Genotype to phenotype distribution at the policing and laying locus. 107

Figure 3.3 Effect of group, individual and genomic levels of selection on the proportion of queen produced offspring. 108

Figure 3.4 Effect of group and individual level selection on the proportion of laying and policing alleles. 109

Figure 3.5 Decision tree of group and individual levels of selection on the proportion of queen produced offspring. 110

Figure 3.6 Effect of group, individual and genomic levels of selection on the proportion of laying alleles. 111

Figure 3.7 Effect of group, individual and genomic levels of selection on the proportion of policing alleles. 112

Figure 3.8 Effect of group, individual and genomic levels of selection on the level of suppression of laying alleles by policing alleles and suppression of policing alleles by laying alleles. 113

Figure 3.9 Decision tree of group, individual and genomic levels of selection on the proportion of male offspring produced by the queen. 114

Figure 3.10 Decision tree of group, individual and genomic levels of selection on the proportion of imprinted laying alleles. 115

Figure 3.11 Decision tree of group, individual and genomic levels of selection on the proportion of imprinted policing alleles. 116

Figure 3.12 Effect of group, individual and genomic levels of selection on the percentage of potential brood lost by a colony due to conflict over male parentage. 117

Figure 4.1 Rate of policing of queen and worker-laid larvae 4 and 24 hours after introduction to test colony. 136

Figure 4.2 Rate of removal of worker and queen-laid larvae of varying ages **a**) 4 and **b**) 24 hours post introduction to test colony. 138

Figure 5.1 Haplodiploid pedigree. 159

Figure 5.2 Effect of age and ancestry of larvae on **a**) average level of ovary activation and **b**) average number of ovarioles. 160

Figure 5.3 Effect of ancestry on level of ovary activation and ovariole number. 161

Figure 5.4 Effect of hybrid vs purebred ancestry on level of ovary activation and ovariole number. 162

Figure 5.5 Effect of ancestry and **a**) ovary development and **b**) ovariole number on worker subcaste. 163

Figure A1.1 Protocol for worker policing experiment. 182

LIST OF TABLES

Table 1.1 Traits defining the different levels of selection from solitary to eusocial. 23

Table 1.2 Calculation of relatedness coefficients in diploid organisms. 25

Table 1.3 a & b Calculation of relatedness coefficients in haploid organisms 28

Table 2.1 Linear, quadratic and cubic model fits to ovary activation rates in European and African honey bees. 68

Table 2.2 Wilcoxon tests for differences in ovary activation level of African and European honey bees aged 0, 8, 16 and 24 days. 69

Table 2.3 Wilcoxon tests for differences in ovary activation of African and European honey bees aged 0, 8, 16 and 24 days, reared in **a**) a colony that had only just become queenless and **b**) a colony that had been queenless for 2 weeks and had commenced egg laying. 70

Table 2.4 Two-way ANOVAs predicting ovary development in 8 and 16 day old workers, of African and European ancestry, in colonies that had been queenless for long and short durations. 73

Table 2.5 Wilcoxon tests for differences in ovary activation level of African and European honey bees aged 0, 4, 8, 12, 16 and 20 days. 74

Table 2.6 Three-way ANOVAs predicting ovary development in 4, 8, 12 and 16 day old workers, of African and European ancestry, who had been reared in queenright African or European colonies, and were placed into queenless African or European colonies upon emergence. 77

Table 3.1 Main model parameters. 105

Table 4.1 Diets fed to male larvae from day 1-4 and day 5-7. 135

Table 4.2 Logistic regression of larval age and mother (queen or worker) on policing rate. 137

Table 5.1 Multiple logistic regression model of ancestry and **a**) level of ovary development and **b**) number of ovarioles in determining worker subcaste. 164

Table A1.1 Transplant distribution for worker policing experiment. 183

Table A1.2 Diets fed to male larvae from day 1-4 and day 5-7. 184

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VITA

Education

2007	M.Sc. Evolutionary and Behavioural Ecology, University of Exeter, UK
2005	B.Sc. Combined Honours Science in Psychology and Zoology, University of Liverpool, UK.

Employment

2013-2014	Teaching Fellow, Department of Ecology & Evolutionary Biology, UCLA, Los Angeles, USA.
2010-2013	Teaching Associate, Department of Ecology & Evolutionary Biology, UCLA, Los Angeles, USA.
2008-2010	Teaching Assistant, Department of Ecology & Evolutionary Biology, UCLA, Los Angeles, USA.
2007-2008	Postgraduate Research Assistant, Institute of Zoology, London, UK.
2006	Research Assistant, University of Liverpool, Liverpool, UK.
2005	Volunteer, CERCOPAN, Calabar, Nigeria.
2002	Farm Assistant, Matauri Bay, New Zealand.
2001	Volunteer, Operation Wallacea, Borneo.
1998-2000	Volunteer, Heath Veterinary Practice, London, UK. Volunteer, Waterlow Park Bird Aviary, London, UK. Volunteer Stable Assistant, London Equestrian Center, London, UK. Volunteer Lab Assistant, Royal Veterinary College, London, UK. Volunteer Farm Assistant, Kentish Town City Farm, London, UK.

Awards

2014 IUSSI Travel Award
2014 EEB Travel Award
2013 EEB Travel Award
2012 EEB Travel Award
2011 NSF DDIG
2011 CSHL Award
2011 NAS Student Travel Award
2010 EEB Travel Award
2009 Sigma Xi

Committee Positions

2012-2013	Department of Ecology & Evolutionary Biology, Graduate Funding Student Representative, UCLA, Los Angeles, USA.
2011-2012	Seminar Committee Student Representative, UCLA, Los Angeles, USA.
2010-2011	Darwin Evolving Committee Student Representative, UCLA, Los Angeles, USA.

Publications & Presentations

Narraway, C. L., & Nonacs, P. 2014. Altruistic Worker Policing in Honeybees: A Multi-Level Approach, Oral Presentation at the IUSSI International meeting, Cairns, Australia.

Narraway, C. L., & Nonacs, P. 2013. The Hidden Cost of Altruistic Policing, Poster Presentation at the Animal Behavior Society meeting, Boulder, Colorado, USA.

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CHAPTER 1

Introduction

1.1 Introduction to the Study of Social Behavior

Any individual that interacts with another individual engages in a social interaction. What distinguishes social organisms from solitary ones is that an increase in the frequency of interactions, normally between conspecifics, leads to the formation of permanent groups of adults living together, maintaining relationships from one encounter to the next.

Societies vary in the extent of organization of their social behavior, and, as such, the reliance of group members upon each other. Levels of sociality extend from solitary to eusocial. Whilst solitary animals only associate for courtship and mating, eusocial societies are characterized by overlapping adult generations, reproductive division of labor, cooperative care of young and a caste system (see Table 1.1, Michener, 1974).

The advantages of group living are numerous: 1) groups can modify conditions allowing individuals to inhabit otherwise hostile environments (Allee, 1938), 2) provide predator protection through increased vigilance (Elgar, 1989; Saino, 1994), dilution (Foster and Treherne, 1981), or mutual defense (Göttmark and Andersson, 1984), 3) improve foraging success through increased probability of finding (Krebs *et al.*, 1972) and capturing food (Stander and Albon, 1993; Funston *et al.*, 2001), 4) help rear offspring (Roulin, 2002; McRae, 1996), 5) protect against parasites and diseases (Boecking and Spivak, 1999; Feener Jr. and Moss, 1989). But, living in a society also brings with it many challenges: 1) groups are more conspicuous to predators, 2) there may be increased competition for food (Janson, 1988), 3) group members may interfere with an

individual's reproductive efforts (cuckoldry: Davies, 1985; Power *et al.* 1981, infanticide: Campagna *et al.*, 1988; Crook and Shields, 1985), 4) disease and parasite transmission rate may be higher (Godfrey *et al.*, 2009), 5) some individuals may not contribute to the group's functioning (Clutton-Brock and Parker, 1995; Hauser and Marler, 1993), 6) energy is required to maintain group membership and status which could have been used to perform other tasks (Saplosky, 2005).

Three types of social interaction promote the formation of groups. With mutualistic interactions individuals gain an immediate benefit. Alternately, individuals may be manipulated to join or stay in groups, suffering a cost themselves whilst the manipulator benefits. Altruistic interactions are more difficult to explain from an evolutionary perspective because the actor freely performs an action which confers a fitness cost, whilst the recipient benefits. Yet we regularly observe altruistic interactions in nature.

Despite the inherent costs and conflicts associated with social groups they are prevalent in nature and the species that form such groups are among the most successful. For example, in most areas of the terrestrial world ants, of which all known species are eusocial, monopolize 15-20% of the animal biomass, with that increasing to 25% or more in tropical regions (Schultz, 2000). Furthermore, the Argentine ant, *Linepithema humile*, is found on six continents (Suarez *et al.*, 2001), and forms mega-colonies, the extent of which are paralleled only by human society (Tsutsui *et al.*, 2000; Giraud *et al.*, 2002).

1.2 Models of Sociality

1.2.1 Reciprocal Altruism

Inherent within altruism is a negative fitness effect on the altruist. If an individual always behaves altruistically, his/her fitness diminishes, and the genes for altruism are lost from the population. What Trivers (1971) realized was that altruism could be favored if roles later reversed and the recipient provided aid to the altruist. As such, reciprocal altruism can favor the evolution of altruism between unrelated individuals (Axelrod and Hamilton, 1981).

One of the most famous examples of reciprocal altruism in the animal kingdom, barring that of *Homo sapiens*, is food sharing by vampire bats. Vampire bats feed solely on blood and die if they do not feed every 70 hours. As such, sharing a meal is highly costly to the actor but of greater benefit to the recipient. What Wilkinson (1984) showed is that vampire bats only share meals with individuals that they regularly associate with and who could one day reciprocate.

Reciprocal altruism may only evolve when individuals interact repeatedly and if a mechanism of detecting and punishing cheats (individuals who fail to cooperate) is in place. With the possibility of long time lags between cooperative acts being performed and returned, and the possibility of many potential partners per individual, reciprocal altruism is most likely to be found in species with higher cognitive abilities, or when the benefit is mutual and immediate; e.g. horses grooming each other.

1.2.2 Group Selection

As with most concepts in the study of animal behavior it was Darwin (1882) who first posited the concept of group selection:

'There can be no doubt that a tribe including many members who, from possessing in a high degree the spirit of patriotism, fidelity, obedience, courage, and sympathy, were always ready to aid one another, and to sacrifice themselves for the common good, would be victorious over most other tribes'.

Theories promoting selection on groups existed throughout the 20th century but it was in 1962 when Wynne-Edwards published his book, *Animal Dispersion in Relation to Social Behavior,* that discussions about if and how selection acts on groups really came to a head. Wynne-Edwards claimed that populations and groups had characteristics that were lacking in individuals. For example, food tends to be the factor that regulates population growth and free contest for food would lead to its over exploitation and the depletion of future yields. Through substitution of food for rewards such as territories and social rank, that act to keep population density at the optimum level for the quantity of food available, over-exploitation and the associated future depletion of resources would be avoided. He stated that such adaptations could only be obtained by selection acting at the level of the group because they involve a cost to the individual to provide a benefit to the group. As such, groups that used appropriate behavioral mechanisms to regulate their population density would out live groups that lacked such controls and over-exploited their resources (Wynne-Edwards, 1962).

Support and condemnation of Wynne-Edwards (1962) theory came from several quarters. Fellow Oxford ornithologist David Lack (1966), whose research had inspired Wynne-Edwards, argued that rather than selection acting on groups to temper resource exploitation, natural selection acted on individuals in a density-dependent manner to control population size. Other critics of Wynne-Edwards theory attacked his credentials, claiming that his lack of field experience had led him to oversimplify the way animals live and ignore important factors such as parasites and predation (Elton, 1963). Williams' (1966) book *Adaptation and Natural Selection* popularized a gene centric approach to the study of animal behavior. In reference to group selection Williams concurred with Lack, that group level adaptations did not exist, and that they were either not an adaptation or there was an individual level explanation. The basic tenet was that individual organisms are more plentiful than groups, that individuals are born and die more rapidly than groups can form and become extinct, and that as selection requires differences in birth and death rates between groups, individual level selection would occur at a faster rate than group level selection and as such would be the dominant selective force. Wynne-Edwards' model of group selection floundered whilst inclusive fitness theory (Hamilton, 1963), published only 1 year after Wynne-Edwards' book, gained credence.

1.2.3 Inclusive Fitness

Hamilton (1964a & b) was the first to demonstrate, mathematically, the role of kinship in explaining the evolution of altruism, however, several others had come exceptionally close, stopping just shy of formalizing the theory mathematically.

In 1922, Sewall Wright developed the coefficient of relationship, *r*, that Hamilton would later use. The coefficient of relatedness expresses the degree of kinship between two individuals by calculating the average proportion of genes that they share. For example, the coefficient of relatedness for two random individuals is 0. In diploid organisms full siblings will each obtain 50% of their genes from their mother and 50% from their father, as such, the coefficient of relatedness between the siblings will be 0.5 (*r = (0.5+0.5)/2 = 0.5,* see Fig. 1.1 and Table 1.2*)*, as inbreeding increases in a population the relatedness coefficient will approach 1. Remarkably, Wright (1945) also built a group selection model of altruism but failed to link it with his work on relatedness.

Fisher (1958) also came close to explaining the evolution of altruism in a chapter of his book *The Genetical Theory of Natural Selection* on the evolution of distastefulness. Whilst most predator avoidance mechanisms are easily explained by natural selection the evolution of nausea inducing flavors posed a problem for Fisher as it was only effective in deterring predators once a larva had been bitten; for larvae, which have a soft outer skeleton, the first bite would lead to an early death.

Fisher determined that if larvae were more often congregated in groups, and if their noxious taste dissuaded a predator from eating anymore larvae in that group, an individual might benefit genetically through being eaten and thus, saving the life of related individuals. After which, Fisher says very little more about altruism and kinship.

Finally, JBS Haldane probably was the most explicit in his linking of kinship and altruism. He is famously quoted as quipping:

"*Would I lay down my life to save one brother? No! But I would to save two brothers or eight cousins"*

In fact, Haldane wrote about his ideas in 1932 and 1955, and clearly stated that if a gene coding for altruism benefits kin it could spread through the population due to natural selection. What Haldane never did was to formalize his thinking into a mathematical equation.

In 1963, Hamilton published the first of three papers that did what Haldane, Fisher and Wright never did. He put into a mathematical form how kinship, interacting with the costs and benefits of altruistic acts, can lead to the evolution of altruism. Not only was his theory significant in the fact that it could explain the swathes of altruistic behaviors observed in the animal kingdom from the level of the individual, it was also remarkably simple:

$$rB - C > 0$$

Where r is the relatedness of the actor to the recipient, B is the benefit to the recipient, and C is the cost of the act to the altruist. As such, when the benefit to the recipient, weighted by genetic relatedness, is greater than the cost to the actor of performing the

act, altruism will evolve (Hamilton, 1963, 1964a & b). Hamilton referred to his theory as inclusive fitness theory, intimating that an individual's fitness was comprised of direct fitness from personal reproduction and the indirect fitness obtained by helping other, related individuals to reproduce extra offspring. Maynard-Smith (1964) later coined the term kin selection.

Hamilton's papers have spawned thousands of research articles. His 1963 article has been cited 700 times and his 1964 papers 6,908 and 1,625 times, according to web of science. His theory has been termed the most important contribution to the theory of natural selection since Darwin (1859) conceived it. Studies invoking his rule span social organisms from *Homo sapiens*, to bacteria, eusocial insects, to cooperatively breeding birds, and alarm calling in meerkats (Madsen *et al.*, 2007; Santema and Clutton-Brock, 2013; Strassmann *et al.*, 2011; Krakauer, 2005; Rumbaugh, *et al.*, 2012).

1.2.4 Multilevel Selection

Whilst researchers around the world tested the predictions that arose from Hamilton's law, some felt that the case against group selection had been overstated and worked to right this. Sober and Wilson (1998) proposed multilevel selection theory which argues that selection is happening simultaneously at different levels. The lowest level is genomic, then cellular, organismal, and finally the level of the group. Across the different levels of selection the selective forces acting on a trait will either concur or oppose each other.

Multilevel selection theory does not favor any single level of selection but rather can be used to evaluate the balance of different levels on a case by case basis. However, for a purely group benefiting trait to spread, group level selection must outweigh individual level selection (O'Gorman *et al.*, 2008). For example, in a group of altruists cheaters will do very well, and in a group of cheaters altruists will suffer, however, if groups with a higher proportion of altruists produce more offspring that enter the mating pool and form new groups, altruism can be selected for despite the success of cheats at the individual level (Fig. 1.2).

Examples in nature come from improved weight gain in Japanese quail (Muir *et al.*, 2013), and increased egg productivity in *Gallus gallus* (Muir, 1996; Muir and Craig, 1998), but multilevel selection is most often discussed in terms of human evolution (Wilson *et al.*, 2008), pathogens (Pepper, 2008) and the eusocial insects (Nowak *et al.*, 2010).

1.2.5 Kin, Group and Multilevel Selection Today

With the demise of Wynne-Edwards' group selection, and the publication of Hamilton's inclusive fitness theory, studies of social behavior focused on whether or not social groups were comprised of kin. In fact, one hypothesis, the haplodiploid hypothesis, suggested that the high genetic relatedness between full sisters (r = 0.75, Fig. 1.3 and Table 1.3) could explain the high occurrence of eusociality throughout the Hymenoptera. However, this hypothesis has effectively been rejected because of the high relatedness amongst full sisters is averaged out by the lower relatedness of brothers (r=0.25), there

is no clear reason why the probable ancestors of eusocial species would produce female-biased broods, and the average relatedness between females is often reduced through polyandry. Finally, eusociality also exists in diploid organisms such as termites and naked mole rats.

Whilst few would discount the value of Hamilton's rule in stimulating an entire field of research, the almost singular focus on the relatedness term has led some, initially ardent proponents of kin selection, to criticize it (Wilson and Hölldobler, 2005; Hölldobler and Wilson, 2008). Today, the debate has become quite heated. A critique by Nowak *et al.* (2010) prompted a rebuttal signed by 137 social evolution biologists stating that

'*Nowak et al. … arguments are based upon a misunderstanding of evolutionary theory and a misrepresentation of the empirical literature*'.

Unsurprisingly, the debate has continued to rage with further critiques and rebuttals coming from both sides (Abbot *et al.*, 2011; Liao *et al.*, 2015).

In part the debate seems semantic. Since Hamilton first proposed his rule many theorists, including Hamilton himself, have explored whether or not the same outcomes could be reached even when some of Hamilton's initial assumptions were relaxed. As such, there are many iterations of Hamilton's rule and it is not always clear which iteration is being discussed (Birch and Okasha, 2015). The second claim made by Nowak *et al.* (2010), and Allen *et al.* (2013) is that Hamilton's rule has no advantage

over the standard natural selection models. Hamilton (1964) himself noted that if, instead of denoting the proportion of genes individuals share, *r* expressed the degree to which altruists differentially interacted with each other than with non-altruists, the same results would be found. One problem with multilevel selection is that it is less intuitive than Hamilton's rule (Birch and Okasha, 2015), and the key insight from Hamilton's rule, that organisms maximize their inclusive fitness, has no obvious parallel. Finally, despite kin selection and multilevel selection being fundamentally equivalent models (Lehmann *et al.*, 2007; Frank, 2013), they may not be equally good at representing the causal structure of social interactions (Birch and Okasha, 2015).

Overall, the multilevel selection versus kin selection debate has been mostly theoretical (Wilson, 2005; Foster *et al.*, 2006; Traulsen and Nowak, 2006; Lehman *et al.*, 2007; Wild *et al.*, 2009; Wade *et al.*, 2010). The wealth of evidence supporting individual level selection as driving social evolution, is compromised by the reliance on group level explanations, when predictions from individual level selection are violated (Wilson, 2005). Today, research expressly examining alternate levels of selection to that of the individual level have become more common (Amarasinghe *et al.*, 2014; Oldroyd *et al.*, 2014). However, few studies incorporate more than one level, or consider their interaction. **As such, I proposed empirical and theoretical research examining if and how selection acts on different levels.**

1.3 *Apis mellifera*

Honey bees (*Apis mellifera*) are eusocial, with colonies averaging 25,000 individuals. Colonies are headed by a single queen who mates, on average, 12 times at the beginning of her life and stores the sperm. Queens lay single eggs in wax honeycomb, which is produced and shaped by worker bees. Sex determination, in honey bees and all haplodiploid organisms, depends on whether or not the individual is hetero- or homozygous at the complementary sex determiner (*csd*) gene. However, it is often easy to identify males even at the egg stage, as male eggs are laid in drone cells, which are noticeably larger than worker cells, where female eggs are laid. Eggs hatch after 3 days. Once male larvae reach the pupal stage (day 10) the cell is capped until emergence, day 24. Whilst female worker larvae are capped at day 9 and emerge on day 21, and queens are capped day 8 and emerge day 16.

Any female larvae may mature into either a queen or worker, two castes which are phenotypically disparate. It is nutritional differences during larval development, rather than differences in the genetic code, that determine whether or not a female larva will develop into a queen or worker (Haydak, 1970; Kamakura, 2011). During development most larvae transition from a diet of royal jelly, a proteinaceous substance produced by worker bees, to honey and pollen. However, larvae destined to be queens are fed royal jelly throughout their development (Seeley, 1995).

Bearing in mind that a honey bee worker and queen could bear exactly the same complement of genes, it is outstanding how morphologically discrete they are. For

example, the queen is noticeably larger than the workers, weighing 178-292 mg at emergence as compared to 81-151 mg for workers (Winston, 1987). The queen's abdomen is filled with approximately 320 ovarioles (Jackson *et al.*, 2011) allowing her to lay up to 2000 eggs per day (Winston, 1987). Workers, on the other hand, have an average of 8 ovarioles, although workers rarely activate their ovaries in the presence of the queen (Makert *et al.*, 2006). In fact, as workers never mate, unlike the queen, they are unable to lay fertilized eggs and so, due to the haplodiploid sex determination system (Fig. 1.3), are only able to produce males. The queen mates multiply, on average with 12 males, during 1-3 mating flights which are taken at the beginning of her life (Tarpy and Page, 2000). After mating, the queen only leaves the colony for swarming. The queen stores sperm within her spermatheca, using it until she dies, approximately 3 years later (Seeley, 1978). Workers live an average of 3-6 weeks during which time they transition from working within the colony to foraging outside it (Ribbands, 1953; Page and Peng, 2001).

The honey bee was the first social insect to have its genome fully sequenced (The Honeybee Genome Sequencing Consortium, 2006). It consists of approximately 10,000 genes with around 236 million base pairs. It is one-tenth the size of the human genome but contains genes coding for orthologues of all vertebrate proteins required for DNA methylation, a key way in which genes are imprinted. Indeed, studies have already identified evidence of methylation and imprinting in honey bees (Kucharski *et al.*, 2008; Elango *et al.*, 2009; Oldroyd *et al.*, 2013, Hunt *et al.*, 2014).

Although humans have cultivated *Apis mellifera* colonies for thousands of years it is unlikely that their socio-biology has become atypical of their wild counterparts. Domestic colonies regularly mate with individuals from wild populations, maintaining gene flow, and several of the behaviors identified in the commonly domesticated honey bee have also been identified in other *Apis* species (Wenseleers and Ratnieks, 2006).

1.3.1 European and Africanized Honey Bee Subspecies.

Honey bees originated in Africa, expanding into Eurasia on at least two occasions (Whitfield *et al.*, 2006). Humans have introduced honey bees to the new world on multiple occasions. The earliest introductions began as early as 1622 and were into North America. *A. m. ligustica*, one of the most commonly bred honey bee species in the United States, was first introduced in 1859. More recently, *A. m. scutellata*, an African honey bee subspecies, was introduced into Brazil in 1956, and rapidly spread throughout Latin America and the south-western United States (Schneider *et al.*, 2004; Whitfield *et al.*, 2006). The expansion of the *A. m. scutellata* range and displacement of European honey bee subspecies is one of the most rapid biological invasions known (Schneider *et al.*, 2004).

African and European honey bees, in the new world, display distinct behavioral phenotypes. African honey bee colonies grow and swarm faster than European honey bee colonies (Winston, 1992), and may usurp struggling European colonies (Danka *et al.*, 1992; Vergara *et al.*, 1993). African colonies also exhibit higher levels of defensive

behavior (stinging, guarding, pursuing) than most European honey bee races (Collins *et al.*, 1982; Breed *et al.*, 2004).

Within the colony, African virgin queens develop faster, emerge sooner and more frequently perform behaviors such as "piping" (a series of pulsed notes), which inhibit the emergence of rivals and enhance fighting success (Schneider *et al.*, 2001; Schneider and DeGrandi-Hoffman, 2003). When colonies become queenless, worker honey bees can activate their ovaries and lay male eggs (Velthuis, 1970). Worker *A. m. scutellata* honey bees develop ovaries and oviposit more rapidly than European races (Ruttner and Hesse, 1981). The environment in which a worker finds herself is instrumental in determining whether or not she lays. Workers rarely activate their ovaries in the presence of the queen (Ratnieks, 1993) or larvae (Trouiller *et al.*, 1991; Arnold *et al.*, 1994; Mohammedi *et al.*, 1996 & 1998; Oldroyd *et al.*, 2001; Pankiw *et al.*, 2003). Likewise, temperature (Lin and Winston, 1998), trophallactic interactions with other workers (Korst and Velthuis, 1982), and the amount of protein received both at the larval and adult stage (Hoover *et al.*, 2006) impact worker ovarial development. The ancestry of individuals that make up the social environment may also be important. For example, anarchistic honey bees activate their ovaries in the presence of the queen and brood (Oldroyd *et al.*, 1994). However, when workers from the anarchistic strain were placed into a queenright colony with wild type larvae only 16% activated their ovaries, compared with 41% when the queenright colony contained anarchistic strain larvae. Likewise, 0% of the wild type bees activated their ovaries when in the presence of wild

type brood compared with 13% when placed with anarchistic brood (Oldroyd *et al.*, 2001).

Initially, I examined the effect of the genetic and social environment on worker reproduction using honey bees from European and African stock. Over a series of experiments I found a dominant role for the effect of social environment on levels of ovary activation, results of which are presented in Chapter 2.

1.4 Reproductive Conflict in *Apis mellifera*

The *prima facie* evidence for kin selection comes from worker policing studies (Whitfield, 2002; Gadagkar, 2004; Sugden, 2004). Worker policing is the removal of worker laid eggs or aggression directed towards workers with activated ovaries. It is predicted when the queen mates multiply because workers are more related to their own sons than they are to their half-sisters, but less related to their half-nephews than they are to their brothers (Fig. 1.3 and Table 1.3). As such, whilst individual workers can gain direct benefits by laying eggs that are reared by the colony, the collective workforce prefer queen-laid eggs over those laid by other workers. Worker policing is the key mechanism for resolving this conflict (Ratnieks, 1988; Ratnieks and Visscher, 1989). As workers may not be able to discriminate between patrilines they act collectively to suppress worker laying (Reeve and Jeanne, 2003). When an effective mechanism of policing is in place the collective workers have more power than the individual workers and control their reproductive behavior (Wenseleers *et al.*, 2004; Visscher and Dukas, 1995). Moreover, the more effective policing is, the less likely it is that an individual

worker will attempt to reproduce, leading to functional sterility, manifest as inactive ovaries (Ratnieks and Visscher, 1989).

Worker policing was first identified in the honey bee (Ratnieks and Visscher, 1989) and has since been found in a wide range of ant, bee and wasp species (Wenseleers and Ratnieks, 2006). Despite the wealth of supporting studies, predictions stemming from kin selection are not always upheld. For example, in monogamous colonies, workers are more related to their nephews than their brothers (Fig. 1.3 and Table 1.3) and so should prefer to assist in rearing nephews over brothers, leading to workers producing all of the males in the colony. Worker policing is still observed in monogamous colonies (Visscher, 1996; Foster *et al.*, 2002; Endler *et al.*, 2004). Secondly, worker policing has been documented in species where workers produce diploid female eggs parthenogenetically through thelytoky (Saigo and Tscuchida, 2004; Pirk *et al.*, 2003). In this instance workers are equally related to the clonal offspring of their sisters as they are to their mothers (in both cases r=0.75) and as such there is an absence of fitness benefits to policing. In both cases colony efficiency has been proposed as the mechanism through which worker policing is selected (Foster *et al.*, 2002; Hammond and Keller, 2004).

1.4.1 Group and Individual Levels of Selection

Where predictions from kin selection fail to explain the full array of behaviors we see in nature other mechanisms are often invoked. In the case of worker policing and reproduction, improving colony efficiency is often the touted mechanism. Pirk *et al.*

(2004) proposed that worker laid eggs are policed because they are less viable than queen laid eggs, for hygienic reasons. These differences could be due to oviposition technique, failure to develop, or competition among workers in queenless colonies (Pirk *et al.*, 2003; Sakagami, 1958; Hölldobler and Wilson, 1990). Also, queens are fed more extensively with a protein rich diet and a greater protein intake has been shown to significantly enhance embryo development in honey bees, increasing egg size and productivity, and increasing resistance to dehydration in eggs (Pirk *et al.*, 2004). This "dead egg hypothesis" was elegantly rejected by Beekman and Oldroyd (2005) and some studies suggest that worker eggs are not less viable than queen laid eggs but rather more or equally viable (Ratnieks, 1988; Beekman and Oldroyd, 2005).

Despite the proximate mechanism, that eggs are policed because they are dead, being rejected, the hypothesis that worker-laid eggs are policed because they are less viable has not. Workers do not need to be able to identify whether or not an egg is alive or dead to be able to discriminate between worker and queen-laid eggs. Queen and worker -laid eggs are thought to be distinguishable due to egg-marking pheromones, although despite extensive research nobody has identified the specific suite of hydrocarbons that distinguishes queen-laid eggs from worker-laid eggs (Zeng and Yves, 2009). It is clear however, that workers can distinguish between worker and queen-laid eggs (D'Ettore *et al.*, 2004). If eggs are cheap to produce it will cost workers very little to replace all worker-laid eggs with queen-laid ones. As such, it is possible for worker policing in a colony with a multiply mated queen to provide two benefits: increased colony relatedness and increased brood viability (Nonacs, 2006).

The two competing theories can be disentangled because once an egg hatches workers start to feed and care for the larva extensively. As such, the further a nephew progresses through development without detection the less benefit can be gained through removing that larva. The viability hypothesis predicts that once an egg has hatched and proven its viability it should face a reduced probability of being policed. However, nepotism via Hamilton's rule predicts that workers should still discriminate between early instar worker-produced larvae as strongly as they do against eggs (Nonacs, 2006).

By rearing queen and worker laid larvae in a single colony, transferring larva of different ages to a queenright colony and observing the proportion and ages at which larvae are removed, I attempt to distinguish between worker policing selected at the level of the individual and the level of the colony. **If worker-produced male offspring continue to be removed post hatching, and queen-produced male offspring remain, this will provide evidence supporting the relatedness hypothesis. However, if the level of discrimination between worker and queen produced larva, and the rate of policing, drops post-hatching, selection is acting at the level of the colony.** I present these results in Chapter 4, showing that workers do not distinguish between queen and worker laid male larva, regardless of age.

1.4.2 Genome Level of Selection

Worker policing may also be the result of intragenomic conflict (Haig, 1992; Queller, 2003; Kronauer, 2008). If the queen lays all of the male eggs in a colony then her mate's genes will not be expressed in any male offspring. There are two ways that a

male can get his genes into future males: if his daughter becomes queen and lays male eggs, or if his daughter becomes a worker and lays male eggs. For any particular male the chances of one of his daughters becoming a queen is relatively low. Secondly, there would be no conflict between the maternal and paternal genomes expressed in queens (both genomes favor the queen producing all the male eggs in the colony). However, in workers, from the point of view of the paternal genome it is selectively advantageous that workers lay all male eggs. If workers were to lay all male eggs it would be at the expense of the maternal part of the genome. As such, there could be a strong selective advantage to a gene, or suite of genes, imprinted in a parent of origin manner (Burt and Trivers, 2006). There is no one outcome to this conflict but rather a continuous dynamic conflict between the two halves of the genome expressed in workers, possibly leading to workers who police and those who lay, as is observed in nature.

Using a series of crosses, of African and European honey bees, I looked for parent-of-origin effects; where **workers with a European mother and African father were predicted to activate their ovaries and have greater reproductive potential than those with an African mother and African father, who in turn have greater reproductive potential than workers with an African mother and European father, and finally a European mother and European father.** Rather, I found evidence that both hybrids out performed pure-bred colonies, and the results are presented in Chapter 5.

1.4.3 Multilevel Selection

Ultimately, within a honey bee colony all three levels of selection are likely to exist, and interact. Models determining reproductive behavior tend to highlight a single level of selection (Ratnieks, 1988; Queller, 2003). The current debate focuses primarily on whether or not kin selection automatically subsumes group selection (Wilson, 2005; Foster *et al.*, 2006; Traulsen and Nowak, 2006; Lehman *et al.*, 2007; Wild *et al.*, 2009; Wade *et al.*, 2010). Whilst, intragenomic conflict is discussed entirely separately. As such, **I constructed a simulation model examining the interaction of two, and then all three levels of selection on worker reproduction and policing.** In Chapter 3, I present results showing a dominant role of intragenomic conflict in predicting whether workers or the queen will dominate male production, with the evolution of imprinted genes being driven primarily by group level selection.

1.5 Summary

Combining a series of experiments and theoretical modeling I examined the interplay across levels of selection on the conflict over the production of males in honey bees. After examining how genetic and environmental factors influenced ovary activation I wrote a theoretical model examining how the different levels of selection interacted and under what conditions each was more prominent. Subsequently, I examined the levels of selection themselves, firstly the group and the individual level and finally the intragenomic.

1.6 Figures and Tables

Degree of Sociality	Parental investment	Cohabitation of adults & offspring	Cooperative care of young	Reproductive division of labour	Caste system	Overlapping adult generations
Solitary	-	-	-	-	-	-
Subsocial	+	-	-	-	-	-
Solitary but social	+	+/-	-	-	-	-
Communal	+	+	-	-	-	-
Quasisocial	+	+	+	-	-	-
Semisocial	+	+	+	+	+	-
Eusocial	+	+	+	+	+	+

Table 1.1 Levels of sociality, from solitary to eusocial, defined by the level of social organization. + indicates a behavior that is present in the species, whilst - indicates its absence. Solitary but social animals forage independently but females and brood may share nests, hence the +/- symbol.

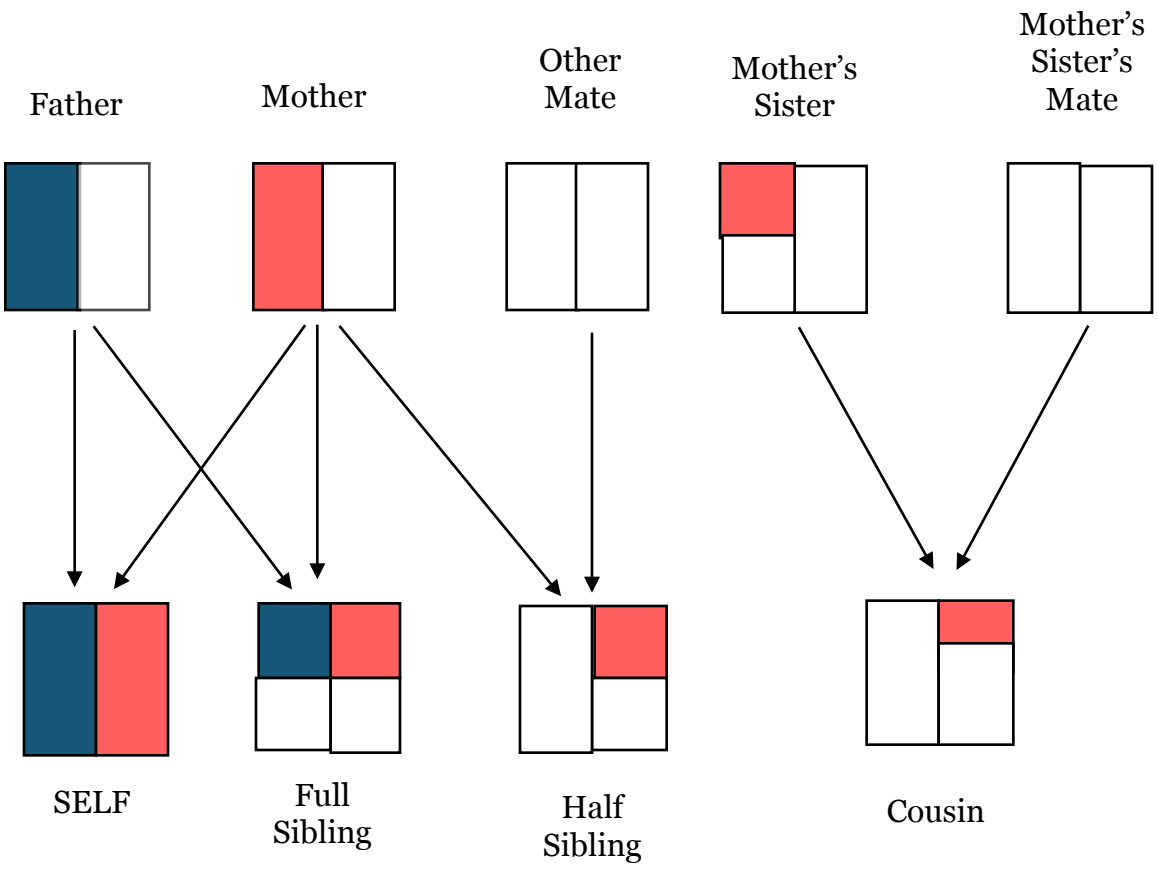

Figure 1.1 A diploid pedigree. The coloring shows the degree of shared genes between individuals and 'SELF'. As such, 'SELF' is 100% related to itself, gaining 50% of its genes from its father (blue) and 50% from its mother (pink). White indicates genes not shared with 'SELF'.

	Paths	**Formula**	***r***
Father	Self -> Father	$(0.5)^1$	0.5
Mother	Self -> Mother	$(0.5)^1$	0.5
Full Sibling	Self -> Father -> Sibling Self -> Mother -> Sibling	$(0.5)^2 + (0.5)^2$	0.5
Half Sibling	Self -> Mother -> Half Sibling	$(0.5)^2$	0.25
Aunt	Self -> Mother -> Aunt	$(0.5)^2$	0.25
Cousin	Self -> Mother -> Aunt -> Cousin	$(0.5)^3$	0.125

Table 1.2 Calculation of the relatedness coefficient (*r*), in diploids, for some common family relationships.

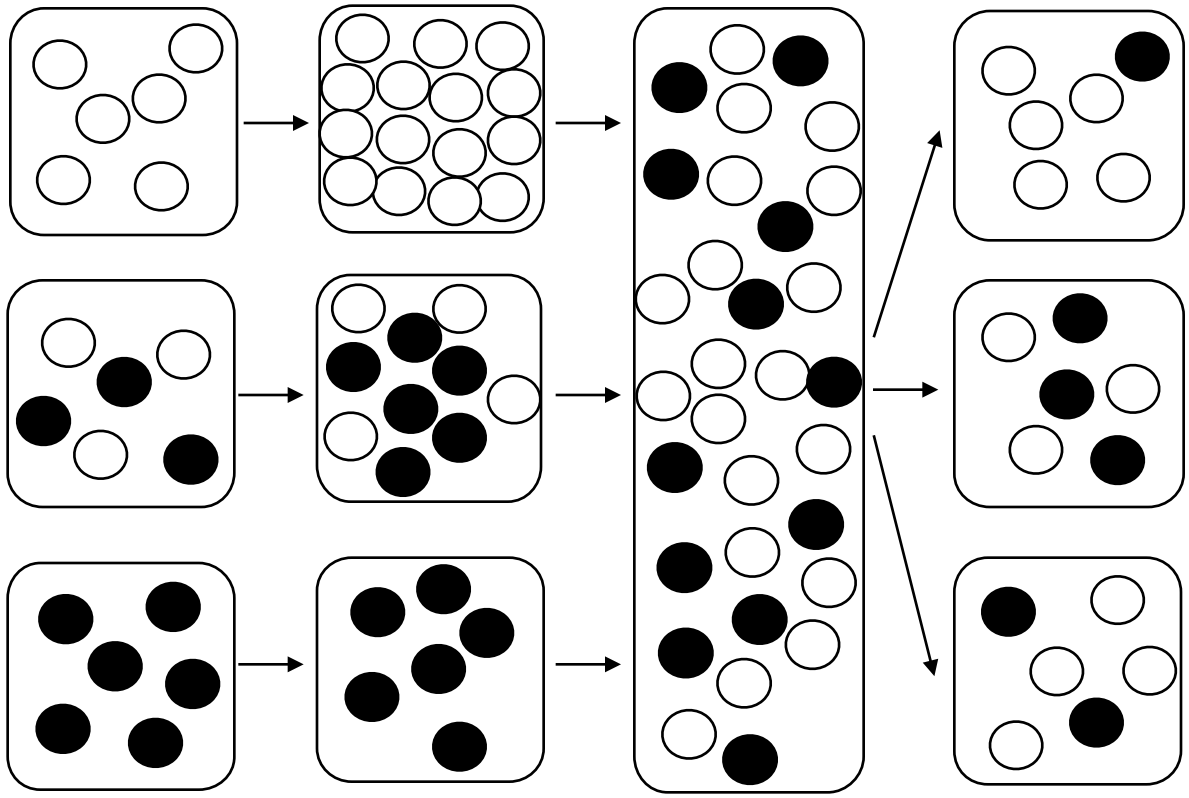

Figure 1.2 Group selection can prevail over individual level selection because whilst cheats (black) outcompete altruists (white) within groups, groups of altruists produce more individuals in total than groups of selfish individuals. Group members enter a common mating pool from which individuals randomly form new groups.

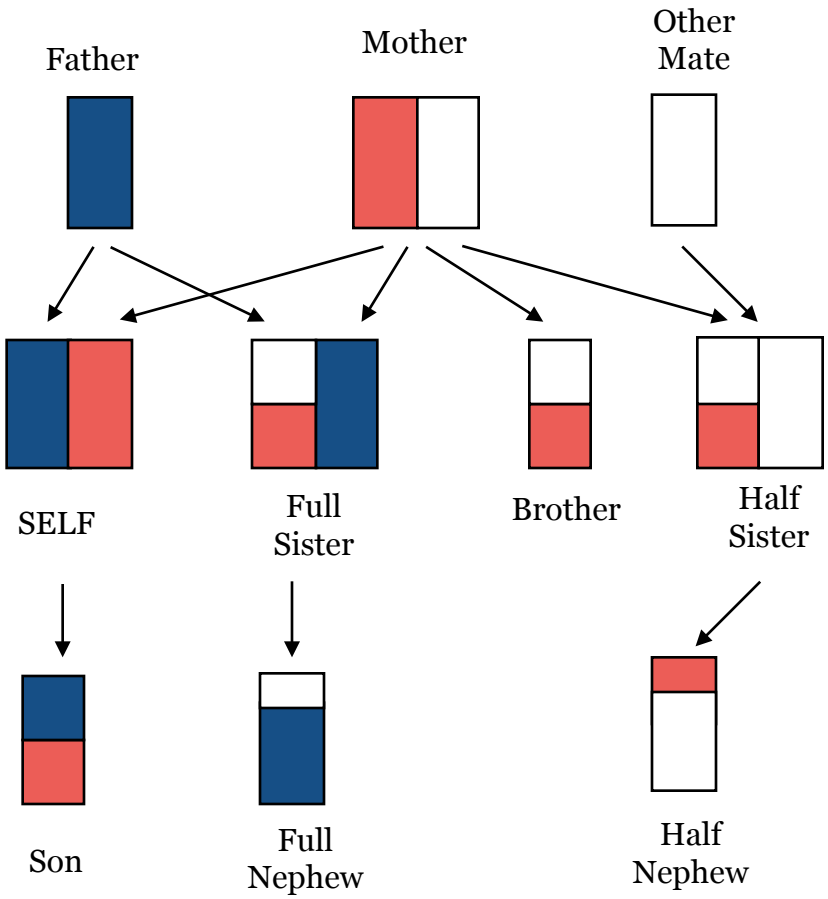

Figure 1.3 A haplodiploid pedigree. The coloring shows the degree of shared genes between individuals and 'SELF'. 'SELF' is female and 100% related to herself. Half her genes come from her father (blue) and half from her mother (pink). As males are haploid, a SELF's son gets all his genes from his mother. On average, 50% of those genes will be his grandfathers (blue) and 50% will be derived from his grandmother (pink). White indicates genes not present in 'SELF'.

a.	**Paths**	**Formula**	***r***
Father	Self -> Father	$(0.5)^1$	0.5
Mother	Self -> Mother	$(0.5)^1$	0.5
Full Sister	Self -> Father -> Full Sister Self -> Mother -> Full Sister	$(0.5*1) + (0.5)^2$	0.75
Half Sister	Self -> Mother -> Half Sister	$(0.5)^2$	0.25
Brother	Self -> Mother -> Brother	$(0.5)^2$	0.25
Son	Self -> Son	$(0.5)^1$	0.5
Full Nephew	Self -> Father -> Full Sister -> Full Nephew Self -> Mother -> Full Sister -> Full Nephew	$(0.5 * 1 * 0.5) + (0.5)^3$	0.375
Half Nephew	Self -> Mother -> Half Sister -> Half Nephew	$(0.5)^3$	0.125

b.	**Paths**	**Formula**	***r***
Father	Father -> Self	$(1)^1$	1
Mother	Mother -> Self	$(0.5)^1$	0.5
Full Sister	Full Sister -> Father -> Self Full Sister -> Mother -> Self	$(0.5 * 1) + (0.5)^2$	0.75
Half Sister	Half Sister -> Mother -> Self	$(0.5)^2$	0.25
Brother	Brother -> Mother -> Self	$(1 * 0.5)$	0.5
Son	Son -> Self	$(1)^1$	1
Full Nephew	Full Nephew -> Full Sister -> Father -> Self Full Nephew -> Full Sister -> Mother -> Self	$(1 * 0.5 * 1) + (1 * (0.5)^2)$	0.75
Half Nephew	Half Nephew -> Half Sister -> Mother -> Self	$(1*(0.5)^2)$	0.25

Table 1.3 Calculation of some important family relationships in haplodiploids from a) 'SELF' in Fig. 1.3 and b) relative to 'SELF'. It is important to note that relationships are not equal e.g. Fathers share a higher proportion of their genes to daughters than daughters are have with fathers.

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CHAPTER 2

Genetic and Environmental Effects on Ovary Activation

2.1 Abstract

Honey bee workers are functionally sterile in the presence of the queen. However, when the queen is lost workers activate their ovaries and compete over production of the final batch of male brood. Whether or not a worker activates her ovaries and lays eggs may be determined both by her genotype and environment. Here, I examine worker ovary development using African and European bees in 3 separate experiments. Firstly, I dequeened African and European colonies and assessed both the presence of eggs and levels of ovary activation on a daily basis. I showed that African bees commence egg laying sooner than European bees. However, I found little evidence that African honey bees activated their ovaries faster than Europeans. Rather, worker reproduction may be constrained through continued policing, despite high levels of ovarial development. Secondly, I examined the effect of queenless duration on ovary development, and found that ovarial development was inhibited in colonies that had been queenless for an extended period of time. Finally, I examined the effect of individual genotype, and the social genotype of the rearing and adult environment on ovary development. All three factors affected ovarial development, with their relative import changing as the bee aged. The significant effect of social environment suggests a possible epigenetic mechanism controlling ovary development whilst, the delayed onset of laying behavior suggests intragenomic conflict may be resolved differently, through policing, in the two subspecies.

2.2 Introduction

Honey bees have been upheld as the exemplar of a harmonious society because workers refrain from reproducing, instead working to rear the brood of a single queen. To achieve this, honey bee workers transition through multiple subcastes throughout their lives (Wheeler, 1986). Subcastes include cell cleaners, nurses who tend to the brood and queen, food storers who receive nectar and pollen from foragers and pack it into cells, and foragers (Seeley, 1982). Nurse bees abdominal fat bodies (functionally homologous to the vertebrate liver and white fat) store proteins and lipids, and the hypopharyngeal glands in the head take up nutrients produced by the fat body to produce royal jelly, the proteinaceous substance fed to brood (Crailsheim, 1990). Worker bees normally remain as nurses for the first 2 to 3 weeks of their life, after which they transition to foraging (Seeley, 1982). Foragers work outside the colony, collecting pollen and nectar for the colony. They tend to only live as foragers for 7-10 days. However, the social environment can delay, promote, or even reverse the transition to foraging. When there are less brood, and little nursing or provisioning behavior is required, workers will develop their fat body protein stores and can live for about 20 weeks, allowing the colony to survive over winter (Omholt, 1988; Smedal *et al.*, 2009). When there are few young, nurse bees in the colony about 20% of the foragers revert to nurse behavior (Page *et al.*, 1992; Robinson, 1992; Huang and Robinson, 1996).

Whilst worker honey bees rarely activate their ovaries in the presence of the queen and larvae (Ratnieks, 1993), when a colony becomes queenless workers develop their ovaries and approximately 10% of the workers have fully formed eggs in their ovaries (Velthuis,

1970). However, worker ovary activation is not only dependent on the absence of the queen. The absence of larvae may also be necessary as larvae produce a variety of pheromones which not only communicate their need for care but also inhibit ovary development (Trouiller *et al.*, 1991; Arnold *et al.*, 1994; Mohammedi *et al.*, 1996 & 1998; Oldroyd *et al.*, 2001; Pankiw *et al.*, 2003). Likewise, temperature (Lin and Winston, 1998), trophallactic interactions with other workers (Korst and Velthuis, 1982), and the amount of protein received both at the larval and adult stage (Hoover *et al.*, 2006) have all been found to effect a worker's ovarial development. The ancestry of individuals that makeup the social environment may also be important. For example, anarchistic honey bees activate their ovaries in the presence of the queen and brood (Oldroyd *et al.*, 1994). However, when placed into a queenright colony with wild type larvae only 16% of the anarchistic strain activated their ovaries compared with 41% when larvae were solely from the anarchistic strain. Likewise, 0% of the wild type bees activated their ovaries when in the presence of wild type brood compared with 13% when placed with anarchistic brood (Oldroyd *et al.*, 2001). As such, results suggest that the rate of ovary activation reflects not only the genotype of the worker bee, but also the "social genotype" of the brood it encounters.

Honey bee subspecies also behave quite differently. Africanized bees (*Apis mellifera scutellata*) are more aggressive than those of European ancestry (*Apis mellifera ligustica* and *Apis mellifera mellifera*; Collins *et al.*, 1982). African colonies bias their foraging efforts towards pollen collection (Page *et al.*, 2000; Fewell and Bertram, 2002), more rapidly convert pollen into brood (Spivak, 1992; Schneider and McNally, 1993),

and devote a larger proportion of the colony to brood rearing (McNally and Schneider, 1992; McNally and Schneider, 1996), leading to increased colony growth rate compared to that of European bees (Otis, 1991; Winston, 1992). Finally, African bees have more ovarioles than European honey bees (Ruttner and Hesse, 1981; Thuller *et al.*, 1996; Linksvayer *et al.*, 2009).

Understanding the interplay of environmental and genetic factors on honey bee behavior is important as it furthers understanding of the mechanisms behind phenotypic plasticity and aids researchers in minimizing confounding effects and establishing effective methodologies. Here, I examine the interplay between present and past environment on ovary development in African and European honey bees. Firstly, I examine ovary activation rate compared to the onset of laying by dequeening African and European colonies, and dissecting their ovaries daily until worker laying commenced. Secondly, I examine the effect of the duration of queenlessness on ovary development. In nature, when colonies first become queenless developing brood will be present. As such, workers may emerge into a colony that has only been queenless for a short while or, up to 3 weeks into the queenless condition. Workers emerging into colonies that have been queenless for an extended period should activate their ovaries more rapidly so as to contribute to the last cohort of drones. To study this I introduced newly emerged workers into colonies which had been either recently dequeened or queenless for two weeks and commenced worker laying. Finally, I examine the effects of ancestry, rearing environment and the queenless colony on ovary development through

rearing African and European brood in African and European colonies, and then placing the emerging workers into queenless colonies of African and European ancestry.

2.3 Methods

2.3.1 Sample Collection

2.3.1.1 Experiment 1 - Rate of Ovary Activation, Prior to Egg Laying, in African and European Honey Bees

Four Africanized (*Apis mellifera scutellata*) and four European (*Apis mellifera ligustica*) honey bee colonies were selected from those available at the USDA's Carl Hayden Bee Research Center, Tucson, Arizona. From these source colonies queenless nucs (small colonies that can hold up to five frames) were established. Each of the 8 nucs was furnished with an empty brood frame and a frame full of capped nectar. Around 5000 bees were shaken into each nuc. The nucs were then closed completely so that the bees could not fly and return to the natal, queenright part of the colony. Mesh covered the top of the colony and the entrance, allowing the bees to breathe, and they were sprayed with water twice a day, providing hydration, for the duration of the experiment.

Every day the colonies were checked for eggs and a sample of ~50 bees removed from the colony and placed in a freezer to store until dissection. Honey bee colonies consist of approximately 12 patrilines and patrilineal effects on ovary activation has been identified (Martin *et al.*, 2004). As such, the sample size of 50 bees per day was chosen to gain a daily average unlikely to be skewed by patrilineal effects. Removing 50 bees

per day from nucleus colonies comprising 5000 bees seems unlikely to significantly impact worker laying onset which has been observed commencing within 6 days in colonies of less than 300 bees (Narraway, unpublished data).

When eggs were located in a nuc they were counted and an empty frame of drone comb was placed between the brood and nectar frame. Twenty-four hours later the nuc was checked for eggs; eggs were counted and which frame they were on was noted. A sample of 50 bees was taken and stored in a freezer until dissection. This was repeated until the bees were laying promiscuously (more than 100 newly laid eggs in a 24 hour period). Observations and collections for that nuc then ceased but continued across all nucs where promiscuous laying had not been observed.

After 10 days of daily checks and collections some colonies were becoming limited in the number of workers. As such, after 10 days samples of worker bees were taken every 3 days whilst checks for eggs continued daily. If eggs were found in a nuc a sample of ~50 workers was taken that day, drone comb was inserted into the colony, and the protocol followed that described above.

2.3.1.2 Experiment 2 - Effect of Queenless Period on Ovary Activation in African and European Honey Bees

Colonies were selected from those available at UC Riverside, and mitotyped to assess ancestry (Crozier *et al.*, 1991). Six African (*Apis mellifera scutellata*) and five European (*Apis mellifera ligustica*) colonies were selected and the queens placed into cages with

empty brood comb for 24 hours to lay eggs. The sides of the cages were comprised of excluder material that allowed worker bees to pass in and out but prevented the queen from doing so. The queens were removed and the frames were placed above a queen excluder (excluder material, placed between hive boxes), in their natal colony, to continue their development. Seventeen days later the frames were placed into an incubator at 35°C, 95% humidity to emerge. Every 24 hours, for 2 days, up to 200 newly emerged workers were marked with colony and date specific paint marks on their thorax and abdomen.

Two weeks prior to marking, a queenless nuc was established containing one frame of empty drone comb, one frame of nectar, and one frame of pollen. By the first day of marking eggs had started to appear. On the day of the first marking a second queenless nuc was also established containing one frame of empty drone comb, one frame of nectar, and one frame of pollen. Half of the marked bees from each colony were placed in the newly queenless nuc and the other half were placed in the nuc that had been queenless for 2 weeks. 10% of the bees were then collected every 8 days for 24 days (4 collections) and stored in a freezer until dissection.

2.3.1.3 Experiment 3 - Effect of Genetic and Social (Rearing and Adult) Environment on Ovary Activation in African and European Honey Bees

Four European (*Apis mellifera ligustica*) and four African (*Apis mellifera scutellata*) colonies were selected from those available at UC Riverside, and mitotyped to confirm ancestry. Empty brood frames were cut in half and stapled back together. They were

then placed above a queen excluder (a metal sheet that sits between hive bodies and prevents the movement of the queen between sections but allows workers to pass freely) for 48 hours so that worker honey bees could fix any gaps in the wax between the two sections. The frames were then placed into cages, the sides of which were comprised of excluder material, with a European or African queen. After 24 hours the frames were removed and the two halves separated and joined with half from a colony of the opposite ancestry. This created two frames both with brood from 2 sources. One of these was placed into the brood nest of a European colony, the other was placed into the brood nest of an African colony, for rearing. The brood nest was located above a queen excluder so that eggs could not be replaced by the resident queen.

Just prior to emergence (17 days after egg laying) the complete frames were removed from the colonies and split in half. As such, both the source and rearing colony were known for each half frame. These frames were placed into an incubator at 35^{o}C and 95% humidity. Every 24 hours, for 2 days, up to 400 newly emerged workers, per half frame, were given a source/rearing colony specific color marking on their thorax and abdomen. Half of the marked workers were placed into a queenless African nuc and half into a queenless European nuc. As such, workers of every origin and rearing colony type would be present in both the European and African nucs.

Every 4 days for 5 collections (until day 20) 10% of the marked workers were collected from both the African and European colony. At the same time both nucs were checked

for the presence of eggs. Samples were placed into a freezer where they were stored until dissection.

2.3.2 Ovary Assessment

The ovary activation levels of the worker honey bees were assessed using an Olympus stereozoom microscope. Ovaries were dissected following Dade (1962) and classified by their level of activation using a 5 level system; 3 levels were classed as inactivated ovaries (I) and 2 as activated ovaries (A, see Fig. 2.1). Level I1 denoted ovaries that were translucent along their entire length. Level I2 was characterized by some whitening of the ovaries and/or the formation of ovarioles at the top of each tube. Level I3 was designated by ovaries that had further whitening with oocytes beginning to form (small, distinguishable globules) at the top of each ovary. These globules are distinct from fully formed oocytes in that the outer lining of the ovariole did not follow their shape, but rather remains straight. The active levels (A1 and A2) were characterized as being white, with clearly defined ovarioles and oocytes. A1 and A2 are distinguished solely by the size of the oocytes: for A1 the largest oocyte is less than half the size of the average egg, whilst for A2 the largest oocyte was greater than half the size of an average egg.

2.3.3 Statistical Analyses

All data analyses were performed using R (version 3.1.2). In all experiments ovary activation was examined using non-parametric Wilcoxon tests. In experiment 1, the average level of ovary activation was plotted per day per colony and linear, quadratic and cubic models fitted to the data. Model fit was assessed using the adjusted R^2, AIC

and BIC scores. Factors predicting ovary activation were assessed using two- and three-way ANOVAs (experiments 2 and 3 respectively). Analyses were performed on bees of each age class independently. Analyses were not performed for days 20 and 24 due to reduced sample size. All p-values are 2-tailed, unless specified, and adjusted by a Bonferroni correction factor when multiple tests are involved.

2.4 Results

2.4.1 Experiment 1 - Rate of Ovary Activation, Prior to Egg Laying, in African and European Honey Bees

Honey bee workers of African origin laid eggs earlier, and commenced promiscuous egg laying earlier, than European workers (First Egg: $W(10) = 0$, $p < 0.01$; Promiscuous Laying: $W(8) = 0$, $p < 0.05$, Fig. 2.2). African honey bees laid their first egg on average 3 days after the removal of the queen, whilst eggs did not appear in European colonies for 2 weeks. African honey bees had higher levels of ovary activation on the first day after dequeening than European honey bees ($W(630) = 57602.5$, $p < 0.0001$, Fig. 2.3), although this result disappeared when only workers from the European colonies that reached promiscuous laying were considered ($W(536) = 3.6453.5$, $p = 0.19$). Once laying had commenced within the colony, no difference in ovary development was detected between African and European honey bees ($W(307) = 10412$, $p=0.45$, Fig. 2.3).

From the first day of dequeening to the end of the experiment African and European honey bee ovary development increased from inactive (approximately 1.5, on a scale of 1-5) to an average level of ovary activation of 3, indicating that some workers had fully

developed ovaries whilst some remained inactive. African honey bees developed their ovaries rapidly (Fig. 2.4), reaching an average ovarial development level of 3 on approximately day 6 and promiscuous egg laying occurred concurrently. African ovarial development was best described using a linear regression, although the difference in fit between linear and quadratic models was minor (Table 2.1). Ovarial development in European honey bees was found to follow a quadratic curve (Fig. 2.4, Table 2.1). In the two European colonies where laying was observed, workers activated their ovaries rapidly, reaching an average ovarial development level of 3 around day 5, which remained constant until egg laying became pervasive on days 18 and 21 (Fig. 2.4). Alternatively, European colonies that did not lay promiscuously reached their highest levels of ovary development of 2.5 on day 13 and 2.3 on day 19. African and European colonies did not differ significantly in the day that average ovary development reached a score of 2.5 ($W(6) = 4.5$, $p = 0.72$).

2.4.2 Experiment 2 - Effect of Queenless Period on Ovary Activation in African and European Honey Bees

Ovarial development increased in European honey bees from day 0 to day 16 (Day 0-8: $W(542) = 29596.5$, $p < 0.0001$; Day 8-16: $W(456) = 21326.5$, $p < 0.0001$; Day 16-24: $W(333) = 13877.5$, $p = 0.39$) whilst, overall, in African bees it increased from day 0 to 24 (Day 0-8: $W(608) = 36721.5$, $p < 0.0001$; Day 8-24: $W(267) = 1260.5$, $p < 0.05$; Fig. 2.5). Level of ovary development was higher in European than African bees on days 0 and days 16 (Fig. 2.5 and Table 2.2). This difference seems to be driven by the long term queenless colony where, overall, average levels of ovarial development did not increase

at all in Europeans (Day 0-24: W(229) = 4123; $p = 0.65$), nor until day 24 in Africans (Day 0-16: W(238) = 5862.5, $p = 0.076$; Day 16-24: W(66) = 146, $p < 0.01$), but European honey bees had significantly higher levels of ovary activation than African honey bees (Fig. 2.6b and Table 2.3b). In contrast, in the short term queenless colony there was no difference in levels of ovarial development between African and European honey bees (Fig. 2.6a and Table 2.3a). However, levels of ovarial development rose in both rose until day 16 (African - Day 0-8: W(298) = 7063, $p < 0.0001$; Day 8-16: W(182) = 2676, $p < 0.01$; Day 16-24: W(66) = 160.5, $p = 0.65$; European - Day 0-8: W(241) = 4490.5, $p < 0.0001$; Day 8-16: W(258) = 5960.5, $p < 0.0001$; Day 16-24: W(207) = 5896, $p = 0.058$).

Queenlessness was the dominant factor in predicting ovarial development in honey bee workers, although a workers ancestry had influence at day 16 but not day 8 (Table 2.4 and Fig. 2.7 and 2.8). Ovary development in workers was lower in colonies that had been queenless for an extended period than those that had recently lost their queen (Fig. 2.6, 2.7 and 2.8).

2.4.3 Experiment 3 - Effect of Genetic and Social (Rearing and Adult) Environment on Ovary Activation in African and European Honey Bees

Overall, levels of ovary development increased in African workers until day 12 (Day 0-4: W(233) = 5044, $p < 0.001$; Day 4-8: W(271) = 7348.5, $p < 0.01$; Day 8-12: W(239) 5225, $p < 0.001$; Day 12-16: W(212) = 5730, $p = 0.783$) and European workers until day 16 (Day 0-4: W(346) = 10976, $p < 0.0001$; Day 4-8: W(440) = 19897, $p < 0.001$; Day 8-12:

W(361) 16350.5, $p = 0.29$; Day 12-16: W(274) = 7323.5, $p < 0.001$; Fig. 2.8). African and European honey bees only differed in their ovary development on day 12 when African honey bees had significantly higher levels of ovary activation than European honey bees (Fig. 2.9 and Table 2.5).

None of the factors were found to have a continuous effect on ovarial development. Rather, the importance of both genetic and environmental conditions changed with the age of the bees (Table 2.6). In 4 day old workers the queenless environment was the only factor predicting ovarial development (Fig. 2.10), with those in the European environment having higher levels of ovarial development than those in the African environment (W(359) = 13980, $p < 0.05$). As the bees aged, the bees rearing environment and ancestry became more important.

On day 8 both the main effects of rearing colony and queenless colony ancestry predicted ovary development. With those in a European rearing or queenless environment having lower levels of ovary development on average than those in an African rearing or queenless environment (Rearing: W(352) = 13034, $p < 0.05$; Queenless: W(252) = 20657, $p < 0.001$). The genetic ancestry of an individual was found to interact with the queenless environment such that workers of African origin were found to experience a greater reduction in ovarial development in European colonies than European honey bees. In African colonies, African workers had an average ovarial development score of 2.33, compared to 1.46 in European colonies. For

workers of European ancestry average ovary development decreased from 1.98 in African colonies to 1.55 in European colonies (Table 2.6 & Fig. 2.10).

On day 12 the queenless colony no longer predicted whether or not a worker would have activated ovaries rather, workers of African ancestry or reared in African colonies generally had greater levels of ovary development than their European counterparts (Ancestry: $W(248) = 9679$, $p < 0.001$; Rearing: $W(248) = 9299$, $p < 0.01$). However, there was also an interaction effect of rearing colony and genetic ancestry as African workers reared in African colonies had much higher levels development by day 12 (mean = 2.47) than in European colonies (mean = 1.81) and this decrease was more than that experienced by European workers (African rearing colony mean = 1.78; European rearing colony mean = 1.68; Table 2.6 & Fig. 2.10).

No factor was an adequate predictor of ovarial development of bees aged 16 days (Table 2.6 & Fig. 2.10). This is probably due to a reduced sample size, as by day 16, marked bees were becoming scarcer within the colony. Likewise, laying had commenced in most colonies by day 16 and levels of ovarial development may have reached their maximum. As found above, ovarial development in African and European colonies at the time of laying is approximately equal, and this may be independent of the social genotype as well.

2.5 Discussion

This study shows that ovariole development is controlled by both genetic and environmental factors. African honey bees lay eggs more rapidly than European bees, once the colony has lost its queen (Fig. 2.2). However, few differences were observed in the timing of ovarial development (Fig. 2.4, 2.5, 2.6 and 2.9). Worker laying in queenright colonies is controlled by worker policing behavior. Here, workers eat the eggs of other workers or aggress upon workers with activated ovaries (Ratnieks, 1988; Ratnieks and Visscher, 1989; Visscher and Dukas, 1995). One explanation for the delayed onset of worker laying in queenless European colonies, despite high levels of ovary activation, may be the persistence of worker policing behavior. In support of this, eggs were observed in some European colonies several days before the onset of promiscuous laying. These eggs often did not survive 24 hours suggesting that workers had begun laying but that the policing mechanism had not yet demobilized.

Worker policing also controls worker laying in queenright African colonies (Calis *et al.*, 2003). The swift onset of worker laying upon ovarial development suggests that the breakdown in worker policing and the development of ovaries occurs simultaneously in African honey bee colonies, whilst in European colonies policing behavior may persist long after a high proportion of workers have activated their ovaries.

Whilst genetic ancestry, as determined from the matriline, may not have a major effect on ovarial development, the social environment does, and these effects accrue both during development and through adulthood. Workers developing in African colonies

had higher levels of ovarial development than those in European colonies. One mechanism through which such a difference may accrue is larval nutrition. African honey bees are known to forage more for pollen than their European counter parts (Pankiw, 2003). The pollen collected is either stored in the colony for later use, fed directly to larvae, or ingested by workers and converted into royal jelly, a high protein substance that is fed to larvae during the first few days of their development (Crailsheim, 1990). Honey bee larvae fed high-protein diets were found to have higher levels of ovary development than those fed low-protein diets (Hoover *et al.*, 2006). Nutritional differences also accrue during adulthood. Workers who were fed high protein diets had higher ovary scores than those fed low protein diets, and these effects were additive with larval nutrition (Hoover *et al.*, 2006).

Pheromones also control ovary development in honey bees. Pheromones produced by the queen, such as queen mandibular pheromone, are commonly credited with suppressing worker reproduction (Butler and Fairey, 1963; Wossler and Crewe, 1999; Slessor *et al.*, 2005). However, honey bee larvae also produce a variety of pheromones, communicating their presence and need for care to the workers (Mohammedi *et al.*, 1996; Pankiw *et al.*, 2000). The presence of these pheromones have been found to inhibit worker ovary development (Trouiller *et al.*, 1991; Arnold *et al.*, 1994; Mohammedi *et al.*, 1998; Oldroyd *et al.*, 2001), primarily in large queenright colonies where the queen is unable to physically control her workers. However, larvae are not only present in the colony when the queen is. Worker ovary development may be delayed by larvae present immediately after queen loss, allowing time for a replacement

queen to be reared. When queen rearing fails and worker reproduction commences ovarial development in, as yet, non-reproductive workers, may be prevented by the new cohort of worker laid larva.

It is notable that even without the presence of larvae not all of the workers in a colony activate their ovaries. Workers are able to detect workers with activated ovaries and may act aggressively towards them (Visscher and Dukas, 1995) or perform a suite of behaviors that are normally reserved for queens, called retinue behavior (Velthuis *et al.*, 1965). Indeed, the presence of laying workers, but absence of the queen, has been found to inhibit ovary development in workers (Sakagami, 1958; Velthuis *et al.*, 1965; Jay and Nelson, 1973).

Finally, laying queenless colonies decline rapidly (Page and Metcalf, 1984) and as such, the probability that worker-laid eggs will develop into drones also diminishes rapidly (Page and Erickson, 1988). As such, developing ovaries, in a colony which has already commenced worker reproduction, may not be beneficial. A worker's fitness may actually be maximized by aiding her sisters and half-sisters in rearing the final cohort of drones, rather than laying her own eggs, which are unlikely to survive to adulthood.

As such, these experiments demonstrate clearly that the social environment in which a worker is raised and ultimately lives, determine her reproductive potential, and suggest that reproductive conflict in queenless colonies may be controlled through maintenance of policing rather than the prevention of ovary development. As workers within a colony

share many of their genes these differences are probably an epigenetic effect. Nutritional differences during the rearing of honey bee larva are already known to lead to the development of queens and workers, through differences in their epigenomes (Kucharski *et al.*, 2008). Differences in protein intake may lead to similar epigenetic differences between workers. Likewise, policing, at least in queenright colonies, is predicted to be selected for on the matriline (Queller, 2003). The observed difference in onset of laying, between African and European colonies, could be the expression of intragenomic conflict, being resolved differently between the subspecies.

2.6 Figures and Tables

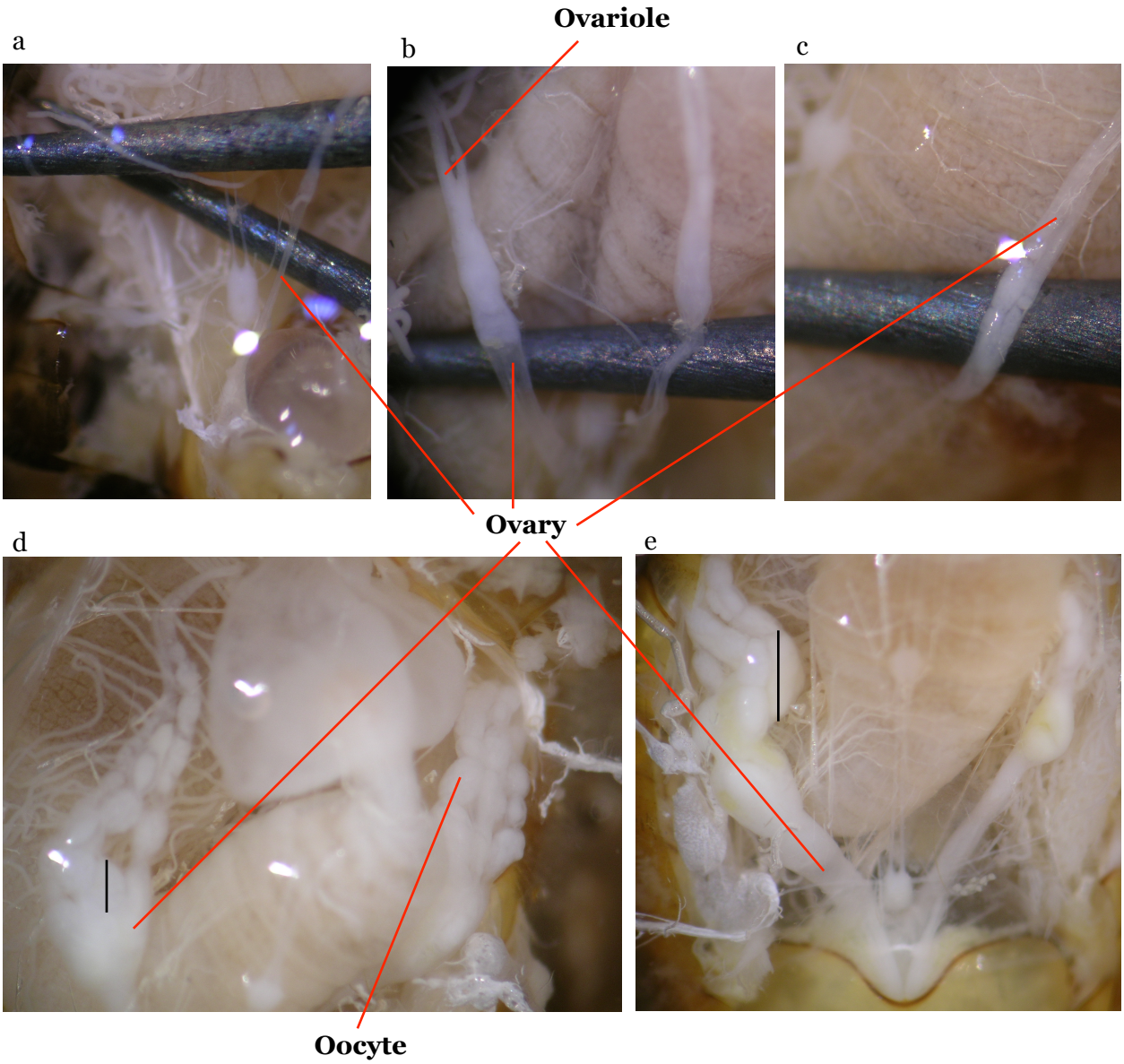


Figure 2.1 Levels of ovary activation. **a-c** are inactive ovaries levels 1, 2 and 3 respectively. **a** is distinguished form **b** in that **b** is whiter and/or the formation of ovarioles at the top of each tube. In **c** oocytes are beginning to form in the ovarioles. **d** and **e** denote activated ovaries levels 1 and 2 respectively. Unlike in **c** oocytes are fully formed. **d** and **e** are distinguished by the length of their largest oocyte (black line).

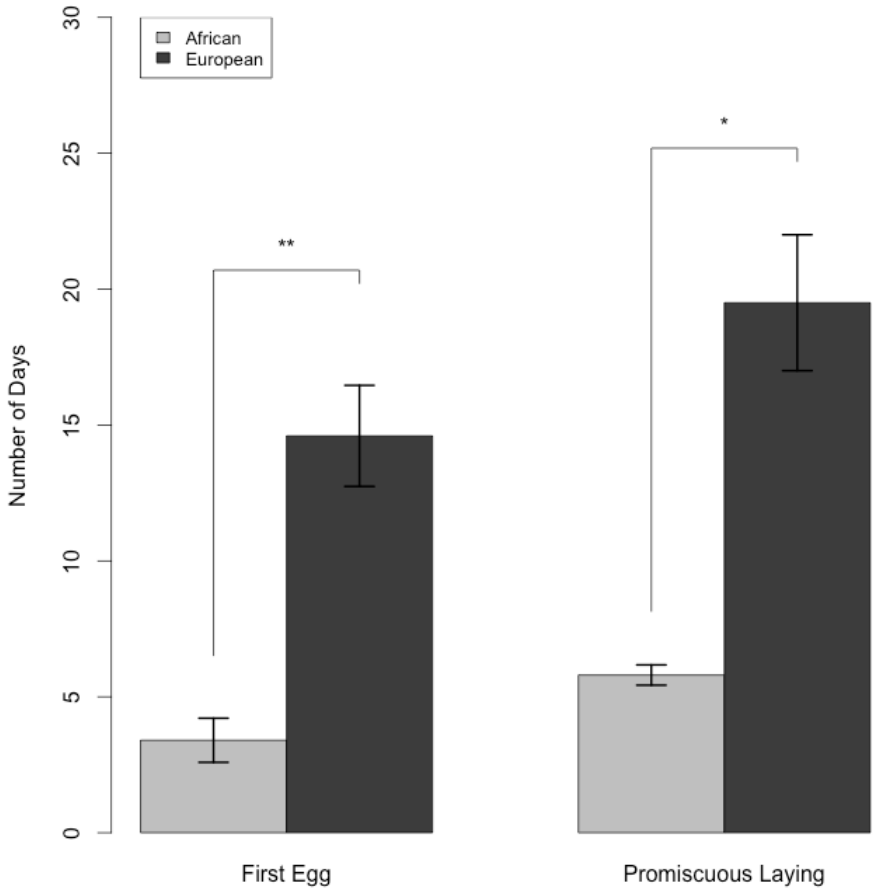


Figure 2.2 Average number of days to the appearance of the first worker-laid egg and to promiscuous laying, defined as more than 100 eggs laid in 24 hours, by honey bees of African (grey) and European (black) ancestry ± standard error. Honey bees of African ancestry began laying eggs, and began laying many eggs, earlier than European honey bees (First egg: $W(10)=0$, $p < 0.005$; $W(8)=0$, $p < 0.05$, one-tailed test).

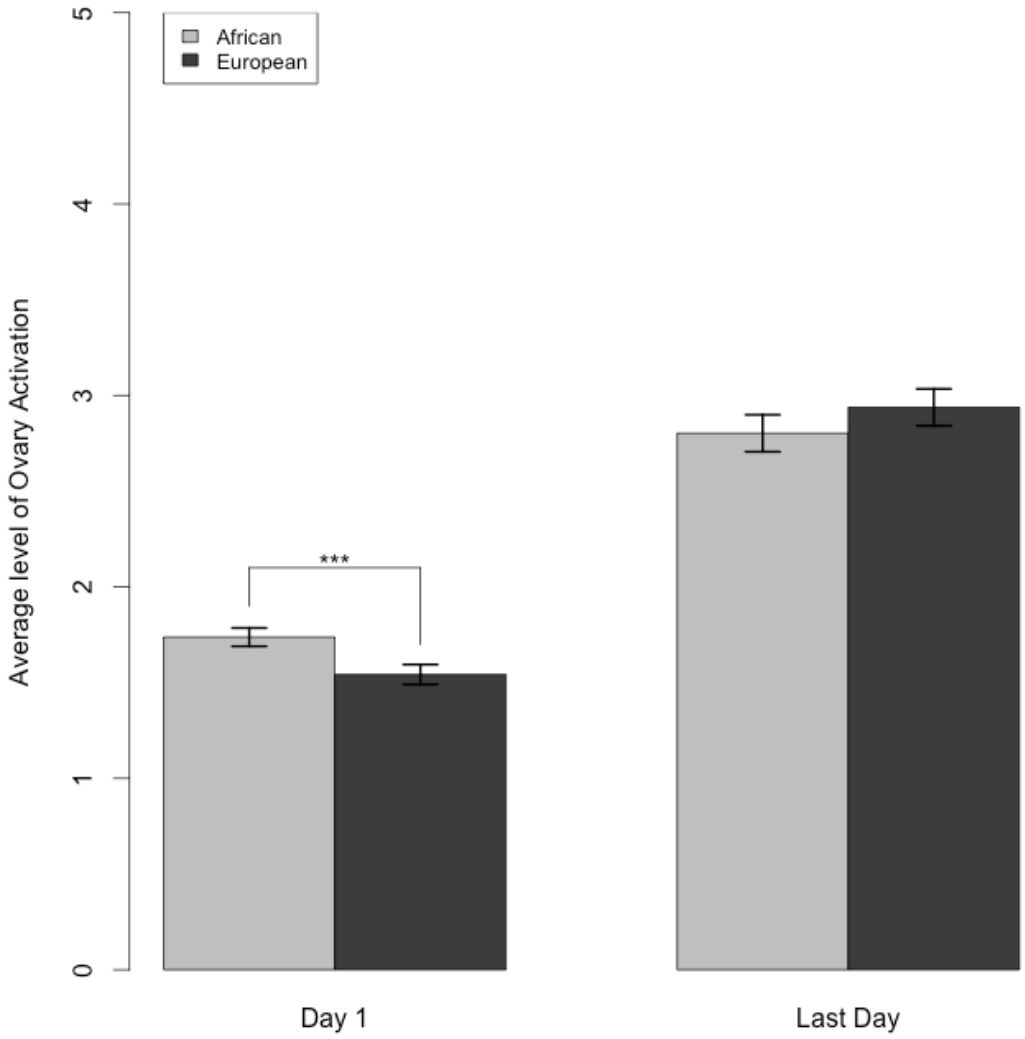


Figure 2.3 African (grey) honey bees had higher levels of OA (± standard error), than European honey bees, on the first day of dequeening, but not once promiscuous laying had commenced (Day 1: $W(630) = 57602.5$, $p < 0.001$; Last Day: $W(301) = 10412$, $p = 0.45$).

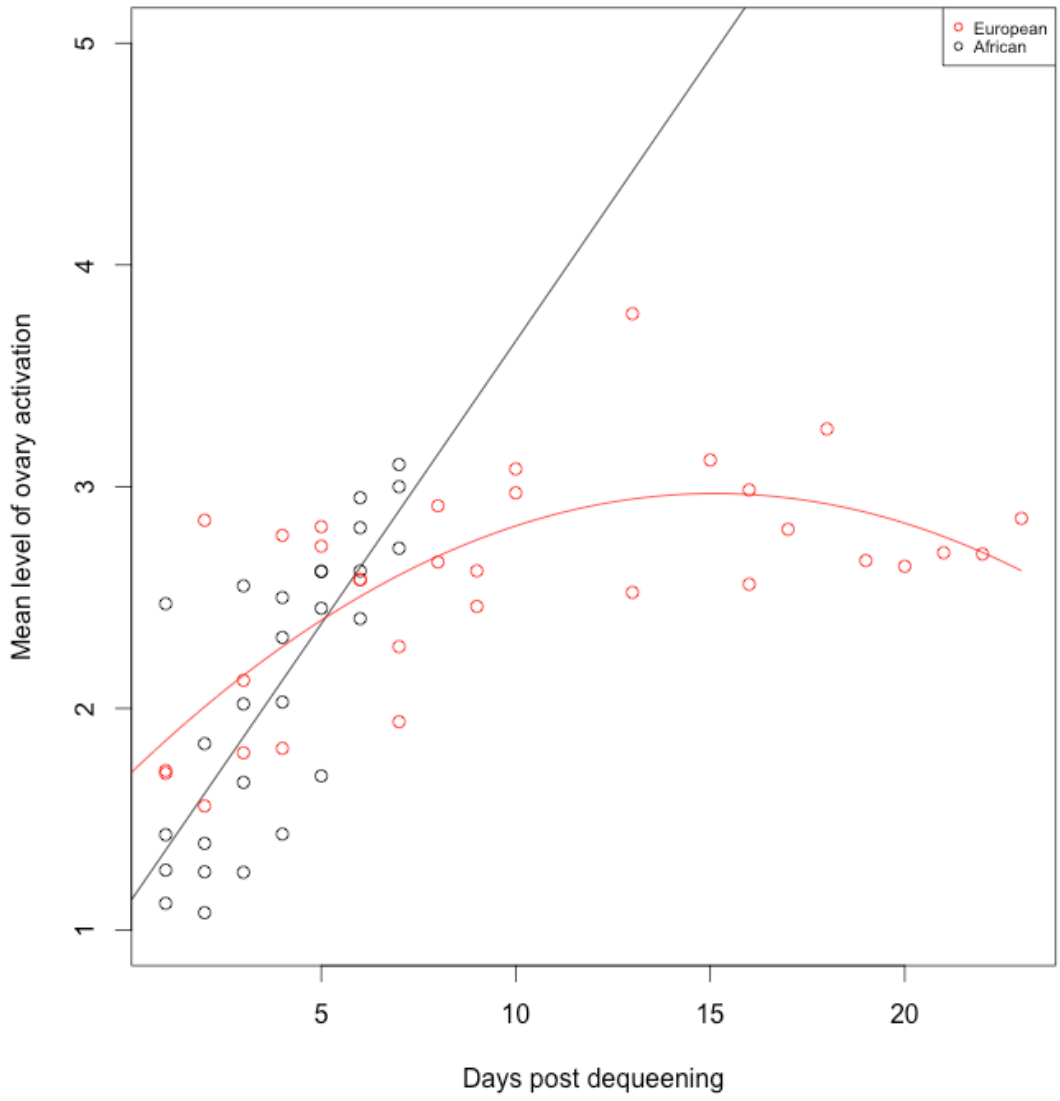
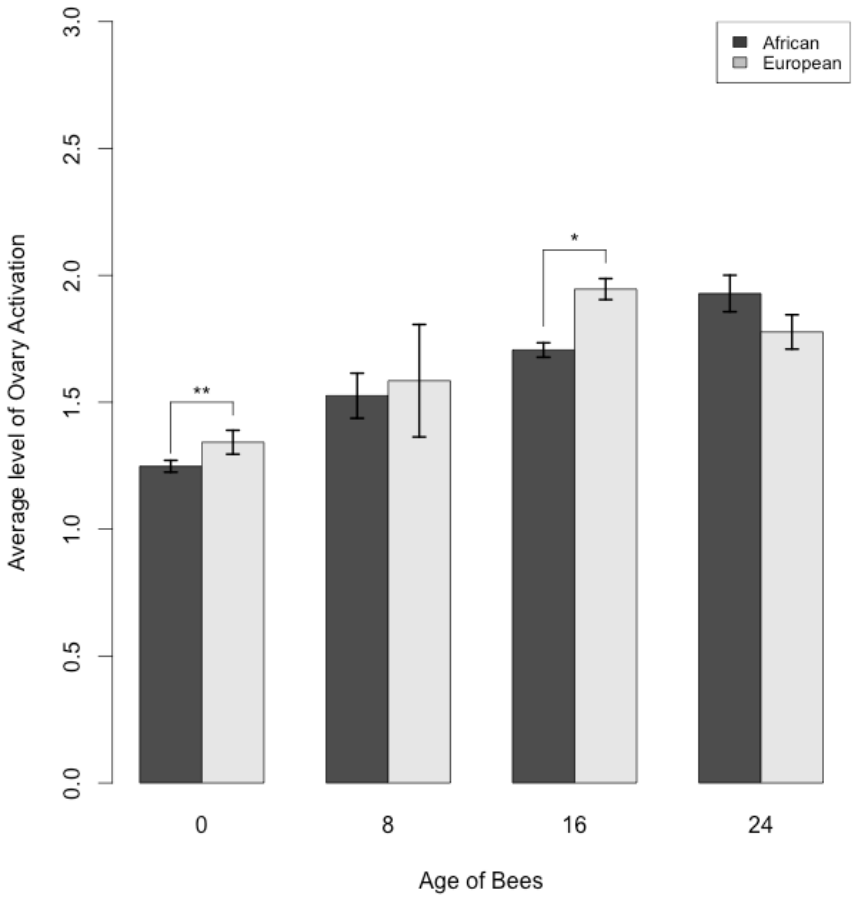


Figure 2.4 Ovary activation in African honey bees (black) is best described by a linear model, whilst in European honey bees (red) the best fit model is quadratic. Data points show the average level of ovary activation per day per African and European colony. Only colonies where egg laying became common is shown.

Ancestry		Linear Model	Quadratic Model	Cubic Model
AHB	Adjusted R^2	**0.5962**	0.592	0.5888
	F	**39.38** ****	19.86 ****	19.61 ****
	df	**1 & 25**	2 & 24	2 & 24
	AIC	**32.65**	33.83	35.11
	BIC	**36.54**	39.01	41.59
EHB	Adjusted R^2	0.2746	**0.4487**	0.4314
	F	12.74 ***	**13.61** ****	8.84 ****
	df	1 & 30	**2 & 29**	3 & 28
	AIC	39.88	**32.02**	33.90
	BIC	44.28	**37.88**	41.21

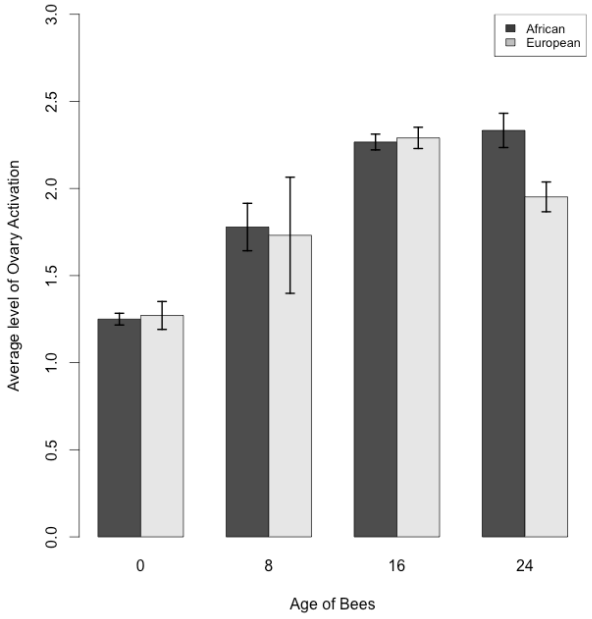
Table 2.1 Ovary activation in African honey bees is best described by a linear model, whilst in European honey bees the model that describes the data best is quadratic. This is determined and confirmed using the adjusted R^2, AIC and BIC.



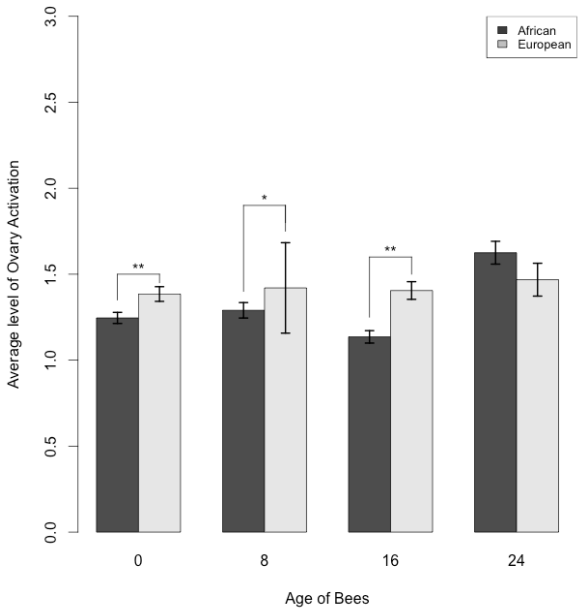
Age	W	n	*p*
0	46479.5	644	0.0089
8	29349.5	508	0.068
16	10230.5	322	0.013
24	1011.5	144	0.46

Figure 2.5 & Table 2.2 Average level of ovary activation (± standard error) was significantly higher in European (grey bars) than African (black bars) workers on days 0 and 16 (table gives the results of the two-way Wilcoxon tests).

a.



b.



Age	W	n	*p*
0	9245	283	0.73
8	8068	256	0.84
16	3692	184	0.93
24	321	89	0.20

Age	W	n	*p*
0	14028	361	0.0048
8	6868	250	0.048
16	1783	138	0.0022
24	211	55	0.54

Figure 2.6 & Table 2.3 Average level of ovary activation (± standard error) for African (black bars) and European (grey bars) workers in **a**) the colony that had only just become queenless when, **b**) a colony that had been queenless for an extended period of time before, workers were introduced. Table beneath give the results of the two-way Wilcoxon tests.

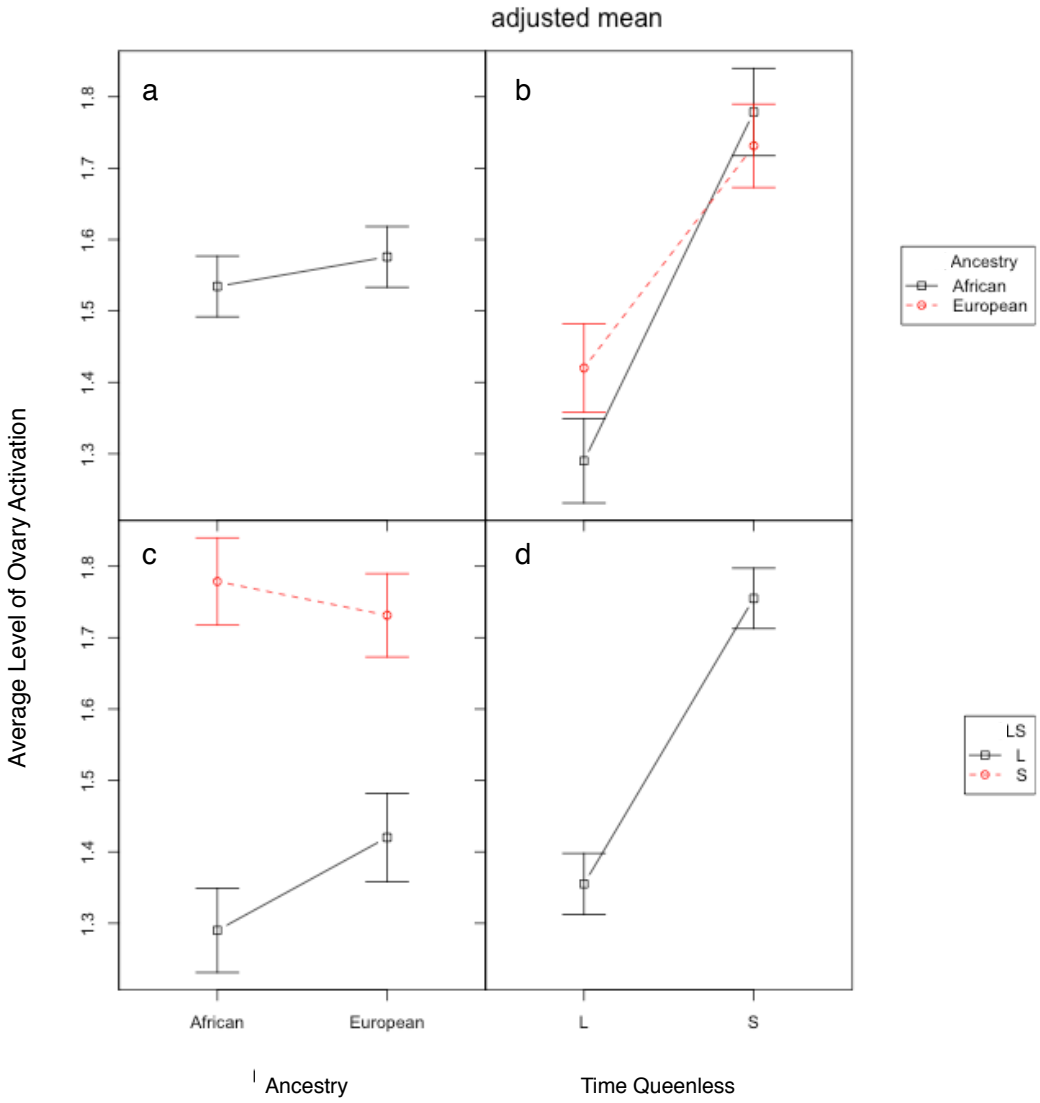


Figure 2.7 Interaction plot showing the level of ovary activation in 8 day old African and European honey bee workers, in colonies that had been queenless for long and short periods of time. Section **a**) depicts the effect of ancestry, without colony status, section **d**) is the effect of colony status without ancestry, section **b**) shows ancestry, by colony status, and section **c**) shows the effect of colony status by ancestry, on ovarial development. Only colony status had a significant effect on ovary development (Table 2.2).

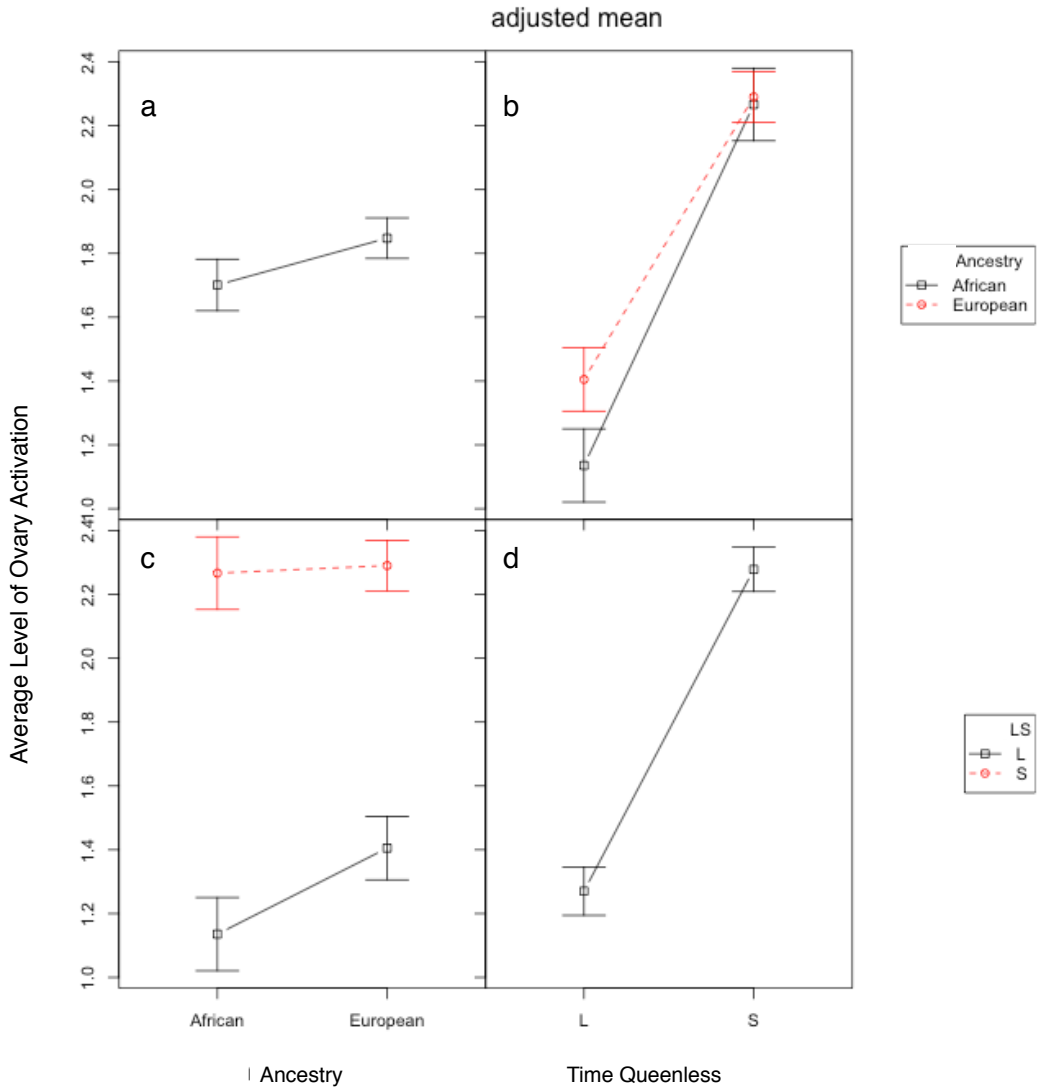


Figure 2.8 Interaction plot showing the level of ovary activation in 16 day old African and European honey bee workers, in colonies that had been queenless for long and short periods of time. Section **a**) depicts the effect of ancestry, without colony status, section **d**) is the effect of colony status without ancestry, section **b**) shows ancestry, by colony status, and section **c**) shows the effect of colony status by ancestry, on ovarial development. Both colony status and ancestry had significant effects on ovary development (Table 2.2).

	Day			
	8		16	
	df	F	*df*	F
Ancestry	1	0.97	1	**5.55***
Time Queenless	1	**44.20*****	1	**96.16*****
Ancestry x Time Queenless	1	2.17	1	1.43

Table 2.4 The dominant factor in predicting whether or not a worker developed her ovaries was the length of time the colony had been queenless. The ancestry of a worker had predictive power at day 16, but not at day 8. However, there was no interaction effect. Model simplification did not alter this result.

Age	W	n	*p*
0	5440	220	0.1792
4	14412	359	0.2511
8	14005.5	353	0.6095
12	9679	248	5.939E-05
16	7241	238	0.5556
20	774	73	0.1617

Table 2.5 Two-tailed Wilcoxon tests revealed no difference in level of ovary development between European and African workers aged 0 to 20, except for bees aged 12 where African workers had significantly higher levels of ovarial development than European workers.

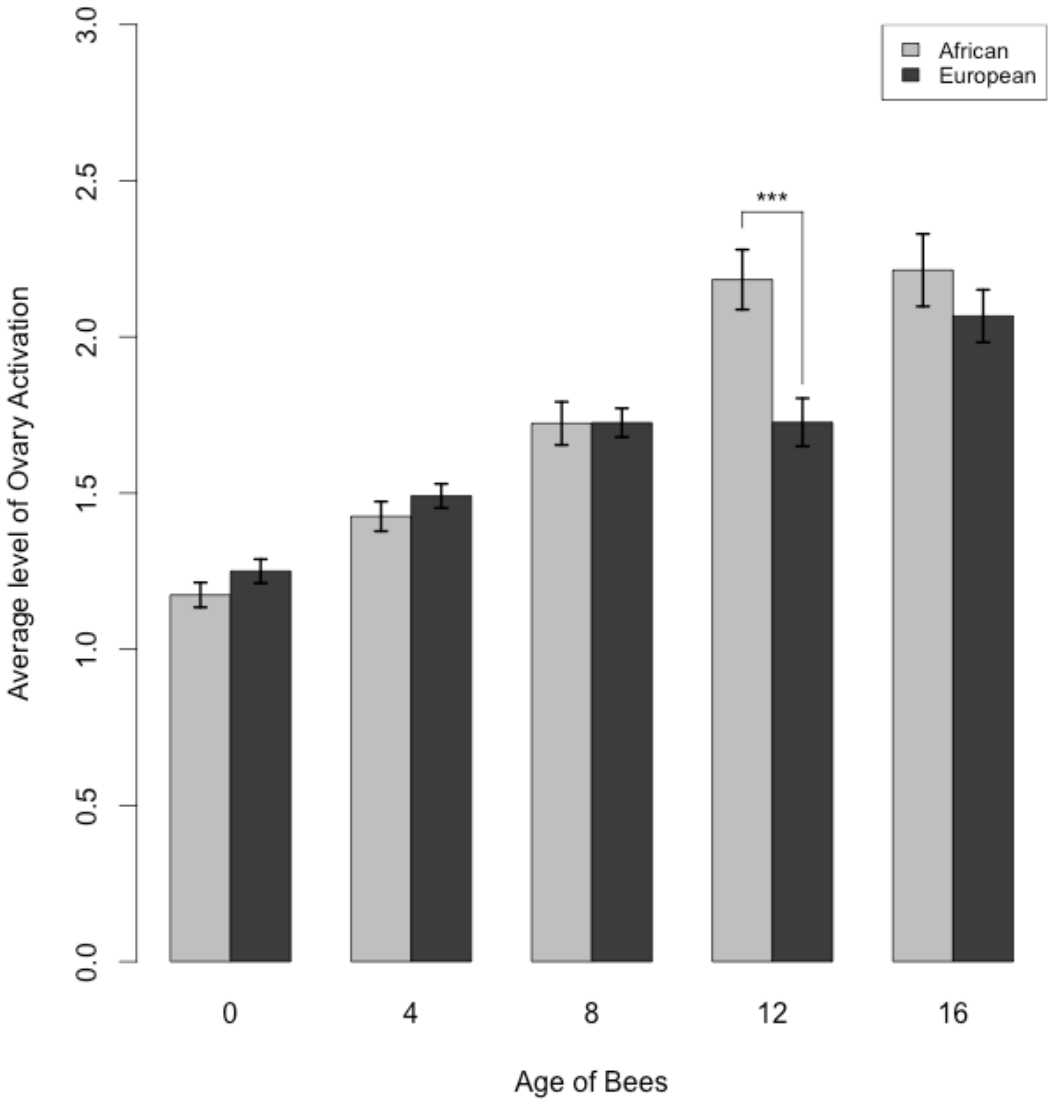


Figure 2.9 The average level of ovary activation (± standard error) for honey bees of African (grey) and European (black) ancestry. There was no difference in level of ovarial development between African and European honey bees except at age 12 where Africans had significantly higher levels of ovarial development than Europeans.

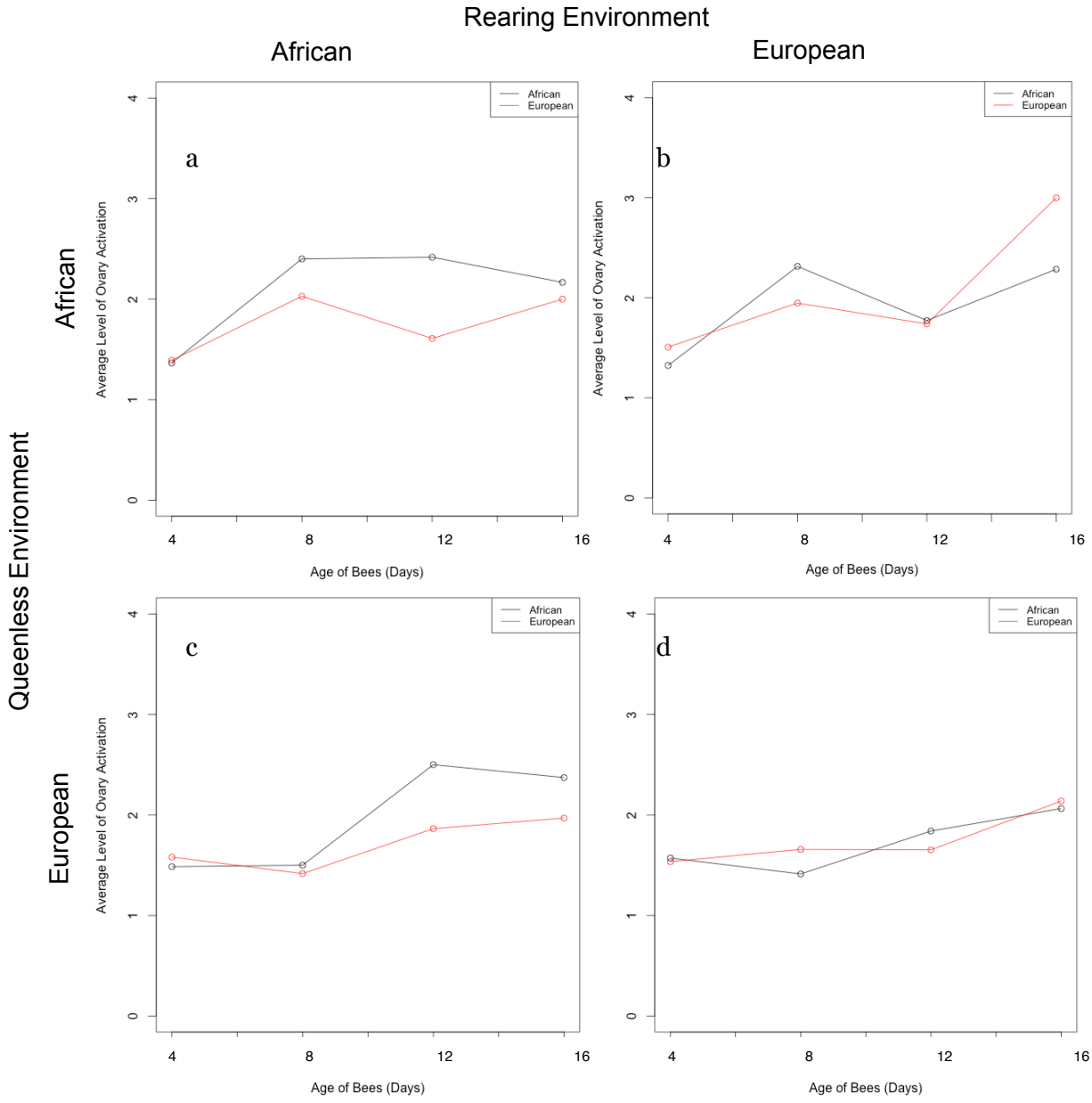


Figure 2.10 Plot showing the effect of ancestry and environment (both rearing and post emergence) on ovary development, over time. Each figure shows the average level of ovary development for African (black) and European (red) honey bee workers **a**) when both reared and emerged into an African colony, **b**) reared in an European but emerged into an African colony, **c**) reared in an African but emerged into an European colony, and **d**) reared in and emerged into an European colony.

	Age							
	4		8		12		16	
	df	F	*df*	F	*df*	F	*df*	F
Ancestry	1	1.14	1	0.00	1	**14.66*****	1	1.10
Rearing	1	0.64	1	**5.51***	1	**8.21****	1	0.00
Queenless	1	**1.47***	1	**58.08*****	1	0.59	1	0.17
Ancestry:Rearing	1	0.08	1	0.26	1	**5.34***	1	2.59
Ancestry:Queenless	1	0.45	1	**8.71****	1	0.00	1	0.28
Rearing:Queenless	1	0.13	1	2.38	1	0.60	1	1.09
Ancestry:Rearing:Queenless	1	1.31	1	0.68	1	0.43	1	0.12

Table 2.6 Factors affecting ovary activation in worker bees was found to change as the age of the bees/ duration of queenlessness increased. Ancestry refers to a workers maternal ancestry (African or European), rearing refers to the ancestry of the colony that the worker was reared in (African or European), and queenless refers to the ancestry of the queenless colony that newly emerged workers were placed into (African or European). The model was analyzed using a three-way ANOVA.

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CHAPTER 3

Multilevel Selection in Social Insect Reproduction

3.1 Abstract

Since Hamilton first proposed his rule, relatedness based kin selection, acting at the level of the individual, has been the dominant paradigm for evaluating the evolution of social behavior. However, selection may also act to maximize colony efficiency (group level selection) or bias behavior towards benefitting one parent's genetic interests over the others (intragenomic level of selection). In colonies of many eusocial insects workers can activate their ovaries and lay male eggs. When the queen is present, other workers may police these eggs by eating them, ostensibly to maximize relatedness within the colony (individual level selection). However, worker policing may also maximize group level efficiency (group level selection) or be the result of conflict between the maternal and paternal portions of a worker's genome (intragenomic level selection). Here we simulate a community comprising 500 nests of a haplodiploid species, over 400 generations, to determine the effect and role of group, individual and intragenomic levels of selection on worker reproductive and policing behavior. Using decision trees to examine the importance of different levels of selection in determining behavioral outcomes we found that all levels of selection impacted conflict over worker reproduction. However, intragenomic and group level effects were always the dominant factors deciding male parentage, with individual level selection being a secondary mechanism. We also found no evidence that worker laying/policing specialization would evolve except when genes were imprinted, in which case paternal effects would reduce the probability that laying workers would also police.

3.2 Introduction

Until about a decade ago Hamilton's inclusive fitness theory appeared to have settled a fundamental question in evolutionary biology: how does altruism, where individuals subordinate their own fitness interests to help others, evolve? What Hamilton realized was that if a trait increases the success (b) of another related (r) individual more than it reduces the reproduction of the trait-bearing individual (c), then evolution of the trait will be favored (Hamilton, 1964a & b). He expressed this in his famous rule: rb > c.

The 2005 publication by E. O. Wilson, an early advocate of inclusive fitness, questioning the usefulness of Hamilton's rule, sparked widespread, and ongoing, debate (Wilson, 2005; Foster *et al.*, 2006; Traulsen and Nowak, 2006; Lehman *et al.*, 2007; Wild *et al.*, 2009; Wade *et al.*, 2010; Liao *et al.*, 2015). The crux of Wilson's argument is that the importance of relatedness, acting at the individual level, has been over emphasized, and that ecological factors occurring at the level of the group are largely ignored but may be of higher importance (Wilson, 2005).

Whilst, the debate centers around the ability of an inclusive fitness approach to sufficiently subsume group level effects, multilevel selection is not confined to the individual and group levels (Sober and Wilson, 1998). Indeed, selection can act at all levels of biological organization (chromosomes to communities), either in opposing or complementary directions, with the strongest selective force dominating alternate levels of selection.

Whilst evidence for inclusive fitness acting at the level of the individual comes from a variety of sources, it is worker policing behavior that has been described as the *prima facie* example (Whitfield, 2002; Sugden, 2004). Here, workers in eusocial insect colonies not only refrain from laying their own eggs, preferring to help rear their mothers offspring, but also punish workers who do attempt to reproduce. Worker policing may take the form of workers removing worker-laid eggs but not queen-laid eggs (Ratnieks and Visscher, 1989), or workers acting aggressively towards workers with activated ovaries (Visscher and Dukas, 1995).

Predictions from relatedness, acting at the level of the individual, state that worker policing should occur in haplodiploid social species when females mate with multiple males. The resultant colony could potentially produce males with four different levels of relatedness to a given worker: 1) $r = 0.5$ for the workers own sons (assumed to be relatively few because workers are not highly fecund), 2) $r = 0.25$ for brothers, 3) $r = 0.375$ for nephews produced by full sisters, 4) $r = 0.125$ for nephews produced by half sisters. If the colony is headed by a single, monogamous queen, then the average relatedness of worker-produced males will always exceed that for brothers. As such, workers should favor themselves and their sisters to reproduce males, over their mother, and therefore, workers should never police. If however, more than two males father the workers, half-nephews will dominate amongst worker-produced males. So, whilst individual workers can gain direct benefits by laying eggs that are reared by the colony, the collective workforce should prefer queen-laid eggs over those laid by other workers. Worker policing is the key mechanism for resolving this conflict (Ratnieks,

1988; Ratnieks and Visscher, 1989). Workers can discriminate between queen and worker-laid eggs, but not between eggs laid by full or half sisters (Reeve and Jeanne, 2003). Therefore, kin selection theory predicts fitness is maximized for non-laying workers by indiscriminately removing all worker-laid eggs (policing) and allowing queens to replace them with brothers (Ratnieks, 1988). All examined species in which queens mate with several males exhibit strong worker policing as predicted by kin selection (Wenseleers and Ratnieks, 2006).

Contrary to predictions based on relatedness, the removal of worker laid eggs is also observed in eusocial colonies where the queen is monogamous (Foster *et al.*, 2002). Here, the collective workforce should maximize their fitness by allowing workers to monopolize male production. In such situations high group level costs, associated with worker laying, are evoked to counteract the benefits from individual level selection. Worker laying can be opposed at the level of the group in two ways. Firstly, worker-queen and worker-worker conflict over male parentage may reduce the total number of reproductives (queens and males) a colony can produce. Therefore, colonies that peacefully cede all reproduction to queens would be at a selective advantage to less harmonious ones (Ratnieks *et al.*, 2006). Second, worker eggs may be less viable than queen-laid ones (Pirk *et al.*, 2004). Thus, at the group level worker policing is predicted to occur whenever worker laying leads to a significant decrease in reproductive output.

A third level of selection acting on worker policing may occur between the maternal and paternal portions of a workers genome (Haig, 1992; Queller, 2003; Kronauer, 2008). As

workers are the equal genetic offspring of a queen mother and a father, all worker-produced males have genetic contributions from both grandparents. Queen-produced sons, however, contain only her genes and none from her mates. Therefore, the only route by which a father's genes can be represented in the colony's male offspring is if workers reproduce. However, if worker-produced males come at the cost of fewer queen-produced males, the paternal portion of a worker's genome is expected to favor worker laying, whilst the maternal portion should favor worker policing (Queller, 2003). As such, there could be a strong selective advantage to a gene, or suite of genes, imprinted in a parent-of-origin specific manner (Burt and Trivers, 2006). Unlike selection at the group level or individual level, intragenomic conflict is an epigenetic effect that would not affect allele frequencies for actual policing or laying behavior (Jaenisch and Bird, 2003). Instead, the imprint would produce an "off" signal for policing or laying across multiple gene loci.

The current debate centers on the interaction of group and individual levels of selection, although singly, all three of the levels of selection mentioned previously, have been evoked to explain particular features of reproductive behavior in eusocial organisms. All three levels of selection are present, concurrently, within every colony. Here, I construct a simulation model to examine the behavioral and genetic outcomes when all three levels of selection are simultaneously acting upon a population. I firstly examine the interplay between individual level and group level selection on the ancestry of the male cohort produced and the level of conflict present in the colony. Secondly, I add intragenomic control, examining when and how it evolves and its effects on male

ancestry and levels of conflict within the colony. Ultimately, I examine the need for a multilevel approach in the study of social behavior.

Finally, worker policing may also present an evolutionary paradox if policing workers are also less likely to be laying workers (i.e. the traits are negatively correlated creating antagonistic pleiotropy). Such self-restraint among policing workers would exclude them from the pool of worker-laid offspring, and could be a strong selective force against the evolution of policing. However, such a bias could be selected for at the intragenomic level, as any reduction in laying behavior would benefit the maternal line, and the paternal line could benefit from reducing policing behavior amongst workers who lay. As such, I examine the evolutionary significance of epistasis in workers both when a mechanism for imprinting is available, and when it is not.

3.3 Methods

3.3.1 Model Description: Group and Individual Level Selection

The model simulated evolution over 400 generations (generation loop), in a community of 500 nests of a haplodiploid species (colony loop). Each nest contained a single, mated queen. Males were selected randomly from the mating pool and mated with the queen. Males could only be selected to mate once. If the total number of males available was less than that required to mate with all of the queens, males could be selected multiple times. This assumes that males could enter the population from nearby populations, and that those populations bore the same traits as the focal population.

Queens could lay both male and female offspring. Female offspring consisted of queens and workers (w). Each colony could produce a maximum of 50 males and 50 queens to enter the common mating pool at the end of each generation (Fig. 3.1, Table 3.1).

In nature, unmated workers are able to produce male offspring (m_w), although they do so rarely in the presence of the queen. Here, the likelihood of them becoming a laying worker was determined by their genotype at a laying locus. Workers could have a laying allele (w_l) or a null allele. The laying behavior was additive, and so workers with two laying alleles would be more likely to lay eggs than those with one laying and one null allele (super lay vs lay, Fig. 3.2). Workers with two laying null alleles would not lay.

Worker policing is the removal of worker-laid males by other workers. Like laying, policing, was determined at a single locus, was additive and comprised of two alleles: policing allele (w_p) and a neutral, non-policing allele. As such, the total number of worker males produced depended on the proportion of the colony with laying alleles, and the proportion of workers with policing alleles. All worker-produced males lost through worker policing were replaced with a queen laid male.

Worker-laid males may be less viable (v) than queen-laid males, and therefore may be less likely to survive to enter the mating pool than queen-laid males. Worker laying may also result in a decrease in the resources available to raise both queen- and worker-laid offspring, of either sex, to adulthood, as laying workers may perform less work in the

colony. As such, before males and queens could enter the mating pool some were lost due to this reduction in resource availability. This was termed the maximum efficiency cost (*e*). It is from this mating pool that queens and drones are selected to begin the next generation.

3.3.1.1 Model Set-up and Starting Conditions

Each run of the model proceeded through a series of loops (Fig. 3.1), and each run was repeated 10 times in TrueBasic (version 5.5).

Firstly the number of mates each queen mated with, the viability of worker-laid males compared to queen-laid males and the maximum loss of brood due to reduced worker helping effort, were set and remained constant throughout the simulation. Queens could be mated to 1, 2 or 10 males. The viability of worker-laid males could be 0, 0.1, 0.2, 0.3, 0.4 or 0.5. The maximum efficiency cost to the colony could be 0, 0.04, 0.08, 0.12, 0.16 or 0.2. As such, the model was run 10 times for each of the 108 different mate number, viability and efficiency cost combinations (a total of 1080 runs).

Secondly, the generation loop ran from 1 to 400 generations, at the end of which the model was terminated. Within the generation loop was the colony loop which ran from 1 to 500. The maternal and paternal genotypes of the first 500 colonies were established prior to the first generation from a pool of 10,000 males and females. The proportion of null alleles was set at 0.98, policing/laying alleles were set at 0.02.

The ability for bias to evolve, such that workers who lay would not police and workers who policed would not lay, was incorporated using two alleles: policing bias and laying bias. There were five different levels of bias: 0, 0.25, 0.5, 0.75, and 1.0. Zero denotes no bias and one denotes that all workers who laid would never police and all workers who policed would never lay. These five levels were randomly assigned at the beginning of each simulation.

Within each colony the queen produced males and females, and workers with laying alleles produced males, as described in section 3.3.1. The probability that a worker-laid male survived to adulthood and entered the mating pool, $P(m_{ws})$, was a function of: 1) whether or not it was policed, determined by the proportion of policing alleles in the population, w_p/w, 2) its viability, v, relative to queen-laid males, 3) whether or not it survived the efficiency cost that laying workers bestowed upon the colony, $e(w_l/w)$. Note, that if the maximum efficiency cost was 0.2 but the proportion of worker laying alleles in the colony was 0.5, the realized efficiency cost was 0.1.

$$P(m_{ws}) = (1 - ((w_p/w) * (e(w_l/w))) * v$$

The probability that any queen-laid offspring survived, $P(m_{qs})$, was reduced solely by the efficiency cost of having laying workers in the colony, $e(w_l/w)$.

$$P(m_{qs}) = 1 - e(w_l/w)$$

Alleles entered the population through *de novo* mutation at a rate of 0.001. At each locus all of the possible allele types had equal probability of being selected.

3.3.2 Genomic Imprinting

The model above was extended to include a third level of selection, the intragenomic level. Here, the phenotypic outcome of an imprinted gene depended upon which parent that gene was inherited from. An extra allele was established at both the laying and policing loci. Likewise, two imprinting bias alleles were established: laying imprinted bias, and policing imprinted bias (Table 3.1).

As theory suggests that worker laying would not be favorable to the maternal line a worker who inherited an imprinted laying allele from her mother and a neutral allele from her father would not lay (Fig. 3.2). However, even at imprinted loci, both copies of the gene are expressed in some tissue, at some stage in development (DeChiara *et al.*, 1991; Pedone *et al.*, 1994). Moreover, imprinting does not always entail a gene's complete inactivation (Sakatani *et al.*, 2001). As such, we did not assume that there would be no effect of a maternal imprinted allele in the presence of a paternally imprinted allele at the laying locus. Instead if a worker were to inherit imprinted laying alleles from both her mother and father she would lay an egg about 50% of the time the opportunity arose. Finally, a worker who inherited an imprinted laying allele from her father and a standard laying allele from her mother would be classed as a super layer, as both alleles would be concordant. The same pattern exists for imprinted policing alleles except that these benefit the maternal line and so an imprinted policing allele inherited

from the mother would promote policing in that individual. Simulations where imprinting was possible started with 98% of alleles being null alleles, 1% being imprinted and 1% being laying/policing alleles.

As above, the model was run 10 times per starting condition combination, totaling 1080 separate runs.

3.3.3 Statistical Analyses

Decision tree models were designed using a training set (50% of the model data). Optimization was achieved through cross-validation with the other half of the model data. The trees were pruned to maximize robustness (determined by minimizing the mean square error), whilst selecting the lowest number of leaves. All statistical analyses were performed in R (version 3.2), and trees were constructed using the package *tree*. The non-parametric Wilcoxon test was also used to assess differences between imprinted and non-imprinted runs.

3.4 Results

3.4.1 Group and Individual Level Selection

When only relatedness costs were present, i.e. the maximum efficiency cost to the colony of worker laying was 0, worker male viability was equal to that of queen-laid males and there was no imprinting mechanism, the model produced similar results to those of previous studies, with the queen monopolizing male production when she had mated with more than 2 males and workers monopolizing male production when the

colony was headed by a monogamous queen (Fig. 3.3). However, workers were also found to monopolize male production when the queen mated with two males. This result is contrary to predictions from kin selection which states that, when the colony is comprised of two patrilines, queens and workers should be indifferent to male parentage. This could be the result of our starting conditions where laying and policing alleles were initially scarce in the population. When the queen mates with two males the colony is equally comprised of full and half sisters, so whether or not the queen or workers dominate brood production is inconsequential. However, workers are always more related to their sons ($r = 0.5$) than their brothers or the average relatedness when worker offspring is equally comprised of nephews and half-nephews ($r = 0.25$). As such, there is strong selection pressure for laying to evolve but weak selection for policing to evolve, unless other factors were involved. Considering this, there is no discrepancy between our results and those of pure individual level selection. What we observe are laying alleles increasing in frequency whenever worker male viability is high, but, policing alleles only becoming prevalent when the group level costs associated with worker laying are strongly opposed to those of relatedness (Fig. 3.4).

This is particularly apparent by the peak in policing alleles when the queen mates with two males (Fig. 3.4). Here, when worker male viability is high workers dominate male production without any effect on colony relatedness, or much negative impact on colony productivity. When worker male viability is low laying never evolves and the queen dominates male production. However, when worker male viability is three quarters that of queen-laid males, and there is little efficiency cost to worker laying (high inefficiency

cost led to worker laying not evolving), policing evolves to maximize the reproductive output of the colony (Fig. 3.4).

With only group and individual level selection, viability of worker laid males was the primary factor in determining whether or not the queen dominates male production (Fig. 3.5, mean squared error between fit of test data to training data = 5.8%). Indeed, if worker male viability was less than three quarters that of the queen's sons the queen always dominated male production. After worker male viability, relatedness was the key factor in determining male ancestry. Low relatedness led the queen to dominate male production (Fig. 3.5). When relatedness was high and worker male viability was more than 85% that of the queen's sons, workers dominated male production unless, efficiency costs were high in which case workers and queens shared male production 42% and 58% respectively. However, when the worker male viability was between 75% and 85% that of the queen's sons the queen only dominated male production if more than 14% of potential reproductives were prevented from entering the mating pool (Fig. 3.5).

The biasing of policing workers not to lay and laying workers not to police did not increase in frequency to more than 5%, amongst non-imprinted alleles. This result was likely due to mutation maintaining the alleles.

3.4.2 Group, Individual and Intragenomic Levels of Selection

When all three levels of selection were interacting, imprinted alleles rather than the non-imprinted alleles controlled the conflict (Fig. 3.6, 3.7, 3.8 & 3.9). When imprinting was present workers never achieved total control over male production (Fig. 3.3). However, workers could still dominate male production, even when relatedness within the colony was low, provided the group level costs associated with worker reproduction were also low (Fig. 3.3). Indeed, when a mechanism for imprinting is in place relatedness appears to play a minor role in determining male ancestry (Fig. 3.3).

Imprinted laying alleles always reached fixation when relatedness within the colony was low (Fig. 3.6). When the queen mated with a single male imprinted laying alleles always reached fixation unless, worker male viability was low and the efficiency cost due to worker laying was high (Fig. 3.6). Here, the null allele maintained its prevalence and worker laying did not evolve (Fig. 3.6).

In contrast, imprinting of the policing allele never reached fixation (Fig. 3.7). When the queen was singly mated imprinting accounted for approximately 50% of all policing alleles when worker male viability was high and there was little impact on colony efficiency due to laying workers. When there were 10 patrilines in the colony, imprinting accounted for approximately 50% of the policing alleles when worker male viability was high, regardless of the decrease in colony efficiency from worker laying (Fig. 3.7).

Whilst biasing of policing workers not to lay and laying workers not to police did not evolve amongst non-imprinted alleles, an imprinted bias of laying workers not to police did evolve when worker male viability was high (Fig. 3.8). When the queen mated multiply and worker male viability was low, imprinting of policing workers to eschew laying was observed (Fig. 3.8).

Only intragenomic conflict played a role in determining the ancestry of males (Fig. 3.9, mean squared error between fit of test data to training data = 3.1%). Indeed, the biasing of laying workers against policing was the dominant factor in predicting male ancestry. When more than 42% of laying bias alleles were imprinted, workers laid the majority of the male offspring in the colony, unless more than 53% of policing bias alleles were also imprinted (Fig. 3.9). When imprinted laying bias was rare the queen tended to dominate male production. However, if the imprinted policing allele reached fixation (in which case 50% of individuals would police and 50% would not police, Fig. 3.2), workers dominated male production.

Whilst imprinted alleles controlled the conflict they were not universally present (Fig. 3.6 and 3.7). Imprinted laying alleles were absent when relatedness was high, worker male survival was low, and efficiency costs were high (Fig. 3.6 & 3.10, mean squared error between fit of test data to training data = 11%). Relatedness had no effect on the evolution of imprinted policing alleles (Fig. 3.11, mean squared error between fit of test data to training data = 12%). Rather, high levels of imprinting of policing alleles only evolved when worker male viability was high. Inefficiency of worker-laid males and the

presence of imprinted laying alleles led to increased prevalence of imprinting at the policing locus (Fig. 3.11).

Ultimately, conflict within the colony, as measured by the percent of brood lost due to the conflict, is highest when worker male viability is high and laying workers contribute least to the colony (Fig. 3.12). Imprinting acts to dampen the level of conflict unless within colony relatedness is low (comparison of the proportion of brood lost to the conflict when imprinting is and is not possible: One father: $W(720) = 73713$, $p < 0.005$; Two fathers: $W(720) = 74386.5$, $p < 0.001$; Ten fathers: $W(720) = 62504.5$, $p = 0.32$). Overall, low colony relatedness reduces conflict when the group level costs associated with worker laying are low, and it is only when the group level costs of worker laying increase that conflict within the colony is apparent (Fig. 3.12).

3.5 Discussion

The model shows that multilevel selection strongly affects worker laying and policing behavior, and that a single level is insufficient when predicting whether or not the queen will dominate male production. Contrary to predictions from kin selection models, group and intragenomic levels of selection seem to be the dominant predictors of male ancestry (Fig. 3.9 and 3.11), with paternal imprinting always evolving to high frequency (Fig. 3.5) and maternal imprinting evolving only when other levels of selection fail to impede worker laying (Fig. 3.6).

Individual level kin selection models predict that when relatedness is high workers will dominate male production and when colony relatedness is low the queen will lay the majority of the males. When species have been identified which violate these predictions, such as *Apis mellifera capensis*, where worker laid female eggs are policed despite being equally related to workers as the queens offspring (Saigo and Tscuchida, 2004; Pirk *et al.*, 2003) or policing of worker laid eggs in colonies headed by a singly mated gamergate (Kikuta and Tsuji, 1999), ecological costs are typically invoked.

My model clearly predicts that worker policing will not evolve in colonies where the queen mates once (and thus, relatedness is high), except when a mechanism for imprinting is in place (Fig. 3.6). Indeed, when available, our model predicts that genomic imprinting should be the dominant force in determining male ancestry (Fig. 3.8). Both relatedness and worker male viability determine whether or not imprinting, particularly of the policing allele, will evolve.

Imprinting mechanisms are known to exist throughout the eusocial Hymenoptera (Kronforst *et al.*, 2008; Yan *et al.*, 2014). Changes in a female's epigenome have been shown to determine her caste (Kucharski *et al.*, 2008), and there are strong suggestions that imprinting of the paternal line may determine a workers ovary size (Oldroyd *et al.*, 2014) and the magnitude of colony defensive behavior (Guzman-Novoa *et al.*, 2005). As such, we would expect imprinting to be pervasive throughout the eusocial Hymenoptera and to be the dominant force determining worker reproductive behavior.

In colonies where relatedness is high and imprinting is not possible, worker policing is only predicted to be present when worker male viability is low. Where genetic relatedness is low, policing is only expected to be selected for when the benefits to individual workers are most in conflict with benefits to the colony as a whole, i.e. when male viability is high. However, colony level costs are more difficult to quantify than relatedness. For example, the viability of worker-laid males can be difficult to assess. For example, in honey bees, worker-laid eggs have been found to be equally (Ratnieks and Visscher, 1989, Beekman and Oldroyd, 2005), and less than half (Pirk *et al.*, 2004), as viable as queen-laid eggs, and the drones produced from worker-laid eggs may also be smaller and less fit than queen-laid males.

Genes do not work independently. Rather, they may work together, co-adapting to produce the optimal behavior. Such co-adaptation is predicted to lead to epistasis between imprinted laying and policing alleles, with laying alleles suppressing worker policing when male viability was high, and policing suppressing laying when both relatedness and male viability are low. Such co-adapted genetic relationships could be disrupted by crossing different sub-species, potentially resulting in extreme phenotypes where laying or policing become more prevalent than in pure colonies (Linksvayer, 2007). Indeed, when backcrossing European and African honey bees, Linksvayer *et al.* (2009) found workers exhibiting ovaries with extreme numbers of ovarioles (up to 147 compared to a normal worker maximum of 20).

Ultimately, conflict seems only to be expressed where group and individual fitness levels are most antagonistic. Imprinting tends to dampen conflict but also increases the range of circumstances in which workers can partake in male production. As such, my model creates many novel predictions, however, in all but a few circumstances any particular behavioral outcome will be determined by multiple interacting levels of selection and the dominant selective pressures cannot be ascertained by examining a singular behavioral trait.

The debate over the importance of kin selection lies in its efficacy in accurately predicting worker behavior. The emphasis on relatedness has been widely criticized but proponents of the theory state that kin selection models can and do account for higher levels of selection (Foster *et al.*, 2006; Marshall, 2011). However, these models do not account for intragenomic conflict, and even if they did, such calculations can be very difficult to compute as accurate estimates of group level effects may be difficult to attain. Here, I have tackled this problem by incorporating all three levels of selection into a single model. I have shown that it is possible to attain worker policing in colonies headed by a multiply mated queen and, when a mechanism of imprinting is in place or group level costs are exceedingly high, in colonies headed by a singly mated female.

Part of the beauty of Hamilton's rule is its simplicity, and there is no doubting the enormous positive impact it has had on the field. But, the pervasive reliance on the relatedness term alone masks a complex and fascinating range of dynamics, only a few of which have been touched here. Future studies could examine the effect of colony size,

and how differences in the translation of imprinting to behavior could impact colony level outcomes. Most importantly, our model demonstrates 1) that levels of selection, other than that of the individual, may be dominant in determining behavior, 2) that all levels of selection interact to maximize fitness across all levels, 3) that simply observing a colony in which the queen mates multiply and lays the majority of the male offspring is not adequate to invoke maximizing relatedness as the key selective force.

3.6 Figures and Tables

Variables	Description
Mates	Number of males a queen mates with. Dictates the average level of relatedness within the colony. If a queen mates only once then all female offspring are full sisters (r=0.75) and are therefore, more related to each others sons (r=0.375) than they are to their brothers. If a queen mates twice the average colony relatedness is (r=0.5) and neither the queens nor the workers sons are favored over the others. Finally, if a queen mates more than twice females will more often encounter half sisters (r=0.25) than full sisters. As such, worker-laid males will more often be half nephews (r=0.125) than full nephews and the queen's sons will be preferred as individual level selection acts to maximize a workers inclusive fitness benefits. Number of mates is set at the beginning of each run and takes the value of either: 1, 2 or 10.
Queen	Head and mother of the colony. Also, describes the female offspring with the potential to become queens and destined for the common mating pool.
Workers (w)	Unmated females in the colony who help to rear the queens offspring. Workers may asexually produce male eggs and/or may eat worker laid eggs, depending on their genotype.
Worker Laying Alleles (w_l)	Count of alleles coding for worker laying. These are the laying allele and imprinted laying alleles derived from the father (Figure 3.2).
Worker Policing Alleles (w_p)	Count of alleles coding for worker policing. These are the policing allele and imprinted policing alleles derived from the mother (Figure 3.2).
Viability (v)	The viability of worker-laid males relative to queen-laid males. Worker male viability is set at the beginning of each run and takes a value of either: 0, 0.1, 0.2, 0.3, 0.4 or 0.5.
Maximum Efficiency Cost (e)	The loss in potential reproductives due to decreased worker helping effort and therefore, resource availability. Maximum efficiency cost is set at the beginning of each run and takes a value of either: 0, 0.04, 0.08, 0.12, 0.16 or 0.2.
Realized Efficiency Cost ($e(w_l/w)$)	The maximum efficiency cost weighted by the proportion of alleles coding for worker laying in the population.
Epistasis	The effect of policing behavior to reduce laying behavior and laying behavior to reduce policing behavior. Can be selected for at four loci (two imprinted and two non-imprinted). The degree of epistasis can take values: 0, 0.25, 0.5, 0.75 or 1.

Table 3.1 Main model parameters.

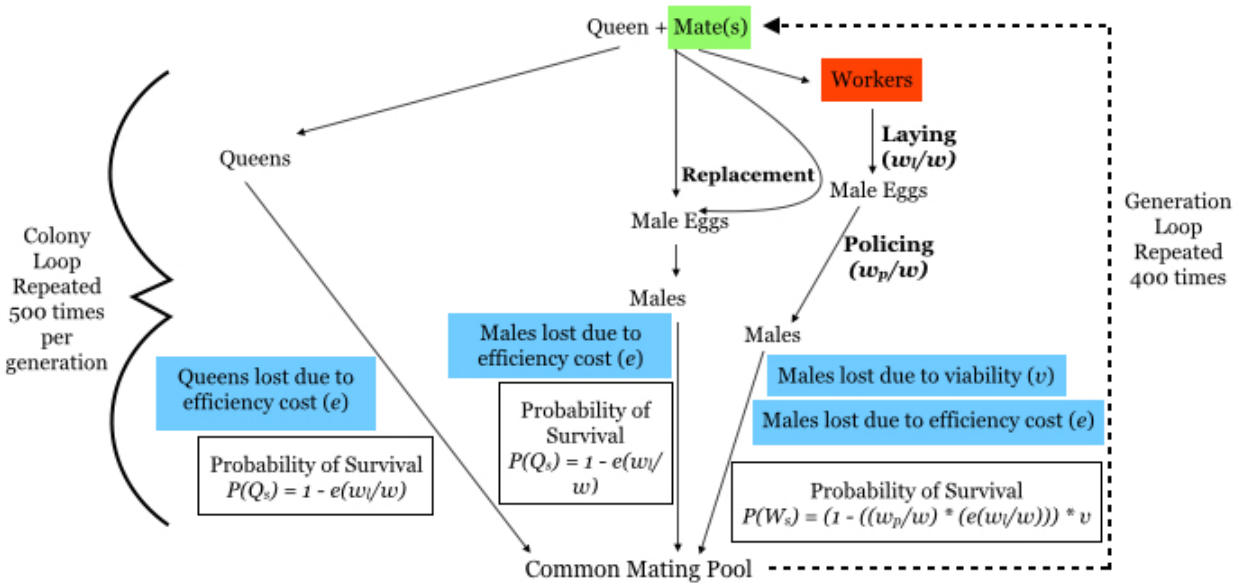

Figure 3.1 Model structure. Genotypes for queens and mates were selected at random, according to the starting conditions. Number of mates, maximum efficiency cost (e) and worker male viability (v) were determined at the start of the run and remained constant throughout. The number of mates a queen has affects colony relatedness. Individual level selection (green) acts to maximize colony relatedness, and therefore maximize inclusive fitness benefits. Each queen reproduces queens, males and workers. Queens and workers receive half of their genes from their mother and half from their father. Males receive their entire genome from their mother. A workers propensity to lay and police is determined by her genotype, where selection acting at the level of the gene may be expressed (red). Workers were selected at random to lay and their genotype determined whether or not they did. The level of policing was proportionate to the number of policing workers. Removed worker-laid eggs were replaced by queen-laid male eggs. Some worker-laid males were lost due to reduced viability in comparison to queen-laid males, a selective force acting at the level of the colony (blue). Laying workers may also work less, decreasing colony efficiency, and leading to the loss of some males and queens (colony level selection, blue). All surviving queens and males enter the mating pool. The mating pool is shared by all colonies of that generation and queens and males are selected from it, at random, to start the next generation.

Blue depicts colony level selection; Green depicts individual level selection; Red depicts gene level selection.

♀ \ ♂	-1	0	1
-1	50% Police 50% Not Police	Police	Super Police
0	Not Police	Not Police	Police
1	Not Police	Police	Super Police

Policing Locus

-1	0	1	♂ \ ♀
50% Lay 50% Not Lay	Not Lay	Not Lay	-1
Lay	Not Lay	Lay	0
Super Lay	Lay	Super Lay	1

Laying Locus

Figure 3.2 Genotype to phenotype distribution at the policing and laying locus. -1 denotes imprinted alleles, 0 null alleles, and 1 police or lay alleles. Paternally derived alleles are the columns whilst maternally derived alleles are the rows.

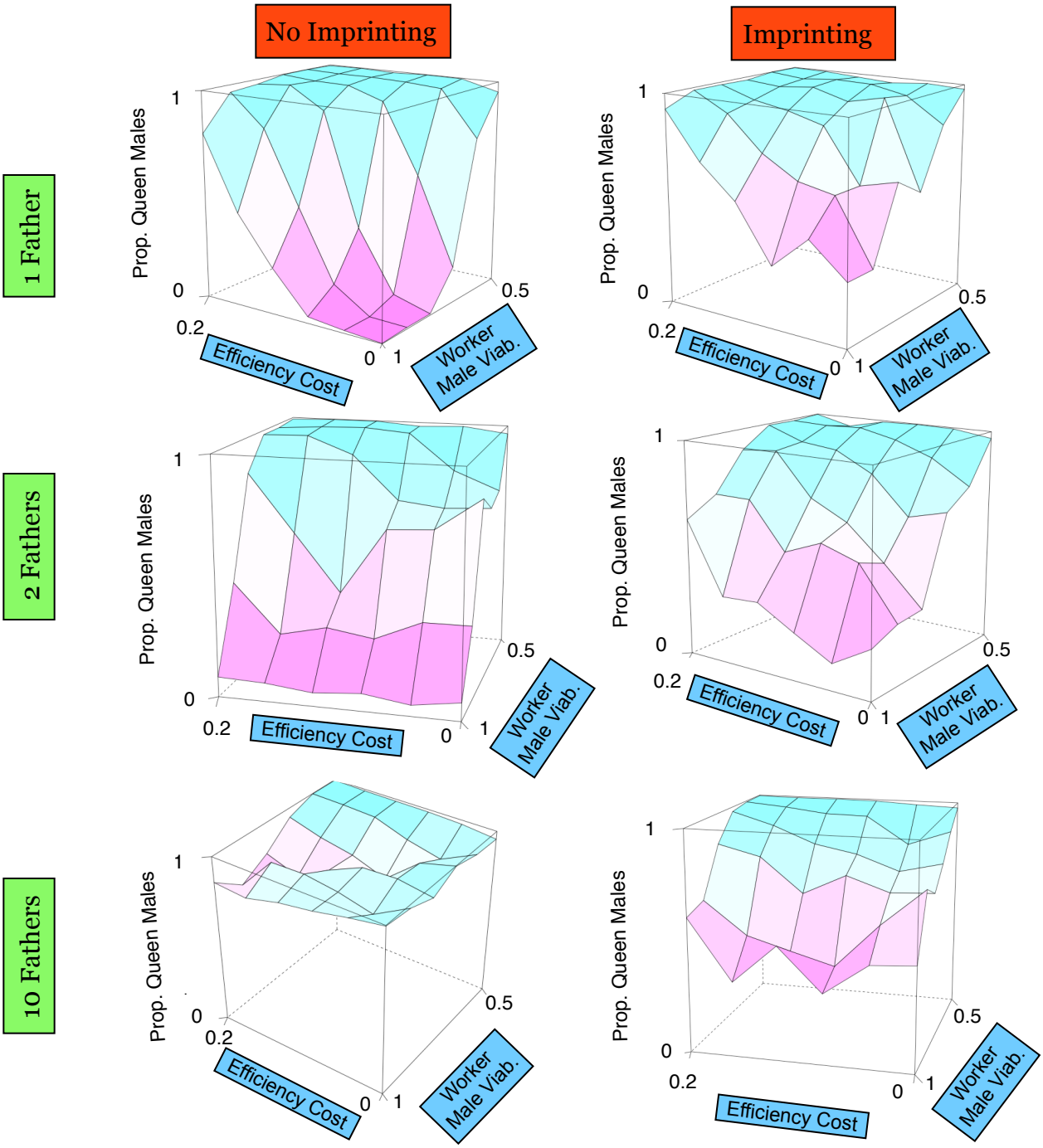

Figure 3.3 Proportion of surviving males that are the queen's sons, with and without imprinting (columns), and 1, 2 and 10 fathers (rows). Efficiency cost is the loss in working effort due to laying workers in the colony. Worker male viability denotes the probability that a worker drone will successfully survive to enter the mating pool compared to queen laid males. Blue depicts selection acting at the colony level, green selection at the level of the individual and red selection at the level of the gene.

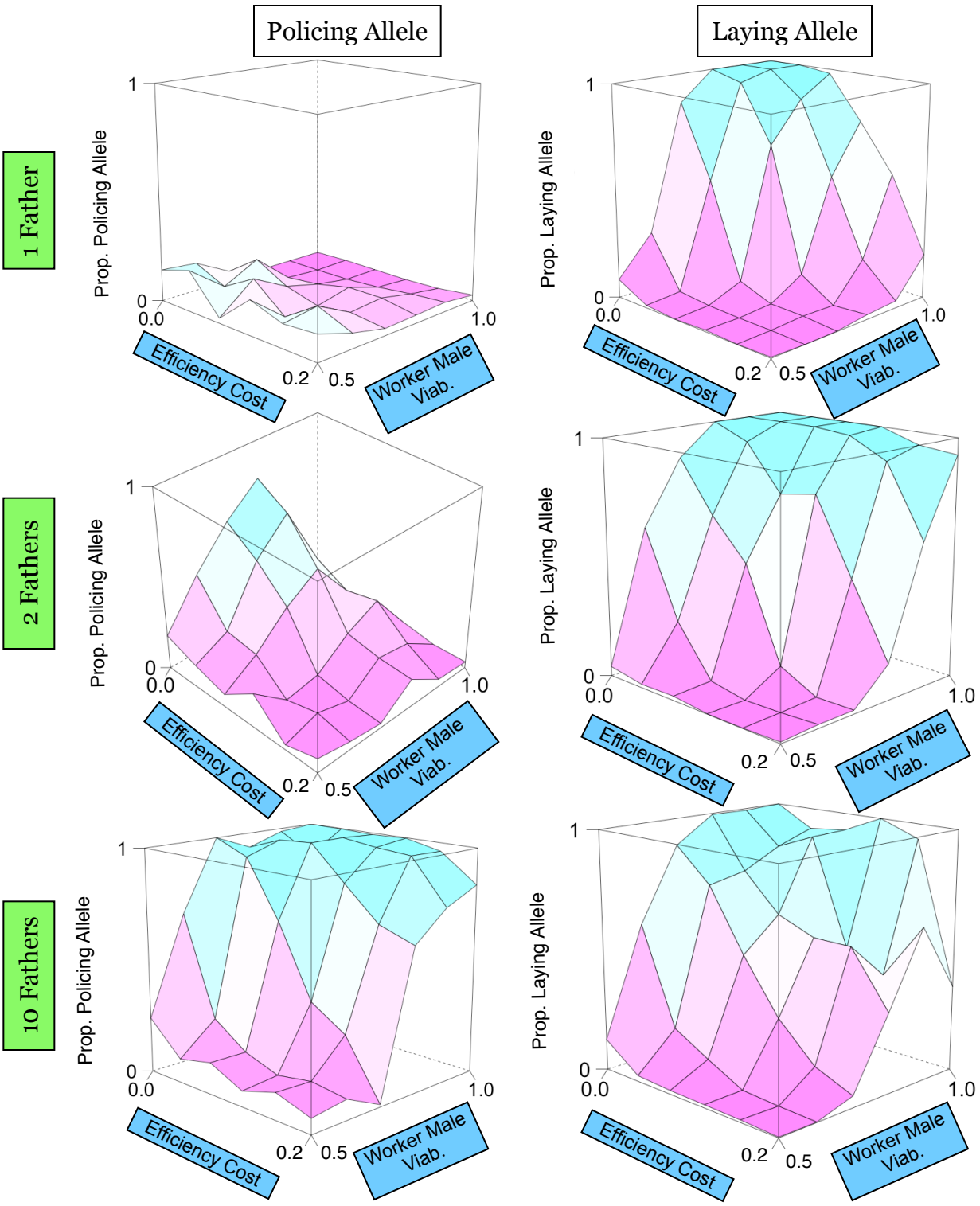

Figure 3.4 The proportion of laying and policing alleles (columns) without imprinting, when the queen mated with 1, 2 and 10 males (rows). At the start of each simulation 2% of alleles coded for laying and policing behavior, the other 98% were null alleles. Blue depicts selection acting at the colony level, green selection at the level of the individual and red selection at the level of the gene.

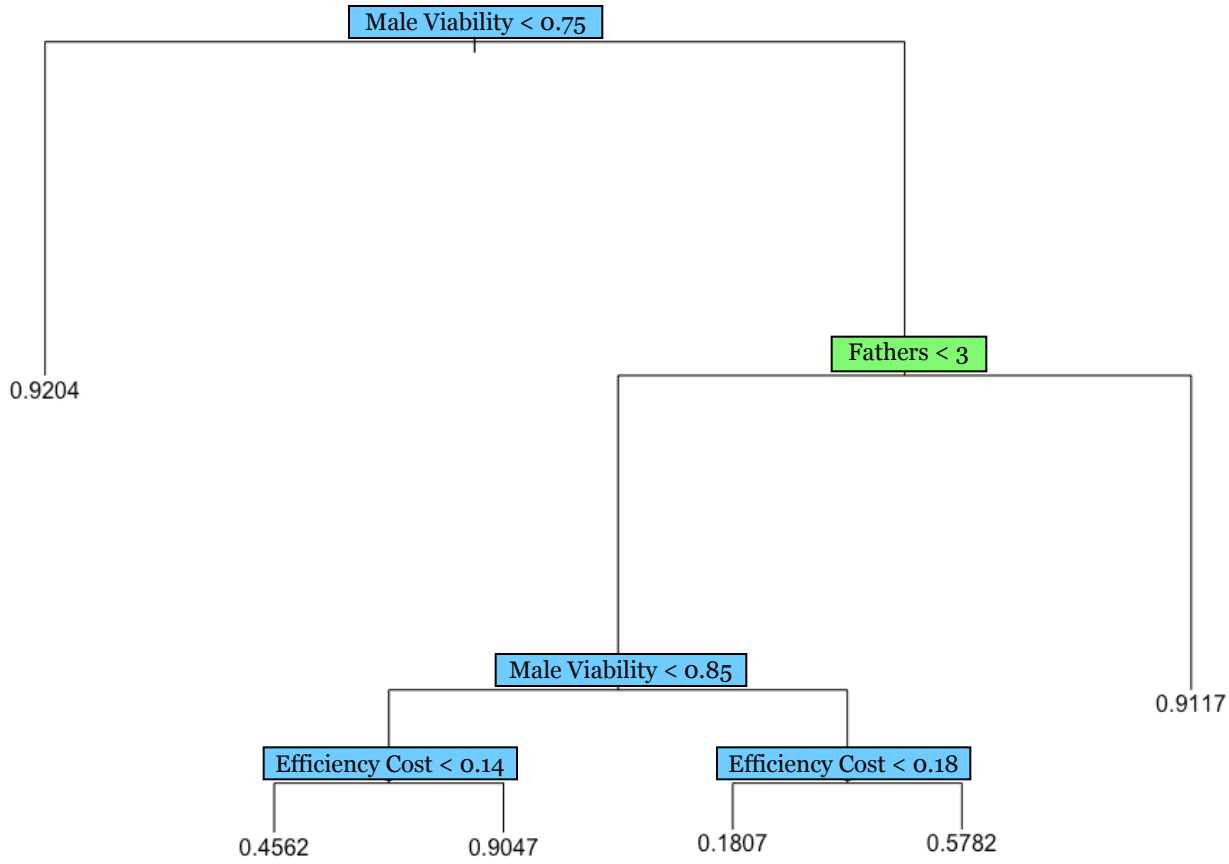

Figure 3.5 Decision tree showing the effect of group and individual levels of selection on the proportion of male offspring produced by the queen. Group level selection is highlighted in blue, individual level selection in green. The model included: number of fathers, worker male viability when compared to queen laid male viability, efficiency cost of worker laying, bias of laying alleles against policing and bias of policing alleles against laying. The end of the leaves denotes the proportion of male offspring belonging to the queen. At each node the left branch is followed when the statement is accepted and the right when it is rejected.

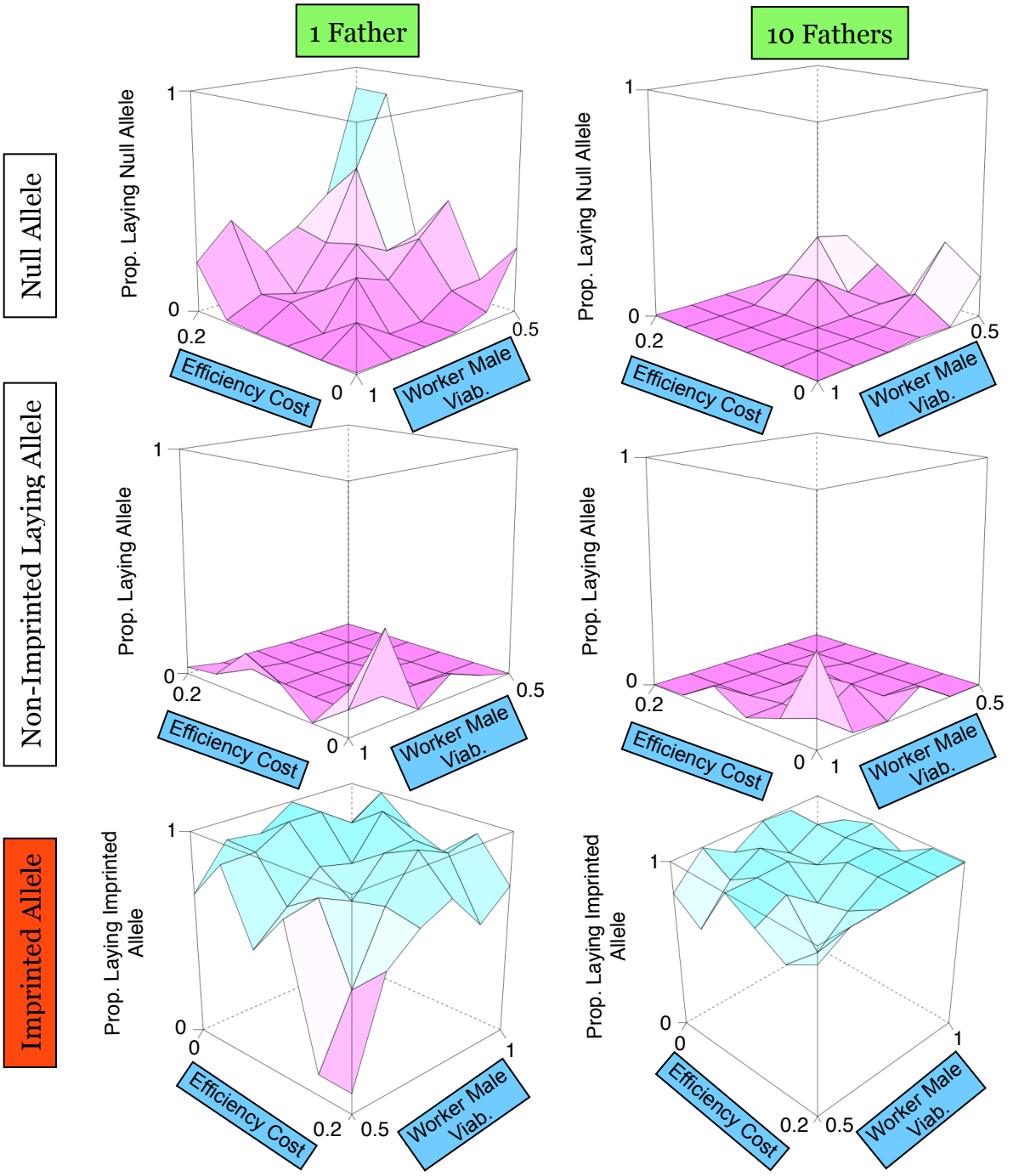

Figure 3.6 The proportion of laying alleles that are: null (never produce laying behavior); non-imprinted (always produce laying regardless of which parent they are inherited from); imprinted to parent-of-origin. Each simulation began with 98% null alleles, 1% imprinted and 1% laying non-imprinted alleles. Blue depicts selection acting at the colony level, green selection at the level of the individual and red selection at the level of the gene. Note the direction of the axes changes between the top two rows and the third row (imprinted alleles) to best display the data.

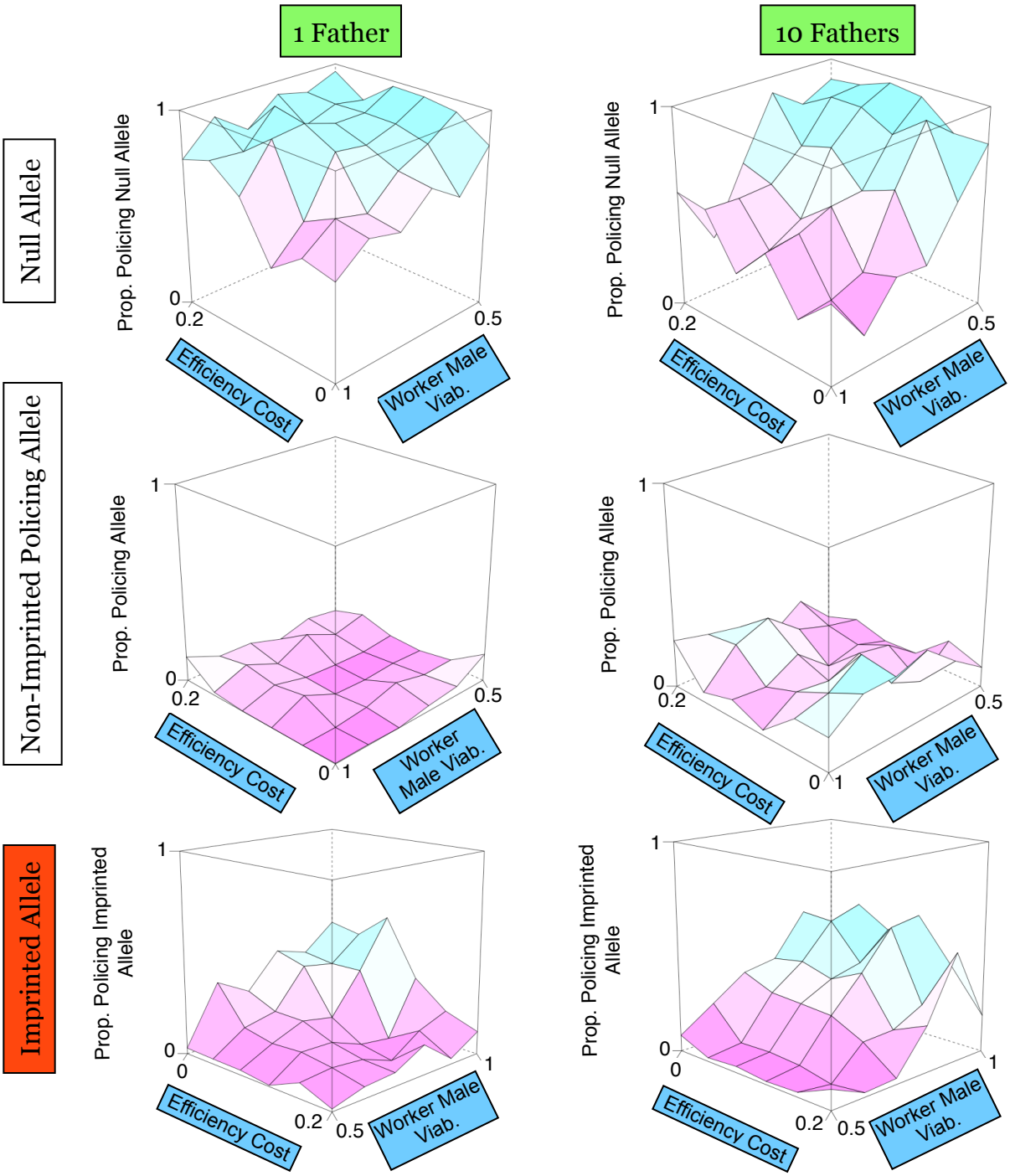

Figure 3.7 The proportion of policing alleles that are: null (never produce laying behavior); non-imprinted (always produce policing, regardless of parent inherited from); imprinted to parent-of-origin. Each simulation began with 98% null alleles, 1% imprinted and 1% policing non-imprinted alleles. Blue depicts selection acting at the colony level, green selection at the level of the individual and red selection at the level of the gene. Note the direction of the axes changes between the top two rows and the third row (imprinted alleles) to best display the data.

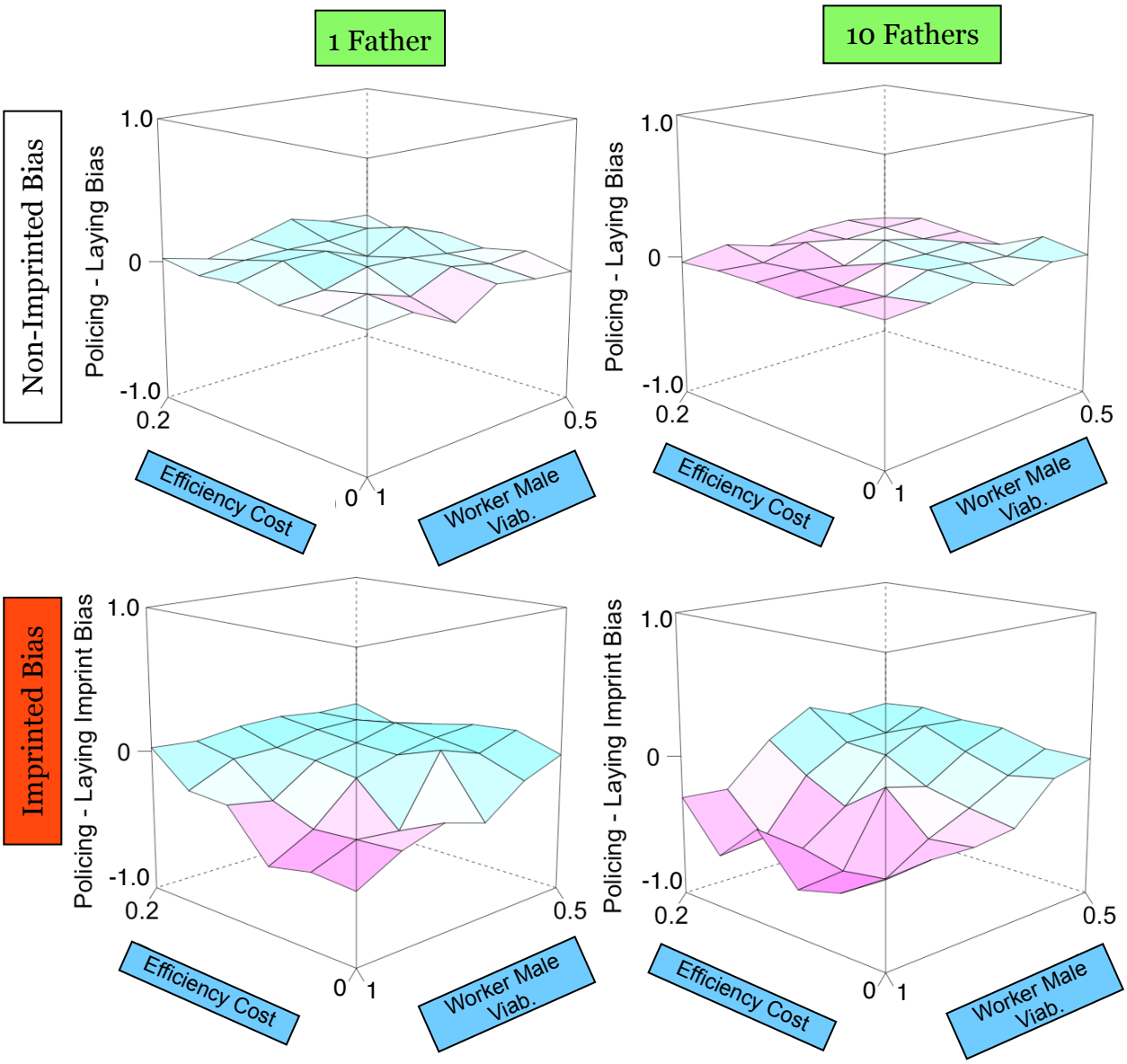

Figure 3.8 The level of suppression of laying alleles by policing alleles, minus the reciprocal effect of laying on policing when the queen mates with one (first column) and 10 (second column) male(s). The top row denotes non-imprinted alleles and the bottom row denotes imprinted alleles. Blue depicts selection acting at the colony level, green selection at the level of the individual and red selection at the level of the gene.

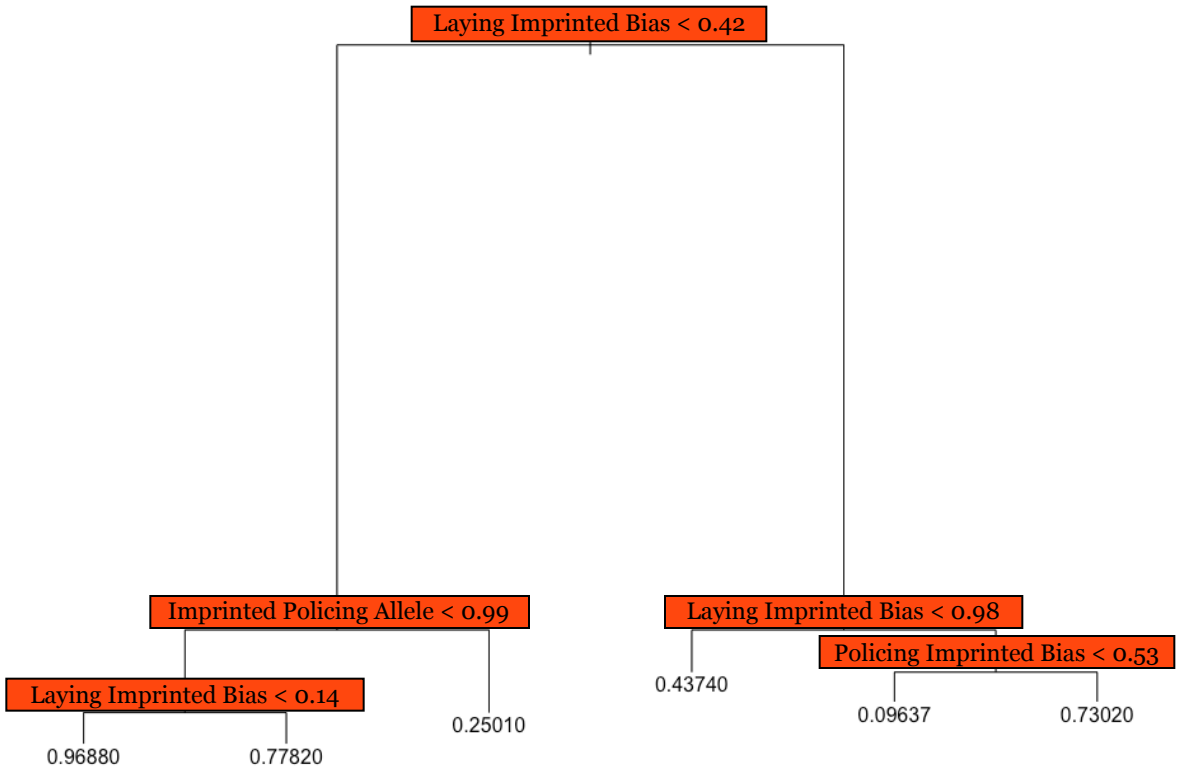

Figure 3.9 Decision tree showing the effect of group, individual and intragenomic levels of selection on the proportion of male offspring produced by the queen. Only selection acting at the level of the gene was predictive of the proportion of queen laid male eggs. Genome level selection is highlighted red. The model included: number of fathers, worker male viability when compared to queen laid male viability, efficiency cost of worker laying, imprinted policing alleles, imprinted laying alleles, bias of laying alleles against policing, bias of policing alleles against laying, imprinted laying bias and imprinted policing bias. The end of the leaves denotes the proportion of male offspring belonging to the queen. At each node the left branch is followed when the statement is accepted and the right when it is rejected.

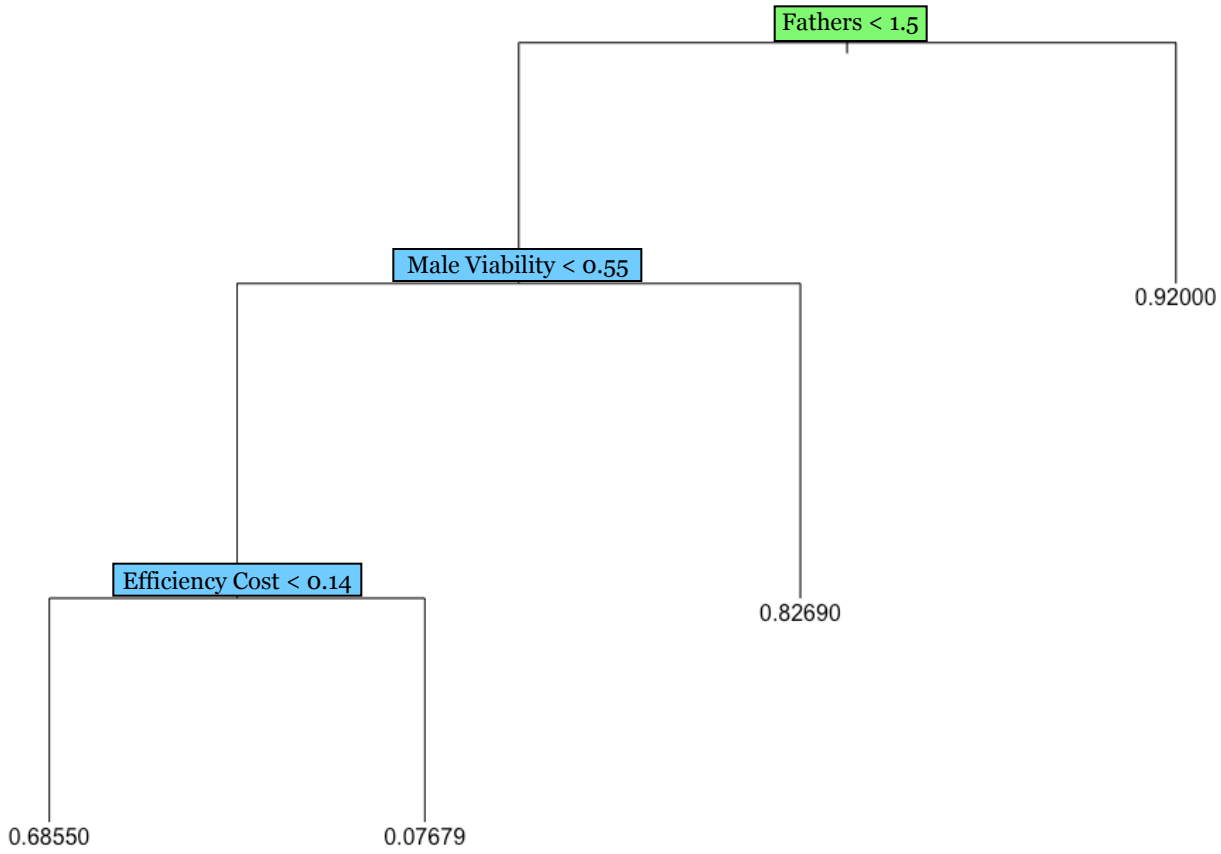

Figure 3.10 Decision tree showing the effect of group, individual and intragenomic levels of selection on the proportion of imprinted laying alleles. Group level selection is highlighted in blue, individual level selection in green. The model included: number of fathers, worker male viability when compared to queen laid male viability, efficiency cost of worker laying and imprinted policing alleles. The end of the leaves denotes the proportion of imprinted laying alleles in the population. At each node the left branch is followed when the statement is accepted and the right when it is rejected.

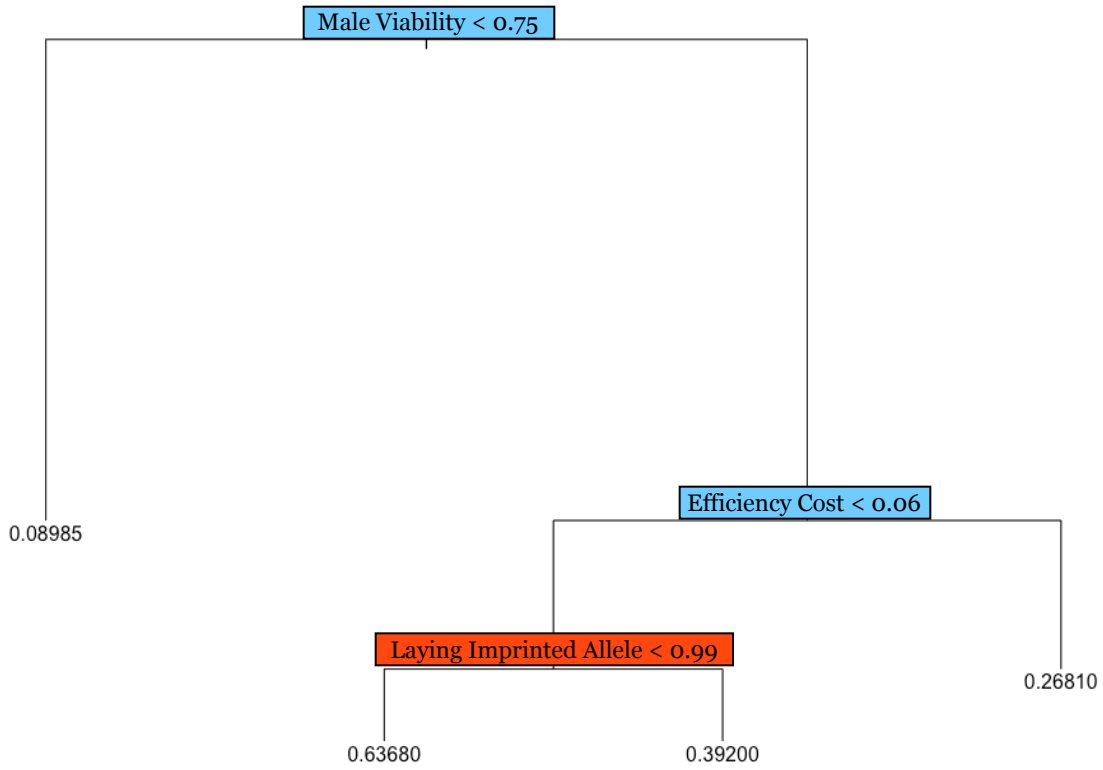

Figure 3.11 Decision tree showing the effect of group, individual and intragenomic levels of selection on the proportion of imprinted policing alleles. Group level selection is highlighted in blue, genome level selection in red. The model included: number of fathers, worker male viability when compared to queen laid male viability, efficiency cost of worker laying and laying imprinted allele. The end of the leaves denotes the proportion of imprinted policing alleles in the population. At each node the left branch is followed when the statement is accepted and the right when it is rejected.

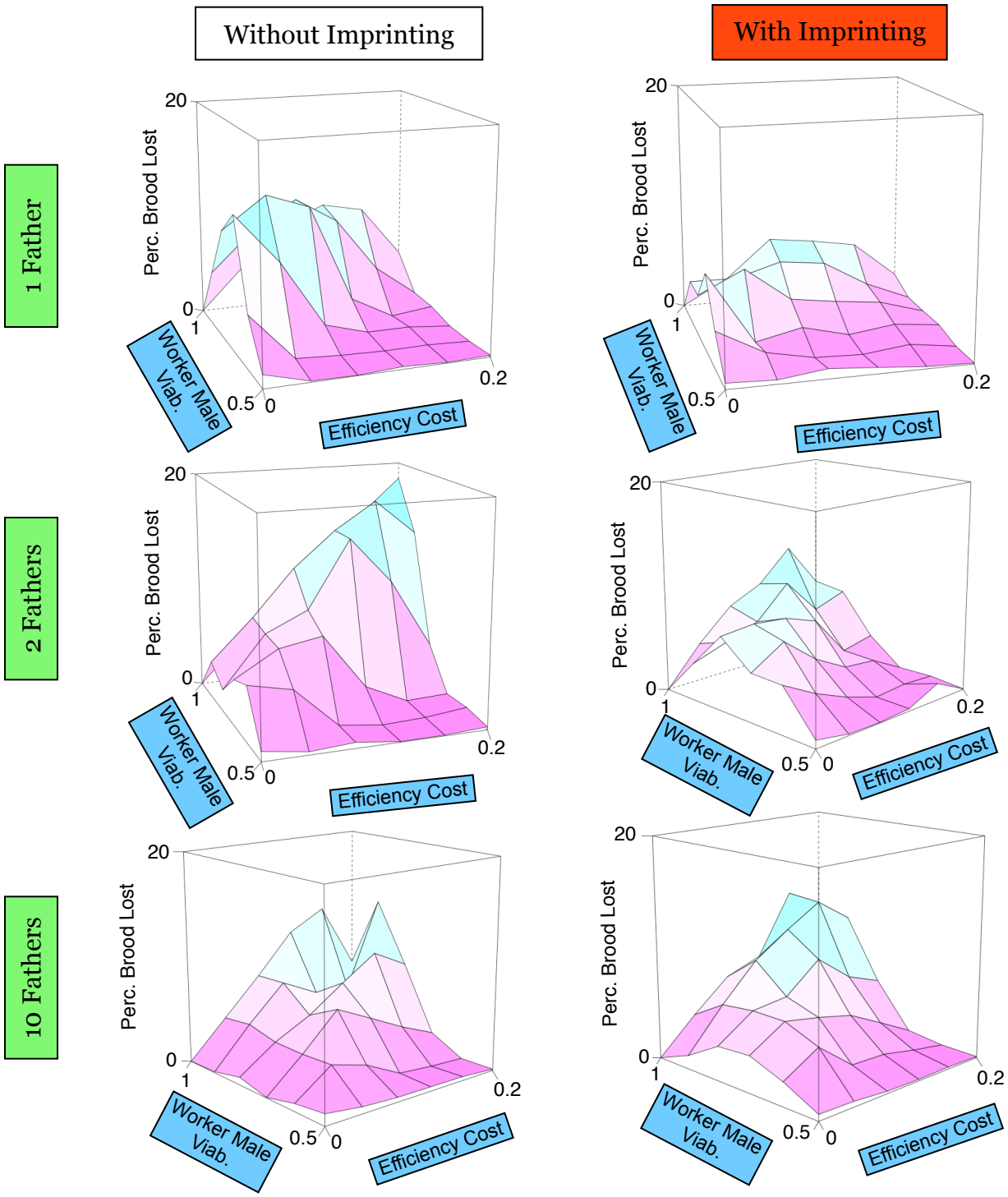

Figure 3.12 Effect of relatedness, worker male viability, decreased work effort by laying workers, and intragenomic conflict on the percentage of potential brood lost by a colony due to conflict over male parentage. Group level selection is highlighted in blue, individual level selection in green and genome level in red.

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CHAPTER 4

Worker Honey Bees Do Not Distinguish Between Queen and Worker-Laid Male Larvae

4.1 Abstract

Worker policing is the *prima facie* example of kin selected altruism where workers eat eggs laid by other workers (r=0.125) to gain benefits from increased relatedness through rearing only the queen's sons (r=0.25). However, another explanation is that workers police eggs to maintain colony efficiency. To distinguish between these two hypotheses, I transplanted queen and worker-laid larvae of varying ages from a queenless rearing colony into the brood nest of a queenright colony. I then recorded the number remaining 4 and 24 hours later. If worker policing evolved to maximize nepotistic benefits worker policing would be expected to continue well into larval development. However, if worker policing evolved because worker-laid larvae are less viable than queen-laid larvae, policing is predicted to cease soon after hatching, once viability is evidenced. No difference was found in the rate of removal of queen and worker-laid larvae, regardless of their age. Policing of worker-laid eggs is very efficient and few hatch into larvae. As such, there may be only a very weak selection pressure to moderate policing relative to age. Alternatively, as only 3.6% of larvae survived 24 hours, either the rearing conditions or transferral mechanism may have rendered all male larvae unacceptable to the colony, regardless of maternal source. Female larvae do not suffer such discrimination after transplantation. One explanation is that male larvae may have a higher acceptance threshold because they can come from multiple sources whilst female larvae can only be queen derived.

4.2 Introduction

Worker policing in the eusocial Hymenoptera is one of the most convincing examples of kin selection in the animal kingdom (Whitfield, 2002; Gadagkar, 2004; Sugden, 2004), not least because it is an example of theory anticipating behavioral observation. I.e., destruction of worker-laid eggs was an unrecognized phenomenon prior to modeling studies that suggested it should exist. Hamilton's (1964) rule suggests that it is possible for a worker to maximize her inclusive fitness benefits through rearing brothers (r=0.25) over half nephews (r=0.125). However, if a colony is headed by a singly mated queen, workers would be more related to their sisters' sons (full nephews, r = 0.375) than to their brothers (r=0.25). Whilst queens control the primary sex ratio, it is workers who control the final sex ratio (Sundström *et al.*, 1996). As such, when a colony is headed by a single monogamous queen, workers will maximize their inclusive fitness benefits by preferentially rearing the sons of other workers. When the queen is multiply mated, the average relatedness of helping to rear workers' sons, provided discrimination across patrilines is impossible, decreases and workers should favor the queen's sons (Woyciechowski and Łomnicki, 1987; Ratnieks, 1988).

Ratnieks (1988) predicted that workers would control the final sex ratio in polygamous colonies through worker policing, a behavior where workers remove other workers' male eggs and/or aggress upon workers with activated ovaries. Worker policing, consistent with nepotism, has been identified across many species (Wenseleers and Ratnieks, 2006). The ultimate result is often sterility among the workers, at least in the presence of the queen.

Worker policing may not always be the result of nepotism. For example, worker policing has been observed in colonies where genetic relatedness patterns would not predict it. Firstly, colonies headed by a single monogamous queen (Visscher, 1996; Foster *et al.*, 2002; Endler *et al.*, 2004), and secondly, where unmated workers parthenogenetically reproduce diploid, female offspring (Saigo and Tscuchida, 2004; Pirk *et al.*, 2003). In such cases, maintaining the efficient functioning of the colony is normally invoked as the evolutionary mechanism through which policing evolved.

Pirk *et al.* (2004) suggested that if worker-laid eggs were less viable than queen-laid eggs, then replacement of all eggs not belonging to the queen could significantly increase the number of males a colony introduces to the mating pool. As such, inclusive fitness would be increased through group-level productivity rather than from an individual's increased relatedness to male brood. Such a preference for queen-laid over worker-laid eggs would be predicted to be present in all colonies, independent of how many times the queen has mated.

Pirk *et al.*'s (2004) suggestion that eggs may be policed because of a viability signal, rather than their source, was elegantly rejected by Beekman and Oldroyd (2005). They showed that *Apis mellifera* workers do not discriminate between eggs that are alive and dead, but do discriminate between worker and queen-laid eggs, no matter their state. Therefore, Beekman and Oldroyd (2005) rejected worker policing based on group level advantages that did not rely on nepotism. Nonacs (2006) pointed out, however, that

nepotism and group benefitting traits do not have to act in opposite directions. Indeed, in the case of worker policing behavior, a worker-laid male need only survive until the level of investment that would be lost through its destruction outweighs either its reduced relatedness or viability.

Worker policing was first identified in honey bees (Ratnieks and Visscher, 1989) and since then research has also linked the maintenance of worker sterility with suites of queen and brood derived pheromones that inhibit ovary activation (Visscher, 1989; Hoover *et al.*, 2003). Even with such a variety of control mechanisms, reproductive workers constitute approximately 4% of the colony (Jay, 1968) and can lay 7% of the male eggs, although only around 0.12% of those make it to adulthood, due to efficient policing of eggs (Visscher, 1996). Results on worker-laid egg viability have also been mixed: from equally as viable (Ratnieks and Visscher, 1989), to less viable (Pirk *et al.*, 2004) than queen-laid eggs. As such, if a worker-laid egg can evade policing, hatch, and survive as a larva until such time as it has proven its viability (at the earliest in honey bees this would be day 2 of larval development; Nonacs, 2006), workers may not gain in fitness by policing it from the colony.

Here, I test Nonacs' (2006) hypothesis, using *Apis mellifera*, by examining the removal rate of worker and queen-produced larvae of different ages. The data showed no effect of mother or development stage on larval removal, suggesting that workers cannot distinguish between worker and queen-produced larvae and may have a low tolerance for imperfect male larvae.

4.3 Methods

A strong honey bee colony that was currently rearing drones, was selected from those available at UC Riverside, courtesy of Professor Visscher, and moved to UCLA. Rearing drones is energetically costly to the colony, so if there are many adult or emerging drones in a colony workers are less likely to rear new ones (Free and Williams, 1975). As such, once at UCLA the drone brood was removed from the colony and replaced with a mixture of pollen, nectar, and empty comb.

The queenright colony was split in two, ensuring that both sections had ample food and access to empty cells, creating one queenright and one queenless section. When eggs appeared in the queenless section an empty frame with large cells used for drone rearing, was introduced for the workers to lay eggs into. The queen in the queenright section was placed into a cage with an empty frame of drone sized cells to encourage the queen to lay male eggs. The walls of the cage were made of excluder material so the queen could not pass through but workers could. Twenty four hours later the frame was removed, covered with mesh so that no bees could access the cells, but so that they could still walk over them, and so that the top of the cells were not damaged by the mesh, and placed into the queenless colony. The drone comb from the queenless colony was also covered in mesh and returned to the queenless section of the colony. Two days later both frames were checked every 4 hours for signs that the eggs were hatching, upon which time the mesh was removed and the frames were placed back into the queenless colony so that the hatched larvae could be reared. As such, both queen and worker-laid

males were reared under the same conditions before being placed into the queenright colony for the policing assay.

Every day 5% of the available larvae were grafted, using a standard grafting tool and a drop of diet 1 (Table 4.1; Wegener, 2009), onto a frame of empty drone sized cells. Larvae were placed in known cells, with source (queen and worker) alternating by row. The queenright test colony chosen for the policing assay was unrelated to that from which worker and queen-laid males were derived. Workers have been found not to distinguish between eggs from different colonies, but only by whether or not they are worker or queen-laid (Ratnieks and Visscher, 1989). The test colony was currently rearing drones and the test frame was placed above the queen excluder, on the outer edge of the brood nest (where drones are reared) between frames of brood. The number of larvae remaining, and their source, were recorded after 4, and 24 hours.

Two other methodologies were attempted prior to the one above. Both were unsuccessful at producing results and are included, in the appendix, for reference purposes (see Appendix A1).

4.3.1 Statistical Analyses

I analyzed whether or not queen-produced larvae were less likely to be policed than worker-produced larvae using a one-way, Wilcoxon rank-sum test after 4 and 24 hours. As after 24 hours almost all larvae had been removed it was decided to continue analysis using only the larval removal data obtained 4 hours after introduction.

As larval removal is binary (0 = present, 1 = absent), I used a logistic regression model to examine the effects of larval age and source (queen or worker), and their interactions on whether or not it was policed after 4 hours. For logistical reasons, policing assays were performed over several blocks (days). Therefore, I have included day as a blocking effect to account for this temporal variation. Block was included as a covariate in the model, rather than as a random or fixed effect, because my aim was to statistically remove this variation, not to explain it. Consequently, even though block was significant in the analysis, I am only interpreting the main effects and their interactions. All analysis was performed using the statistical program R (version 3.1.2).

4.4 Results

There was no significant effect of larval source (queen and worker) on the average percentage of larvae removed after either 4 or 24 hours (4 hours: $W(14) = 24$, $p = 0.5$; 24 hours: $W(14) = 24.5$, $p = 0.5319$, Fig. 4.1).

No effect was found of larval source or age, or an interaction between the two, on whether or not an egg was policed (Table 4.2, Fig. 4.2). The proportion of larvae removed appears to be lower on days 2 through 5 of larval development, peaking on day 1 (Fig. 4.2). However, the age of the larvae had no significant effect on survival so, other factors than age may explain their survival (Table 4.2). All survival however, was short lived as only 3.6% of transferred larvae survived 24 hours.

4.5 Discussion

Honey bee workers neither removed queen and worker-produced larvae at different rates nor varied their removal rate according to the age of the larvae. As such, I find no evidence that male larvae were removed to maximize colony relatedness. Likewise, that the age of larvae has no positive effect on survival is counter to the colony level cost model proposed by Nonacs (2006).

Male larvae were removed at a much higher rate than is usual for female larvae, which can be readily introduced to different colonies (Allsopp, *et al.*, 2003; Pettis, *et al.*, 2004). This is consistent with the findings of Wegener (2009) whose *in vitro* reared male larvae were rarely accepted by colonies. The particularly rapid removal rate of 1, 6 and 7 day old larvae is probably indicative of the difficulties of moving these larvae: newly hatched larvae are exceptionally small, and may be more easily drowned than older, larger larvae, whilst larvae nearing pupation fill the cell and are difficult to remove from, and place into, cells without damaging their skin.

The higher threshold for acceptance of male larvae could be a mechanism to maximize colony efficiency. Workers can only be queen derived whilst drones may be either queen or worker derived. Drones are also much more costly to rear than workers. Drones take 24 days to emerge and a total of 38 days before they are fertile. Workers take between 18 and 22 days to emerge whilst queens develop in the shortest time, emerging after 16 days and becoming fertile 23 days after they were laid. During the larval stage all castes gain a lot of weight, about 900, and 2300 times that of the egg for workers and drones respectively, reaching 140mg and 346mg at capping (Stabe, 1930; Winston, 1991). To reach this weight drone larvae receive around 5 times more food during their development than worker larvae (Haydak, 1970). Once emerged from the cell adult drones are initially fed a royal jelly mixture, during which time they also increase in size and weight (28% increase in dry weight, DeGroot, 1953), after which they consume honey from cells inside the colony (Free, 1957; Haydak, 1970). After emergence worker bees eat pollen, growing and developing internal organs and their hypopharyngeal glands. Worker bees reduce their consumption of pollen on day 10 post emergence (Haydak, 1970). As such, drones are costly to produce and maintain as whilst workers collect food for the rest of the colony and participate in brood rearing and colony maintenance, drones remain in the hive except to defecate and mate. With increased cost of production and maintenance, the cost of mistakenly rearing a less viable drone increases and the threshold of drone acceptance should be higher to maximize colony efficiency. As such, colonies may have a particularly high threshold for drone acceptance as opposed to that of workers, explaining the particularly low acceptance level of transferred larvae (Wegener, 2009). Indeed, even queen-laid male eggs are

removed by workers if environmental conditions are not right (Free and Williams, 1975). Compounding this effect is that male larvae, unlike female larvae, can be worker produced. As such, if worker-laid male viability is lower than queen-laid male viability, the threshold for acceptance would be even higher than policing larvae for purely viability concerns.

No evidence was found that workers favored queen-laid over worker-laid male larvae, indicating that workers do not police to maximize colony relatedness. However, honey bee workers are very successful at identifying and removing male eggs. Whilst 7% of male eggs in a colony may belong to workers only 0.12% of those make it to adulthood (Visscher, 1996). As such, worker policing is highly effective, allowing only 1-2% to develop (Ratnieks and Visscher, 1989; Ratnieks, 1993). Removal is rapid with half of all worker-laid eggs being removed within 2 hours of introduction to a queen right colony and 90% within 6 hours (Ratnieks and Visscher, 1989). With such an effective mechanism of policing in place, and worker laying being rare in the presence of the queen, benefits accrued from being able to distinguish worker and queen laid larvae may be small, and no mechanism may have evolved.

Alternatively, male larvae may have acquired labels or cues that mark them as foreign and are therefore destroyed as unrelated parasites. However, this hypothesis seems less likely than that of reduced quality. Firstly, Ratnieks and Visscher (1989), in contrast to this experiment, achieved high acceptance rates of male larvae, with 82% of queen-derived and 73% of worker-derived male larvae remained 24 hours after having been

introduced to an unrelated colony. Secondly, female larvae laid by *Apis mellifera capensis* workers in *Apis mellifera scutellata* colonies are not only reared, but also receive better nutrition than they do in their natal colonies (Calis *et al*., 2002).

As such, we provide no evidence that worker policing is selected to continue maximizing colony relatedness past the egg stage. The absence of age effects on policing rate also suggests that workers do not police to reduce colony level costs. However, this may be the result of damage during transfer. If larvae were damaged during transfer, and male larvae have a higher quality threshold than female larvae, evidence of age effects on policing rate may have been masked. To elucidate this it may be interesting to compare acceptance thresholds of worker and queen-laid female larvae in colonies of *Apis mellifera capensis*. Because *A. m. capensis* workers reproduce thelytokously there are no nepotistic benefits to policing. There are colony level costs however, as despite being able to lay female eggs, queenless *A. m. capensis* colonies rarely rear a new queen and perish after approximately 4 months (Anderson, 1963). Higher thresholds of acceptance of female larvae, compared to colonies that cannot reproduce thelytokously, would indicate a role of viability in the evolution of policing behavior.

4.6 Figures and Tables

	DIET D1 **Drone Larvae up to 108 hours**	**DIET D2** **Drone Larvae > 108 hours**
Royal Jelly	66.6%	50%
Sterile Water	27.4%	30%
Glucose	3%	14%
Fructose	3%	6%

Table 4.1 Diets fed to male larvae from day 1-4 (Diet 1), and day 5-7 (Diet 2) as per Wegener (2009).

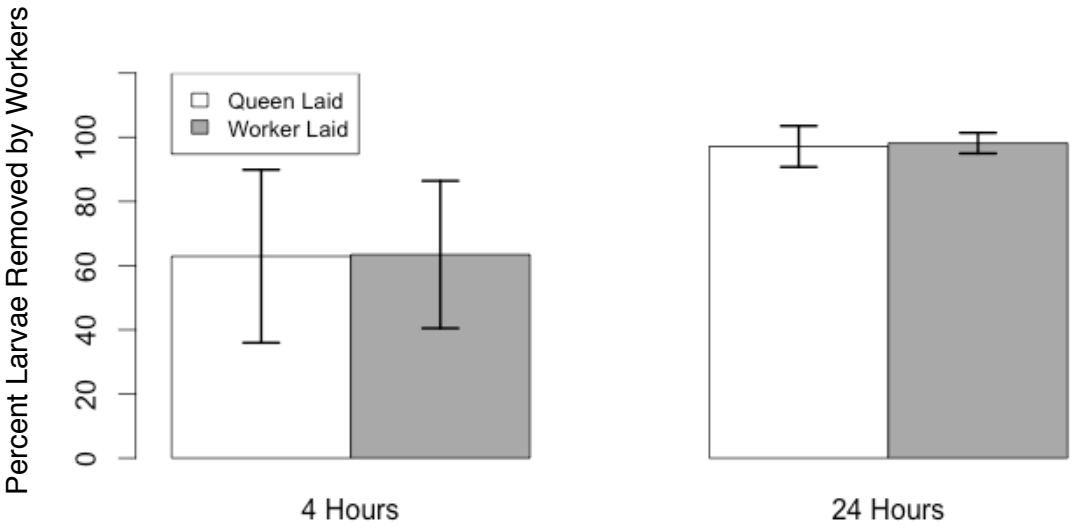

Figure 4.1 Effect of mother on larval survival 4 and 24 hours after introduction to test colony. In both cases neither queen (white) nor workers (grey) conferred a survival advantage to their larvae (4 hours: $W(13) = 24$, $p = 0.5$; 24 hours: $W(13) = 24.5$, $p = 0.5319$)

	d.f.	Wald χ^2	*p*
Block	1	28.5	9.4E-08
Age	1	0.33	0.56
Mother	1	2.1	0.15
Age x Source	1	0.52	0.47

Table 4.2 Neither female source nor age of larvae influences policing rate in honey bees. Larval removal, four hours after introduction, was analyzed with a logistic regression. The interaction model showed no effect of either mother or age on larval removal. Model simplification did not alter this result.

a

b

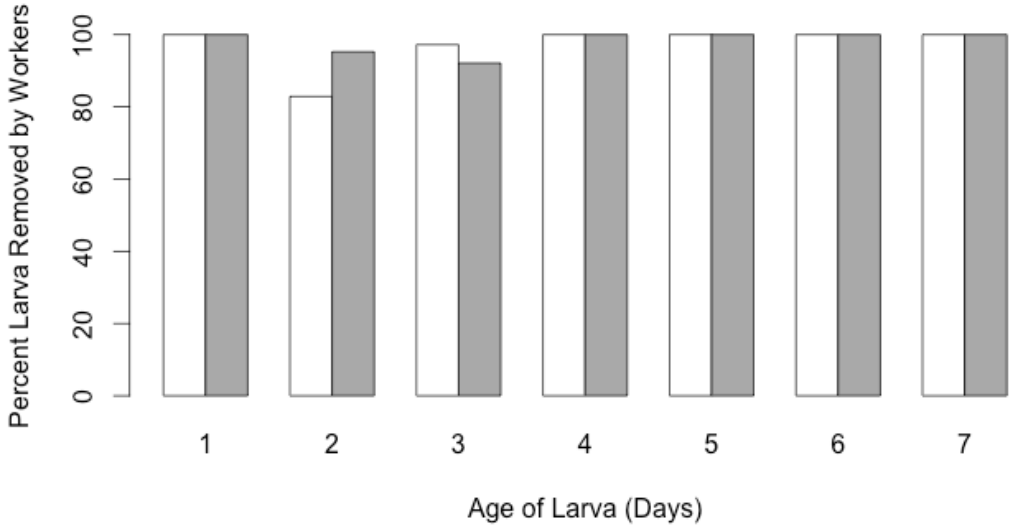

Figure 4.2 Worker (grey bars) and queen (white bars) laid larvae are removed at similar rates regardless of their age (see also, Table 4.2). Percent larval removal is shown **a**) 4 hours and **b**) 24 hours post introduction to the test colony.

4.7 References

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CHAPTER 5

Intragenomic Conflict Over Worker Laying in the Honey Bee

5.1 Abstract

Workers in honey bee colonies can activate their ovaries and compete with the queen to produce male offspring. Due to the haplodiploid sex determination system, paternal genes may only enter male offspring if daughters reproduce males. As such, males may enhance their reproductive success by imprinting genes that promote worker reproduction. African honey bees activate their ovaries faster than European honey bees. Here, I examine parent-of-origin effects on ovary activation by crossing African and European queens with African and European drones, in single drone inseminated crosses. Workers were then placed in queenless colonies and their behavior recorded daily from day 8 to 28. On day 16 and 28 workers were collected and dissected for ovary development. I found no evidence of paternal effects on ovary development. Rather, both hybrids had higher levels of ovary development than pure bred individuals. Behavioral data provided support the ovarian ground plan hypothesis, which proposes a link between maternal behaviors and ovary activation, as individuals with high levels of ovary development participated in brood rearing over foraging.

5.2 Introduction

The members of a eusocial insect colony fall into three different castes: queen, worker, and male. Queens and workers are female castes and may range from having only minor statistical differences in body size to being completely distinct morphologically, and behaviorally (Wheeler, 1913). Queens produce all of the offspring in the colony whilst workers help to rear, feed, defend, and structurally maintain the colony. Indeed, in colonies of the primitively eusocial wasp, *Polistes versicolor*, subordinates perform 81.4% of the total foraging activity (Zara and Balestieri, 2000). Males across all social Hymenoptera almost universally perform no brood care or foraging for the colony. Their only function is to search our mating opportunities (Roig-Alsina, 1993). Workers can be classified into subcastes. As with queens and workers, these subcastes may be both morphologically and behaviorally distinct, although an individual worker may transition through different subcastes as they age (Wheeler, 1986). In honey bees, worker subcastes include cell cleaners, nurses who tend to the brood and queen, food storers who receive nectar and pollen from foragers and pack it into cells, and foragers (Seeley, 1982). Despite queens producing all of the offspring in eusocial insect colonies workers often maintain their reproductive potential. For example, the ovaries of honey bee workers are undeveloped in the presence of the queen, but when she is removed workers activate their ovaries and lay male eggs (Oster and Wilson, 1978).

The ovarian ground plan hypothesis (West-Eberhard, 1996) links individual behavior and ovary development. The model proposes that ovarian development, brood care and foraging became uncoupled in a solitary ancestor of the eusocial insects, leading to ovary

development being maintained in queens, brood care in young workers and foraging in older workers. Reproductive potential is also predicted to be greatest in brood rearing workers, diminishing as a worker ages and transitions to become a forager. Brood rearing workers have also been shown to be more physiologically similar to queens than foragers. For example, honey bee queens and nurse bees have low levels of juvenile hormone, while foragers have high levels (Robinson *et al.*, 1991).

Evidence has also been presented that caste determination has a genetic underpinning (Grozinger *et al.*, 2007; Linksvayer and Wade, 2005; Toth and Robinson, 2007). Reproductive workers were found to have a more queen-like gene expression pattern than sterile workers, and both queens and reproductive workers were found to up-regulate genes associated with the nurse bee behavioral states (Grozinger *et al.*, 2007). These expression patterns may be driven by epigenetic mechanisms (Lyko *et al.*, 2010; Herb *et al.*, 2012). Indeed, honey bee subcastes were found to have substantially different DNA methylation patterns, and that if foragers returned to the nurse subcaste the pattern of DNA methylation would also revert. As differentially methylated regions can influence global gene expression patterns, DNA methylation may regulate the differences in gene expression between foragers and nurses previously observed (Herb *et al.*, 2012).

Genomic imprinting is one form of epigenetic control where the expression of a gene copy depends on whether or not it originated from the maternal or paternal line (Crouse, 1960). To control gene expression in a parent-of-origin specific manner genes

are modified, silencing them, and it is this genetic modification that is termed genomic imprinting. The pattern of imprinting is set for the life of an individual and is erased and reset during egg and sperm formation, a maternal imprint in the egg and a paternal imprint in the sperm. There are two major mechanisms by which these imprints are established: histone modification and DNA methylation (Li *et al.*, 1993; Reik and Walter, 2001).

Genomic imprinting is hypothesized to have resulted from conflict between males, in species where females mate multiply, over maternal resources. As such, males would imprint genes that retarded offspring growth, ensuring that his progeny would grow larger and more rapidly. In response, maternal imprinting would evolve to silence alleles involved in promoting offspring growth as severe overgrowth by embryos can be a disadvantage to the mother's fitness (Haig and Westoby, 1989; Moore and Haig, 1991; Haig, 1996; Haig, 2000). Examples of this evolutionary arms race exist in mice (Guillemot *et al.*, 1995; Frank *et al.*, 2002), humans (Isles and Holland, 2005; Haig, 2010) and birds (McElroy *et al.,* 2006; Tuiskula-Haavisto and Vikki, 2007).

One interesting extension of Haig's (2000) theory concerns that of haplodiploid organisms (Queller, 2003). Here, males have one set of chromosomes whilst females have two and thus males and females are differentially related to kin (Fig. 5.1). A father shares his entire genome with his daughter (r=1), whilst a mother only shares half her genome (r=0.5). Females contribute half of their genes to their sons (r=0.5), which makes up the entirety of his genome (sons relatedness to mothers: r=1). As such, males

do not contribute any of their genome to the male offspring of his mate, the queen. The only way a male may pass his genes to the next generation of males is if his daughters lay male eggs. This can happen in two ways, firstly the daughters may become a queen and as such, both father and mother gain fitness benefits through the production of offspring. Secondly, a worker may activate her ovaries and lay unfertilized male eggs. The laying of male eggs by workers negatively impacts the fitness of the queen as worker laid males replace the queens male offspring. Queens are more related to their own sons (r=0.5) than they are to their daughters' sons (r=0.25) and as such, when workers and the queen are competing, in the same generation, to produce male offspring the queen favors her own reproduction over that of her daughters. A behavior called worker policing helps to mediate the conflict over worker reproduction in hives (Ratnieks, 1988). As such, the paternal imprint would be expected to silence genes involved with worker policing, and maternally imprinted genes would be those that silence worker laying.

Here, I perform crosses of African and European honey bees to test for paternal effects on ovary development. Africanised honey bees are known to activate their ovaries faster and lay eggs more rapidly than European honey bees (Chapter 2). Imprinting in African bees appears to be dominant over imprinting in European honey bees (Guzman-Novoa *et al.*, 2005). As such, workers with an African father and European mother would be expected to activate their ovaries earlier, and have more ovarioles, than individuals of pure European descent. Secondly, I performed behavioral observations to assess

whether or not nurse bees have higher levels of ovary development than foragers, in accordance with the ovarian ground plan hypothesis.

5.3 Methods

One European and 1 African colony were selected for drone and queen rearing from those available at UC Riverside, CA, and ancestry was confirmed through mitotyping (Crozier *et al.*, 1991). Unfortunately none of the European larvae grafted into queen cells survived to adulthood and so 20 virgin European queens were purchased from Jackie Park-Burris Queens, CA. Single drone inseminations were performed by Susan Cobey such that African queens were mated with a single African or European drone and European queens were mated with a single African or European drone. These queens were then introduced to small nucleus colonies and allowed to establish for 2 months.

5.3.1 Behavioral Data & Sample Collection

Queens were placed into a cage with empty brood comb, to lay eggs, for 24 hours. 17 days later these frames were removed from the colonies and placed into an incubator at 37^{o}C and 95% humidity to emerge. Up to 100 bees per colony were individually marked with a distinctive colored number on their thorax and paint mark on their abdomen. All bees were then placed into a queenless five frame hive (nuc) that had recently commenced egg laying.

From day 8 to day 28 behavioral data was collected for 2-4 30 minute periods per day, spaced across both the morning and afternoon. The behaviors of marked bees was recorded. Foragers were identified as those returning to the colony with a visible pollen load or distended abdomen. Bees were determined to be laying an egg if they were observed to be backed, abdomen first, deep into a cell, and if an egg was present in that cell upon their emergence. Workers were determined to be brood rearing if they were observed to be head first down a cell containing larva. For frames containing worker laid eggs, up to 20 individuals per frame were identified and classified as being in an area of the hive where egg laying was occurring (near eggs). Individuals classified as foraging or egg laying were marked, with a paint marker, on their abdomen. Approximately half of the marked, and half of the unmarked bees were collected 16 days after introduction to the colony, and all remaining marked bees were collected on day 28. Bees were flash frozen and stored at -80^{o}C for future dissection.

5.3.2 Ovary Dissection

Samples were moved to RNA Later Ice and stored at -20^{o}C for a minimum of 2 days before dissection. Samples were dissected following the methods of Dade (1962) and the level of ovary activation, of the most advanced ovariole, was scored on a four point scale. The first two points, I1 and I2, denoted inactivated ovaries. I1 was defined as having translucent tubes with very little evidence of ovariole formation, and no evidence of oocyte formation. I2 ovaries were more opaque, with clear evidence of either ovariole formation at the top of the ovaries, and/or the beginnings of oocyte formation. Activated ovaries A1 and A2 were opaque, often had multiple ovarioles, and oocytes

(distinct globules in the ovarioles) were clearly present. A1 and A2 were distinguished by the size of their largest oocyte: A1's was less than half the size of the average egg whilst A2's was more than half the size of an average egg. Apart from ovary activation level the number of ovarioles was also recorded.

5.3.3 Statistical Analyses

Firstly, a two way anova was used to assess the effect of collection day on ovary activation level and ovariole number for each ancestry. Both ovary activation level and ovariole number were box cox transformed so as not to violate the normality assumption, although all figures are presented using raw data for ease of interpretation. The Kruskal-Wallis one-way anova was used to examine whether or not the level of ovary activation or number of ovarioles varied between the different crosses. The Wilcoxon rank sum test with holm correction was used to examine differences between each of the crosses.

As behavior could be binary (0 = rear brood, 1 = forage) I used a multiple logistic regression model to examine the effects of ancestry, ovary activation level and their interaction on this task performance. All analyses were performed using R (version 3.1.2).

5.4 Results

5.4.1 Ovary Activation and Ovariole Number.

There was no effect of collection on the average level of ovary activation ($F(3,622)=0.2094$, $p = 0.72$, Fig. 5.2a) or ovarial number ($F(3,303) = 1.029$, $p = 0.25$, Fig. 5.2b) and so, unless otherwise stated, all data were analyzed together. There was a significant effect of ancestry on both ovary activation and ovariole number (ovary activation: $KW(3) = 20.72$, $p < 0.001$; ovariole number: $KW(3) = 20.83$, $p < 0.001$, Fig. 5.3). However, post hoc analysis using the Turkey HSD test showed little evidence of patrilineal effects, with only pure European workers differing in their ovary development from those with a European queen and African father (Tukey HSD; $p < 0.001$, Fig 5.3). No other ancestries differed in their level of ovary activation. The number of ovarioles in workers with activated ovaries did not follow the same pattern as seen with ovary activation level. Tukey HSD post hoc analysis revealed that individuals with an African mother and European father had significantly more ovarioles compared to all other crosses (AA: Tukey HSD; $p < 0.001$; EA: Tukey HSD; $p < 0.01$; EE: Tukey HSD; $p < 0.01$). Instead, we found that hybrids as a group were more likely to have activated ovaries, and more ovarioles, than purebred colonies (ovary activation: $W(626) = 338116$, $p < 0.0001$, ovariole number: $W(307) = 185462$, $p < 0.0001$, see Fig. 5.4)

5.4.2 Subcaste Determination

Logistic regression analysis was used to measure the effect of ovary development and ancestry on a workers behavior. Both a worker's ancestry and its level of ovary activation affected it's behavior (Table 5.1), however there was no interaction between

the two predictors. All ancestries had more developed ovaries when brood rearing as opposed to foraging (Table 5.1, Fig. 5.5). Pure bred African workers were the less likely than a worker of any other ancestry to rear brood (Table 5.1).

When only workers with activated ovaries were considered there was no effect of ovariole number on ovary activation and pure European workers were the only cross more likely to be nurses than foragers. When hybrid and purebred cross data were combined hybrids were no more likely to be nurses or foragers, and only the level of ovary activation predicted it's behavior (Table 5.2).

5.5 Discussion

The results of this study show increased reproductive potential in hybrid workers over the purebred lines (Fig. 5.4). Hybrid progeny were superior in both ovary development and ovariole number compared to their purebred counterparts. Heterosis is common amongst animals and plants and can be the result of genetic or epigenetic mechanisms (Eggan, *et al.*, 2001; Ni, *et al.*, 2009).

Hybridization leads to large regulation changes among the two different genomes, which is equivalent to 'genomic shock' (Comai *et al.*, 2003; Wang *et al.*, 2006). Hybridization can lead to disruption of imprinting in hybrids often leading to over-expression of one parents genes, and under-expression of the other parents genes (Vrana *et al.*, 1998). This disruption of imprinting may explain the patterns of hybrid vigor that we have observed. Whilst in pure bred individuals patterns of imprinting may have become

balanced, controlling the rate of ovary activation such that both parents interests are represented. Upon crossing however, the pattern of imprinting is disrupted and becomes mismatched, with either the paternal imprint dominating or the maternal imprint being lost, ultimately favoring selfish laying. Indeed, hybridization, and mismatched imprinting, may lead to the over-expression of other paternal traits such as stinging behavior (Guzman-Novoa *et al.*, 2005) and queen rearing (DeGrandi-Hoffman, 2003). Both before and after emergence honey bee queens produce a high pitched audible sound known as piping. Piping has been shown to prevent the emergence of more than one queen at a time (Frisch, 1965) and aid in assessing fighting ability (Visscher, 1993). Compared to their purebred counterparts, hybrid queens pipe more and eliminate a higher percentage of their rivals than their purebred counterparts (DeGrandi-Hoffman, 2003).

Not all African-European crosses lead to fitter hybrids. Schneider and Hall (1997) found no effect of hybridization on colony size, diet selection, pollen foraging activity, foraging distance, flight activity nor comb use. In North and South America negative hybrid vigor, in which the hybrid falls below the range of parental variation with respect to a given trait, has been linked to the loss of European alleles from feral bee populations (Smith *et al.* 1989; Hall, 1990; Clarke, *et al.*, 2001, Schneider *et al.* 2004). Hybrid workers have been found to have the same or lower mass-specific metabolic rates as European and African honey bees (Harrison and Hall, 1993) and have higher fluctuating asymmetry compared to African bees, regardless of rearing environment (Schneider, *et al.*, 2003).

Our results, however, are consistent with a positive effect of hybridization on the reproductive potential of workers and could help to explain the rapid colonization by Africanized honey bees of North and South America, and the loss of European alleles from feral populations (Schneider *et al.*, 2004). Honey bee colonies often become queenless in nature (McNally and Schneider, 1992) and if the colony fails to rear a replacement workers activate their ovaries. As workers of some hybrid colonies have more developed ovaries and more ovarioles, they will introduce more drones into the mating pool. Whilst the viability and mating success of worker produced drones is unknown any increase in the proportion of African genes in an otherwise European dominated mating pool would lead to an increase of those genes in the next generation. Other research into African and European honey bee reproductive behaviors has found: 1) that hybrid workers backcrossed with an African queen produce offspring with extreme ovariole counts (Linksvayer *et al.*, 2009), 2) that queens with African paternity are more likely to survive and inherit a colony (Schneider and DeGrandi-Hoffman, 2003), 3) that African colonies grow faster than European colonies and therefore tend to reach high densities (McNally and Schneider, 1996), 4) that drone production is higher in African versus European nests (Rinderer *et al.*, 1987), and 5) that African spermatozoa outcompetes European sperm (DeGrandi-Hoffman *et al.*, 2003). Considered together, such findings elucidate the underlying mechanisms contributing to the rapid supersession of African alleles in previously European populations.

The result that European bees had, whilst not significantly, higher ovariole number than that of the African workers, is somewhat surprising. Linksvayer *et al.* (2009) found that African workers had significantly more ovarioles than Europeans. This discrepancy in findings could be due to experimental design. In our experiment, due to the rejection of European queens during the rearing process, but acceptance of African queens, European queens were purchased from a breeder in California. As such, European queens and European drones originate from different colonies, introducing more genetic diversity into those colonies, and protecting against negative effects of inbreeding (Wright, 1932).

The second key result from this study is that honey bee subcaste was a product of both a workers ancestry and ovarial development, providing support for the ovarian ground plan hypothesis. Worker behavior is highly flexible. In colonies young workers tend to focus their efforts rearing brood, and maintaining the internal structure of the colony. As workers age they transition to roles outside the hive, guarding the entrance and ultimately foraging (Winston, 1987). These behavioral changes are accompanied by large changes in hormone levels (Jaycox *et al.*, 1974; Jaycox, 1976; Robinson, 1987) and DNA methylation and gene expression (Herb *et al.*, 2012). Despite this subcaste is neither controlled by age nor are they permanent as changes in colony structuring, such as too few forgers or not enough nurse bees, can result in age-matched workers being able to nurse or forage, and foragers reverting to brood rearing tasks (Robinson *et al.*, 1992; Calderone and Page Jr., 1996; Herb *et al.*, 2012).

My results also provide support for the ovarian ground plan hypothesis as workers with activated ovaries were more likely to engage in brood rearing. I did not find evidence of higher numbers of ovarioles in nurses or foragers, possibly because I only examined ovariole number in already activated ovaries. Very few workers were observed pollen foraging and so it wasn't possible to determine if pollen foraging individuals had higher levels of ovary activation than those who foraged for nectar.

My results concur with the findings of Grozinger *et al.* (2007) that reproductive workers gene expression pattern was more queenlike compared to that of sterile workers. These genes, that were up-regulated in reproductive as opposed to sterile workers, were also found to be those up-regulated in nurse bees over forager bees. As such, there is a strong link between reproductive status and behavior and many are likely derived through epigenetic changes. Divergent patterns of DNA methylation have been found between the queen and workers (Lyko *et al.*, 2010), and reversible DNA methylation patterns have been found in worker subcastes (Herb *et al.*, 2012).

Honey bee workers are able to adapt both behaviorally and physiologically to a changing environment. The loss of the queen, the amount of pollen and nectar stored and being brought into the colony, the age structure of the work force all affect how a worker will behave and if and when she should activate her ovaries. When the queen dies and workers compete over the final brood selection will act such that workers who are most capable of producing viable offspring develop their ovaries and reproduce. The others should focus on maximizing their inclusive fitness benefits through maintaining the

colony as long as possible. With the possible role of epigenetic mechanisms controlling such behaviors in honey bees and of imprinting mismatches driving a naturally occurring invasion, honey bees are a key model organism for epigenetic research, and epigenetic mechanisms of speciation (Michalak, 2009).

5.6 Figures and Tables

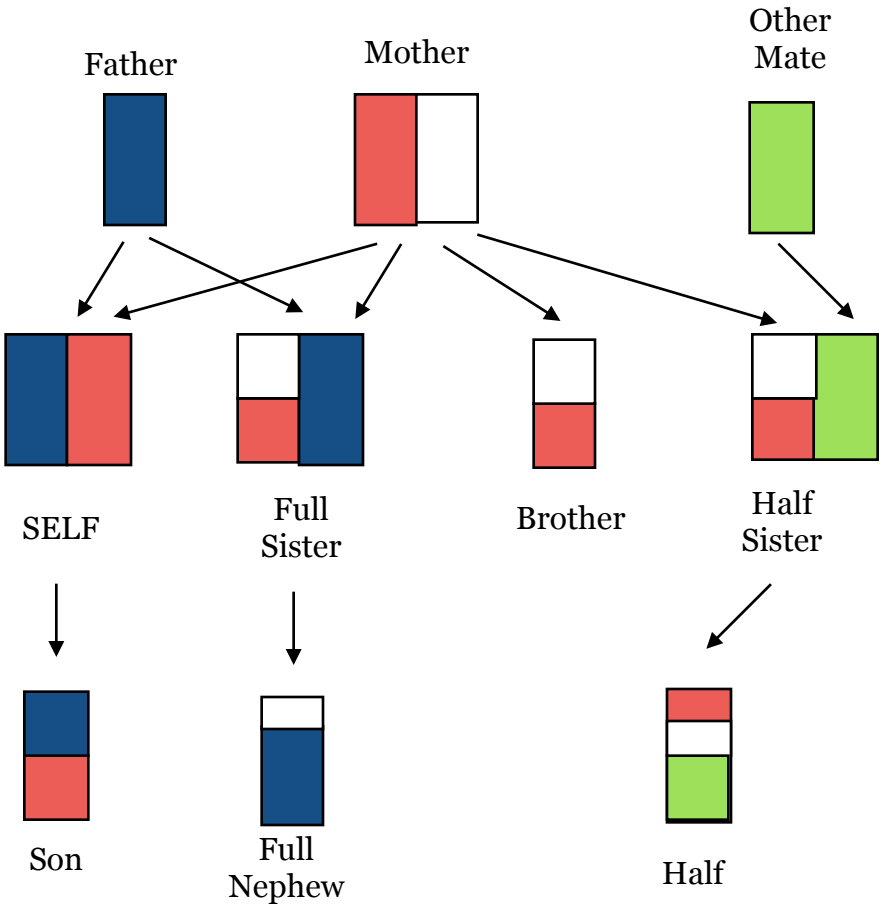
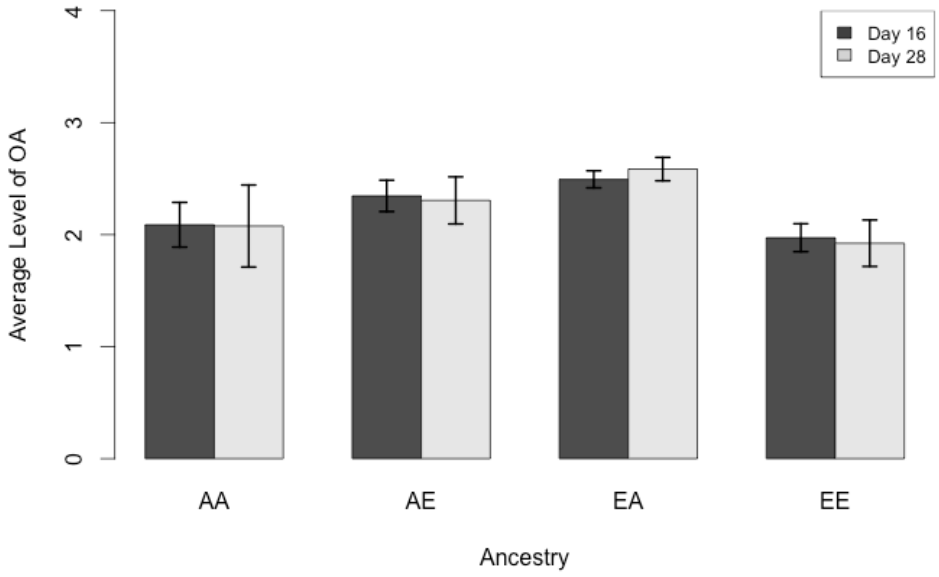


Figure 5.1 A haplodiploid pedigree. The coloring shows the degree of shared genes between individuals and 'SELF'. Note that the fathers genes (blue) only enter the second generation through his daughters male offspring whilst the mothers genes (pink and white) are present in her daughters, her sons, and her daughters grandsons.

a.



b.

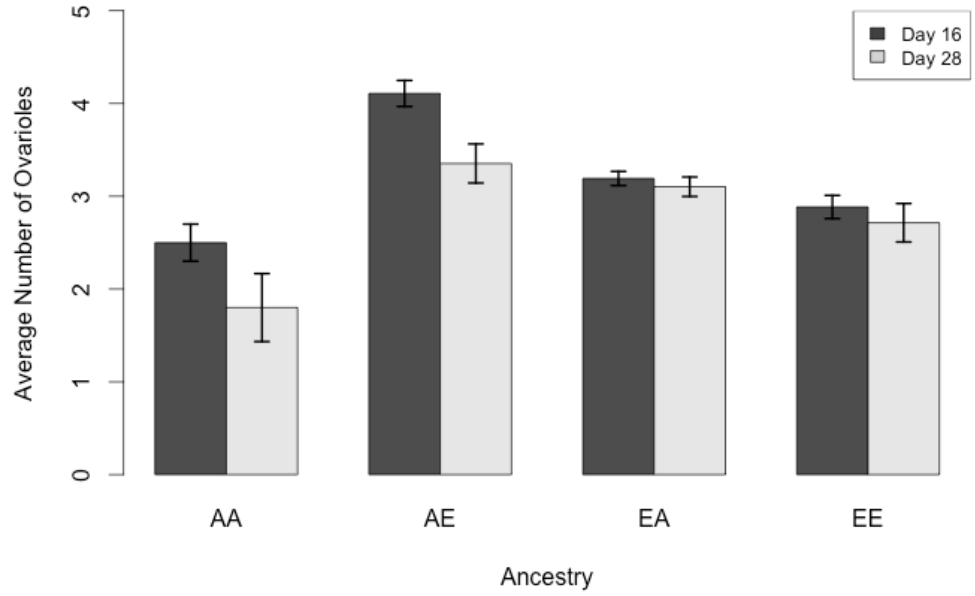


Figure 5.2 Collection day had no effect on **a)** the average level of ovary activation ($F(3,622)=0.2094$, $p = 0.72$) or **b)** the average number of ovarioles ($F(3,303) = 1.029$, $p = 0.25$, Fig 5.2b) regardless of the ancestry of the individual.

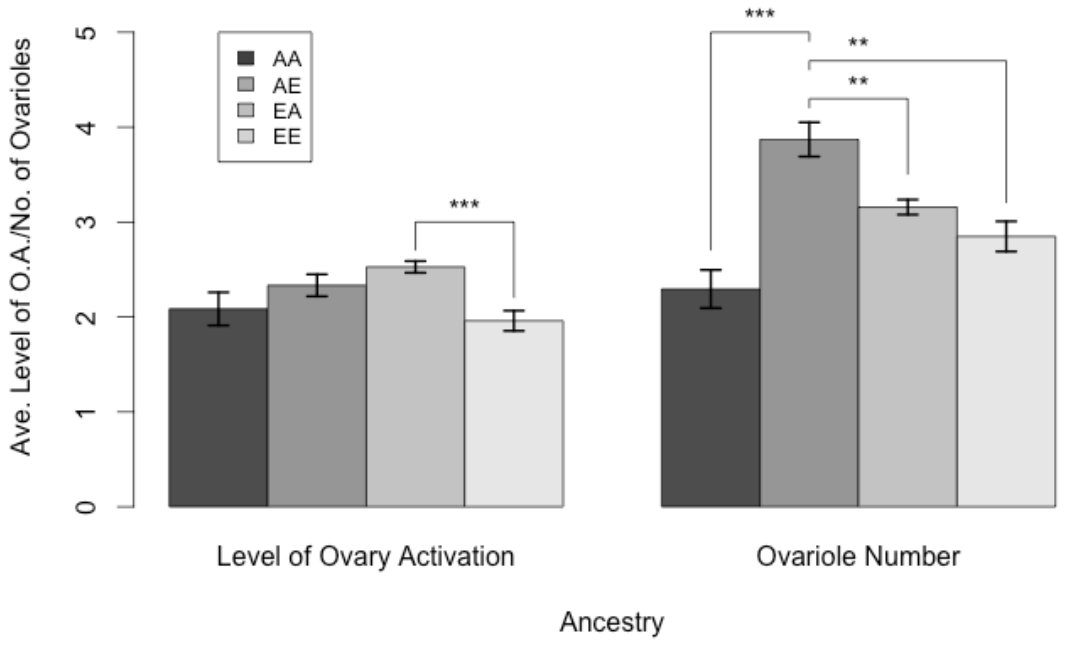


Figure 5.3 Both ovary activation level and number of ovarioles differed with ancestry (Ovary Activation: KW(3) = 20.72, $p < 0.001$; Ovariole Number: KW(3) = 20.83, $p < 0.001$). Individuals with a European mother and African father were more likely to have activated ovaries than individuals of pure European descent (W(466) = , $p < 0.001$). Ovary activation did not differ between any of the other ancestries. Ovarial number was higher in individuals with an African mother and European father than in individuals of any other ancestry (AA: W(162) = , $p < 0.001$; EA: W(481) = , $p < 0.01$; EE: W(213) = , $p < 0.01$). Ovariole number did not differ between individuals of the other crosses.

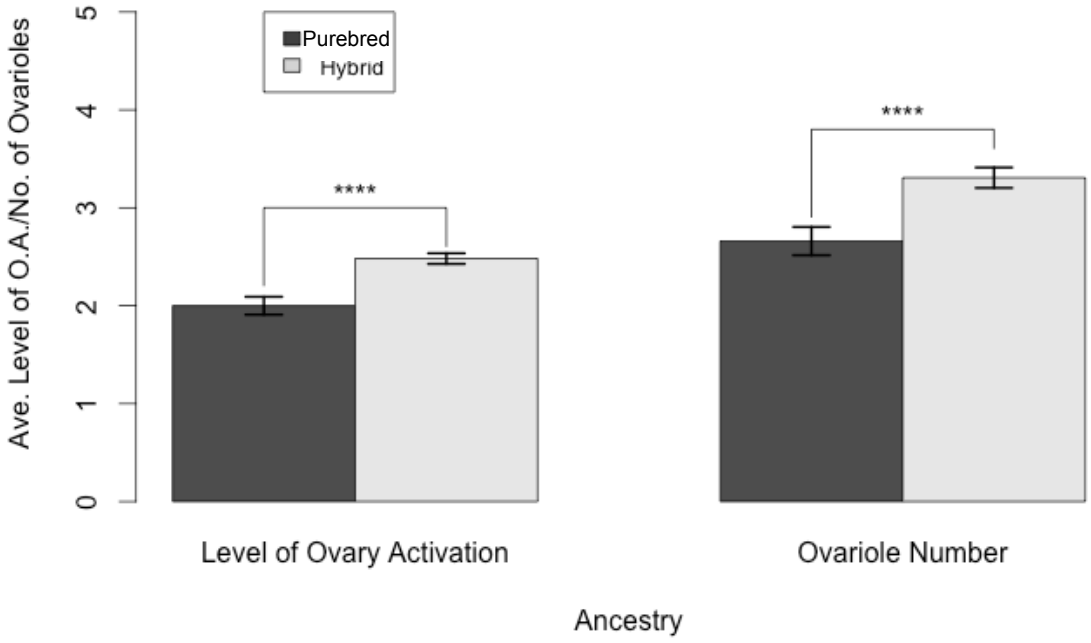


Figure 5.4 African-European and European-African workers (hybrid) had higher levels of ovary activation and more ovarioles than their purebred African or European relatives (ovary activation: $W(626) = 338116$, $p < 0.0001$, ovariole number: $W(307) = 185462$, $p < 0.0001$).

a.

b.

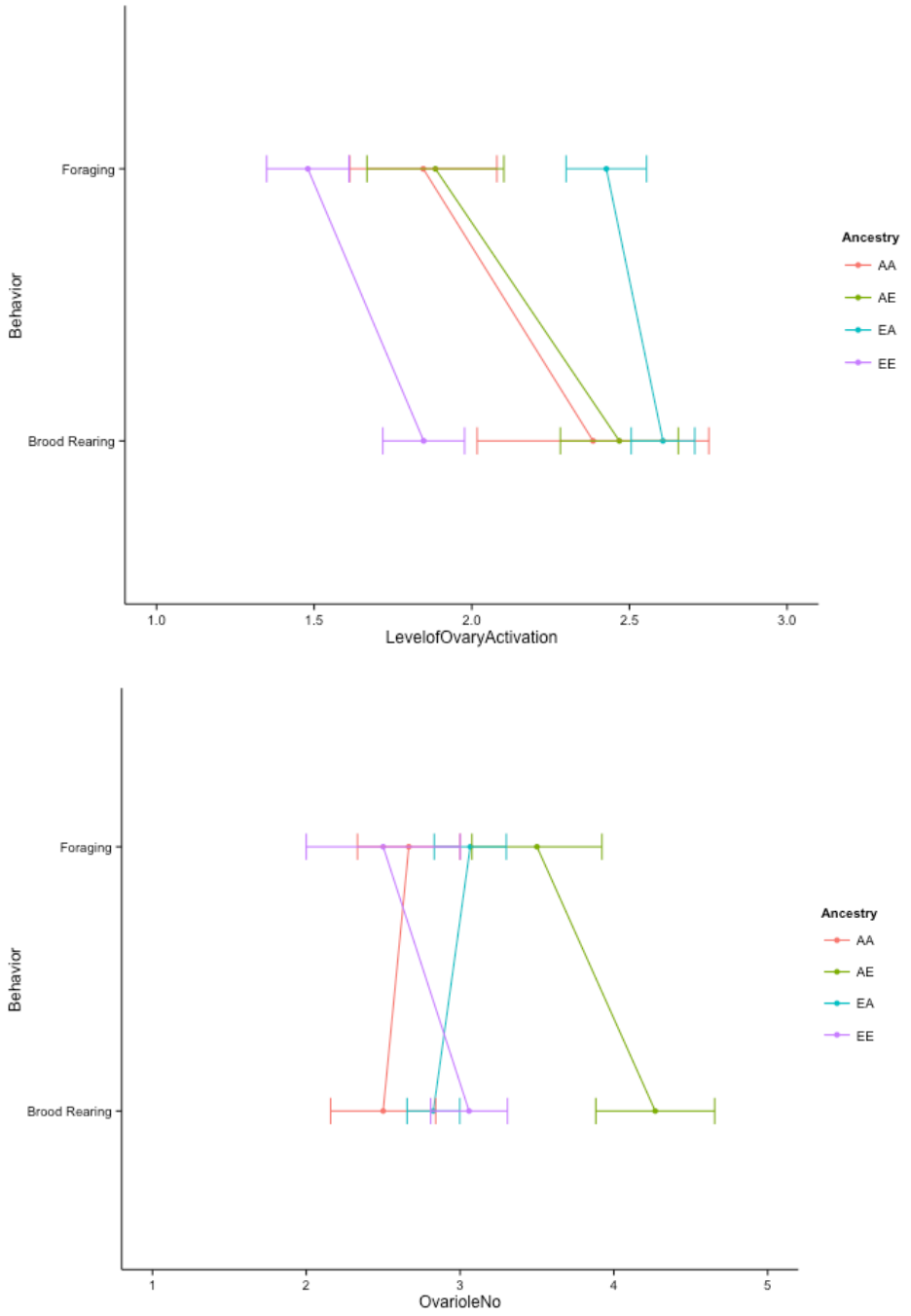


Figure 5.5 The effect of ancestry and **a**) ovary development, **b**) ovariole number, on worker subcaste. Each line represents one ancestral cross. Ancestry and level of ovary activation predict subcaste whereas ovariole number had no effect on an individuals behavior.

a. **Ovary Activation**

		β	*df*	Wald's χ2
Intercept		0.88	1	6.3**
Ancestry	**AE**	-1.22	1	8.3***
	EA	-1.00	1	7.1 ***
	EE	-1.62	1	14.9 ****
Ovary Activation		-0.56	1	6.5 **

b. **Ovariole Number**

		β	*df*	Wald's χ2
Intercept		-0.05	1	0.005
Ancestry	**AE**	-1.21	1	2.8
	EA	-0.52	1	0.72
	EE	-2.15	1	5.2 **
Ovariole		0.02	1	0.03

Table 5.1 Behavior was analyzed using a multiple logistic regression model to test the effect of ancestry and **a**) level of ovary development, and **b**) number of ovarioles in determining worker subcaste.

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CHAPTER 6

Conclusion

The aim of this study was to examine the effects of three levels of selection on worker laying and policing behavior in the honey bee. Despite the honey bee being the model eusocial organism and worker laying and policing behavior being first predicted and identified in honey bees, the potential for such altruism to have evolved through levels of selection other than that of the individual had not been adequately explored. Combining a series of behavioral manipulations with theoretical modeling I addressed this issue.

The simulation model presented in chapter 3 demonstrates that higher and lower levels of selection can dominate individual level selection. Past research tended to only invoke group level selection when the predictions of individual level selection were not met, such as the presence of worker policing in colonies headed by a monogamous queen (Foster *et al.*, 2002; Endler *et al.*, 2004). My model predicts that worker policing, in such situations, would likely be the result of intragenomic conflict. Indeed, genome level selection tended to dominate both individual and group levels, an group level selection was more significant than the individual level in determining when queens dominate male production.

The results from behavioral manipulations were less convincing than those from the model. I first attempted to examine the effect of the social environment and ancestry on worker laying behavior. Whilst workers of African ancestry laid eggs earlier than European workers, ovary activation occurred at similar rates, a novel finding. I found a significant effect of both the juvenile and adult social environment on ovary activation

suggesting that environment has a larger effect on the propensity to activate ovaries than subspecies.

To explicitly examine the role of selection at the level of the group and at the level of the individual in the evolution of worker policing behavior I transferred queen and worker laid larvae of differing ages into a queenright colony. I found no evidence that workers discriminate using either larval source or age. As such, I could not distinguish between selection acting at the level of the group or the individual. However, the model presented in chapter 3 predicts that when a mechanism of imprinting is in place selection at the level of the genome would dominate either group or individual level selection.

As such, I performed a series of single drone inseminated crosses of African and European honey bees. The resulting offspring were then placed into queenless colonies and their levels of ovary development assessed. Workers with an African father were predicted to have higher levels of ovary development than workers with an European father. The predicted results were not realized, rather hybrid workers had higher levels of ovary development than their purebred counterparts suggesting potential mechanisms such as imprint mismatching where the maternal imprint is lost and only the paternal imprint is expressed, or increased heterozygosity in hybrid workers compared to purebred workers. A future study could examine the methylome of hybrid vs purebred worker bees to distinguish whether or not the maternal imprint is indeed lost.

Overall, these results do not identify any single level of selection as producing worker laying and policing behavior. Rather, the clear predictions from my model suggest that individual level selection is at best a secondary factor in determining the evolution of worker policing, and the inconclusive results from my behavioral studies identify this as a complex problem requiring further research. Examining the acceptance of female queen and worker-laid larvae in colonies of *Apis mellifera capensis* could shed light on whether or not the apparent higher threshold for accepting drone larvae is a consequence of colony level selection due to worker laying or the extra resources required to rear drones. Secondly, examining the methylomes of hybrid and purebred workers would elucidate whether or not imprinting controls worker laying and policing behavior from the level of the gene.

6.1 References

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APPENDIX I

Unsuccessful Methodologies from Chapter 4

A1.1 Full Factorial Design

To induce worker laying behavior a queenless 5-frame nucleus (nuc) colony (W, see Fig. A1.1) was established. When the first, worker-laid eggs, appeared in the colony a second queenless nuc (R) was established in which male brood would be reared. The queen was placed into a cage with drone comb that was refreshed daily for 20 days. Drone comb was also placed in, and exchanged daily from, W. Every day 50 eggs per colony were transferred, using Taber (1961) forceps, to 10 small sections of drone comb and placed into R. Each day, one of the sections was moved to the Test colony, and the number removed was counted 4 and 24 hours after transplantation.

The purpose for moving all newly-laid eggs into R was so that both worker and queen produced eggs and larvae experienced identical rearing conditions until tested for worker policing. Any egg that failed to hatch when expected in R was replaced by a new egg. Staggered age distributions across subsections (Table A1.1) allowed us to check if brood quality declined in W as the period of queenlessness increased. The queen was separated from the test frame by a queen excluder to prevent her from eating or adding eggs.

A1.1.1 Problems with the Full Factorial Design

All worker laid eggs that were placed into R were policed until workers in R started laying eggs themselves. At this point it became impossible to know the ancestry of the eggs being reared and too few worker laid eggs survived to hatching to continue with the

experiment. Secondly, after a few days the queen in section Q stopped laying male eggs and a new queen had to be used. As such, the policing assay could not be performed.

A1.2 *In vitro* Rearing of Male Larvae

A rearing plate was set up following Protocol 10 of Wegener *et al.* (2009). Here, four layers of tissue paper were placed onto the upper side of the lid of a 24 well tissue culture plate. The paper was soaked with larval food, and beeswax coated nylon strings were stretched across the lid such that they were approximately 0.5mm from the surface of the lid but touching the soaked wipes. Cotton strings were then stretched over the lid on both sides of each nylon string to prevent the eggs from falling into the food prematurely.

Male eggs were obtained by confining the queen of a strong colony to a comb with drone-sized cells by means of a cage made of excluder material. The queen was freed after 24h and the egg comb placed in an incubator at 35°C and 50% Relative Humidity. After 29h, a group of 100 eggs was removed from the comb using Taber forceps (Taber, 1961) and placed on the nylon string of the rearing plate. These were then placed in an incubator at 35°C and >95% relative humidity and checked 3 days later.

Diets were made up as per Wegener *et al.* (2009, Table A1.2). Queen cups were filled with 300µl of diet D1 and larvae placed individually into each well using a grafting tool. Care was taken not to submerge the larvae. Queen cups filled with sterilized water were placed on either side of the queen cups containing larvae such that there was a column of water, column of larvae, column of water, column of larvae etc. Larvae were transferred into fresh, pre-warmed food daily. From day 5 diet D2 was used instead of diet D1.

This process was repeated daily for 10 days. Both queen and worker laid male larva were then transferred into a frame of drone comb on 100µl of diet D2. The frame was then placed into a queenright colony and checked 4h, 12h and 24h later.

A1.2.1 Problems with the *in vitro* Rearing of Male Larvae Design

No larvae successfully hatched from the eggs. After several attempts I contacted Dr Wegener to get some help and was furnished with his PhD thesis which stated:

"Rearing eggs into larvae before introducing them into colonies did not improve their survival. The removal of the larvae was not caused by the caste of their mother, since control larvae stemming from queens were also removed. Survival of worker-laid eggs in laying worker colonies was equally low. Results of methods 1 and 2 seem to confirm the conclusion of Nonacs (2006) that queenright colonies apply strict standards for accepting male brood for rearing. These standards appear to be stricter

than those applied to female larvae, since female larvae hatched in vitro were accepted by rearing colonies. In conclusion, rearing offspring of selected workers inside normal queenright or queenless colonies is probably not an option, unless new methods are found to protect them from cannibalism." (Wegener, 2009)

As such, this method was aborted.

A1.3 Figures and Tables

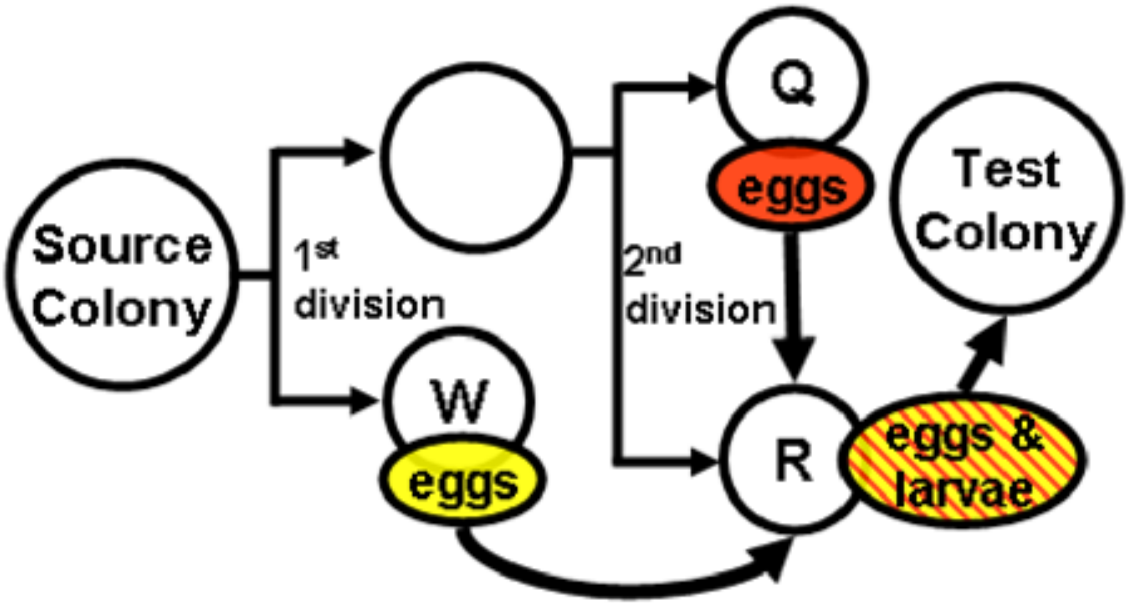

Figure A1.1 Protocol for worker policing experiment. Queen-produced and worker-produced eggs come from sub colonies (Q) and (W) respectively. They were introduced into (R) to be reared under identical conditions. At various times in their development (see Table 4.1), they were moved into (Q) and rate of removal measured.

Frame Section	na	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r
Egg Transfer																			
Day 1 (50e)	5e	5e	5e	5l	5l	5l	5l	5l	5l	5l									
Day 2 (50e)		5e	5e	5e	5l	5l	5l	5l	5l	5l	5l								
Day 3 (50e)			5e	5e	5e	5l	5l	5l	5l	5l	5l	5l							
Day 4 (50e)				5e	5e	5e	5l	5l	5l	5l	5l	5l	5l						
Day 5 (50e)					5e	5e	5e	5l	5l	5l	5l	5l	5l	5l					
Day 6 (50e)						5e	5e	5e	5l	5l	5l	5l	5l	5l	5l				
Day 7 (50e)							5e	5e	5e	5l	5l	5l	5l	5l	5l	5l			
Day 8 (50e)								5e	5e	5e	5l	5l	5l	5l	5l	5l	5l		
Day 9 (50e)									5e	5e	5e	5l	5l	5l	5l	5l	5l	5l	
Day 10 (50e)										5e	5e	5e	5l	5l	5l	5l	5l	5l	5l
Eggs Moved	5	10	15	15	15	15	15	15	15	15	10	5							
Larva Moved				5	10	15	20	25	30	35	35	35	35	30	25	20	15	10	5

Table A1.1 Transplant distribution from sub colony (Q) or (W) into the rearing sub colony (R). Each day 50 eggs (from both sub colonies) were spread across 10 subsections of drone comb (labeled a-r), with one subsection moved back to sub colony (Q). As an example, on the 10th day of the experiment subsection i, now containing 15 eggs and 35 larvae of staggered ages were moved to (Q). On on the 1st day were 5, day-old eggs transplanted directly to (Q) without being in (R).

	DIET D1 Drone Larvae up to 108 hours	DIET D2 Drone Larvae > 108 hours
Royal Jelly	66.6%	50%
Sterile Water	27.4%	30%
Glucose	3%	14%
Fructose	3%	6%

Table A1.2 Diets fed to male larvae from day 1-4 (Diet 1), and day 5-7 (Diet 2) as per Wegener (2009).

A1.4 References

Taber, S., III. 1961. Forceps design for transferring honey ee eggs. *Journal of Economic Entomology*. **54**: 247-250.

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Wegener, J., Al-Kahtani, S. and Bienefeld, K. 2009. Collection of viable honey bee (*Apis mellifera*) larvae after hatching in vitro. *Journal of Apicultural Research*. **48**: 115-120.