



A University of Sussex DPhil thesis

Available online via Sussex Research Online:

<http://sro.sussex.ac.uk/>

This thesis is protected by copyright which belongs to the author.

This thesis cannot be reproduced or quoted extensively from without first obtaining permission in writing from the Author

The content must not be changed in any way or sold commercially in any format or medium without the formal permission of the Author

When referring to this work, full bibliographic details including the author, title, awarding institution and date of the thesis must be given

Please visit Sussex Research Online for more information and further details

Inter- and intracolony conflicts in societies of honey bees and stingless bees

A thesis presented to the University of Sussex,

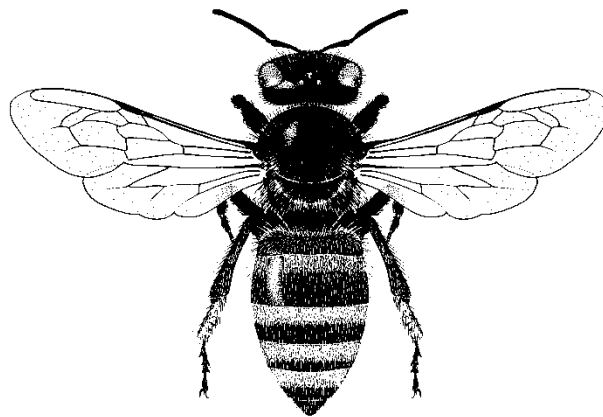
School of Life Sciences,

for the degree of doctor of philosophy

Martin Hans Kärcher

April 2011

Supervised by F. L. W. Ratnieks, J. P. Field and K. Crailsheim



Apis mellifera carnica worker

Illustration by Erwin Scheuchl

Acknowledgements

I am indebted to the Österreichische Akademie der Wissenschaften who was the main source of funding for my PhD (DOC-studentship 22731), the Karl-Franzens-Universität Graz who funded my first few months as a PhD student (Auslandskostenzuschuss), and my generous parents who funded me throughout my studies in Biology, no matter whether I received additional funding or not.

I wouldn't have been able to do any honey bee research, however, without the encouragement of Rupert Gödl who taught me how to successfully keep honey bees in a proper, natural and efficient way, Franz Scherjau who showed me how to rear queen bees and Franz Drescher who has a talent in maintaining an enthusiasm for honey bees in both young and old members of the bee breeders' club of Graz city. Dr. Norbert Hrasnigg and Univ.-Prof. Karl Crailsheim who, when I asked them for someone who is good in honey bees and works in England, advised me to spend one semester after my Bachelor's degree working in Prof. Francis Ratnieks' research group, which is how it all started. Doing a PhD has been tough at times and I thank my family and all my friends in Austria and England who supported me. For advice on statistics I thank Univ.-Prof. Georg Desch, Dr. Heikki Helanterä, Dr. Christoph Grüter and Dr. Francisca Segers. For advice on R graphics I thank Dr. Baptiste Auguie, Dr. Brandon Hurr, Dr. Dennis Murphy, Fernando González Taboada, MSc, Ista Zahn, MSc and Dr. Hadley Wickham of the ggplot2 Google group. For the great support, especially in statistics, R, Excel, EndNote and English, I thank all LASI members from Sheffield and Sussex, especially Dr. Christoph Grüter, Dr. Heikki Helanterä, Dr. Jelle van Zweden, Dr. Sophie Evison, Dr. Francisca Segers, Dr. Margaret Couvillon, Tomer Czaczkes, Sam Jones, MSc, Fiona Ridell, Gianluigi Bigio and Mihail Garbuzov. For additionally checking large parts of my thesis and his patience with my numerous, probably nerve-racking, questions I thank Sam Jones, MSc.

Many thanks also to Dipl.-Ing. Clemens Keil who printed posters for me when I was in Austria, the technician Walter Winkler who modified a pair of forceps for me ideal for transferring honey bee eggs, Univ.-Prof. Franz Neubauer for checking my research proposal for the ÖAW, Erwin Scheuchl for giving me the permission to use one of his bee drawings and the Carl Zeiss AG who provided me with an Axio Imager microscope and a Stemi DV4 microscope including all necessary equipment such as a compatible camera for several months.

Three of the studies of this thesis were conducted in Brazil. I thank Prof. Paulo Nogueira-Neto for the permission to stay at Fazenda Aretuzina, to use the wealth of his perfectly maintained stingless bee colonies and his cordial hospitality.

I am very grateful that Prof. Jonathan P. Bacon and Prof. Andrew F. G. Bourke immediately agreed to be the internal and the external examiner, respectively. Besides, I thank my examiners for their comments which further improved this thesis.

For additional supervision I thank my project supporter from the University of Sheffield, Prof. Ben J. Hatchwell, my second supervisor at the University of Sussex, Prof. Jeremy P. Field, and my third supervisor at the Karl-Franzens Universität Graz, Univ.-Prof. Karl Crailsheim. Finally, I thank Prof. Francis L. W. Ratnieks for choosing me as one of his PhD students and for his understanding in that he agreed that some of the field work could be conducted at my own bee hives in Austria.

Summary

Introduction – Insect societies are well known for cooperation. However, there is a high potential for conflict both over resources (intercolonial) and over reproduction (intracolony). Here I present the key results of my thesis in these two areas.

1. – In our first study we show that *T. angustula* possesses two types of entrance guards, hovering and standing guards, and that they have different tasks. Standing guards, however, can switch to hovering if needed.
2. – Honey bee, *A. m. mellifera*, guards recognise allospecific intruders via “different odours” not “harmful intruder odours”.
3. – Following up on project 1 we demonstrated a relatively clear division of labour in guarding of *T. angustula* where guards either act as standing or hovering guards. This study also adds descriptive data on the natural history at the nest entrances of *T. angustula*.
4. – In our fourth project we found that worker policing in the honey bee (*A. m. mellifera* and *A. m. carnica*) has a low cost because few recognition errors are made, 9.6% and 4.1% of eggs in male and female cells were removed in error, and because these errors are easily rectified.
5. – Virgin queens of *M. quadrifasciata* were only elected in queenless colonies and generally only shortly after the removal of the resident queen. The virgin queens’ behaviour did affect their survival or their life time, respectively. Finally, we described the election process of virgin queens by their colony.

Conclusion – Mainly the finding of two different entrance guards in *T. angustula* generated a series of new questions. In addition, this thesis helped clarifying how social insects recognise each other, it provided the first study that did not measure the benefit but the cost of worker policing, and it shed some light on the bizarre behaviour of queen replacement and execution in *Melipona*.

Publications and manuscripts included in this thesis

Kärcher MH, Ratnieks FLW (2009) Standing and hovering guards of the stingless bee *Tetragonisca angustula* complement each other in entrance guarding and intruder recognition. *Journal of Apicultural Research* 48: 209-214.

Kärcher MH, Ratnieks FLW (2010) Honey bee guards recognise allospecific intruders via “different odours” not “harmful-intruder odours”. *Journal of Apicultural Research* 49: 270-277.

Grüter C, Kärcher MH, Ratnieks FLW (2011) The natural history of nest defence in a stingless bee, *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae), with two distinct types of entrance guards. *Neotropical Entomology* 40: 55-61.

Kärcher MH, Ratnieks FLW: Killing and replacing queen-laid eggs: measuring the cost of worker policing in the honey bee. (Manuscript ready to submit to *The American Naturalist*)

Kärcher MH, Menezes C, Alves DA, Beveridge OS, Imperatriz-Fonseca V-L, Ratnieks FLW: Factors influencing virgin queen survival duration and choice in the stingless bee *Melipona quadrifasciata*. (Manuscript in preparation for submission to *Animal Behaviour*)

Publications and manuscripts from my time as a PhD student, but which are not included in this thesis

Couvillon MJ, Caple JP, Endors SL, Kärcher MH, Russell TE, Storey DE, Ratnieks FLW (2007) Nest-mate recognition template of guard honeybees (*Apis mellifera*) is modified by wax comb transfer. *Biology Letters* 3: 228-230.

Kärcher MH, Biedermann PHW, Hrassnigg N, Crailsheim K (2008) Predator-prey interaction between drones of *A. m. carnica* and insectivorous birds. *Apidologie* 39: 302-309.

Perez-Sato JA, Kärcher MH, Hughes WOH, Ratnieks FLW (2008) Direct introduction of mated and virgin queens into queenless hives using smoke: a method that gives almost 100% acceptance. *Journal of Apicultural Research* 47: 243-250.

Couvillon MJ, Barton SN, Cohen JA, Fabricius OK, Kärcher MH, Cooper LS, Silk MJ, Helanterä H, Ratnieks FLW (2010) Alarm pheromones do not mediate rapid shifts in honey bee guard acceptance threshold. *Journal of Chemical Ecology* 36: 1306-1308.

Ratnieks FLW, Kärcher MH, Firth V, Parks D, Richards A, Richards P, Helanterä H (2011) Acceptance by honey bee guards of non-nestmates is not increased by treatment with nestmate odours. *Ethology* 117: 655-663.

Wenseleers T, Bacon JP, Couvillon MJ, Kärcher MH, Nascimento FS, Nogueira-Neto P, Robinson EJJ, Tofilski A and Ratnieks FLW: Bourgeois behaviour and freeloaders in the colonial orb-web spider *Parawixia bistriata* (Araneae, Araneidae). (In preparation for submission to *Animal Behaviour*)

Table of contents

Chapter 1 - Introduction	1
1.1 Background	1
1.2 Conflict over male parentage.....	3
1.3 Conflict over female caste fate	4
1.4 Nestmate recognition	5
Chapter 2 - How the thesis evolved	8
2.1 Time before the beginning of the PhD.....	8
2.2 Year one of the PhD (October 2006 – September 2007)	10
Training that I received	10
2.3 Year two of the PhD (October 2007 – September 2008)	11
Training that I received	11
2.4 Year three of the PhD (October 2008 – September 2009)	11
Training that I received	12
2.5 Year four of the PhD (October 2009 – September 2010)	12
Training that I received	12
2.6 Year five of the PhD (October 2010 – April 2011)	13
Training that I received	13
2.7 Projects conducted at LASI which I plan to publish following the submission of this thesis	14
Chapter 3 - General methods and study species	15
3.1 <i>Apis mellifera</i>	16
3.2 <i>Tetragonisca angustula</i>	17
3.3 <i>Melipona quadrifasciata</i>	18
3.4 From classical to modern statistics in social insect research.....	20
Chapter 4 - Standing and hovering guards of the stingless bee <i>Tetragonisca angustula</i> complement each other in entrance guarding and intruder recognition	22
4.1 Summary	22
4.2 Introduction	23
4.3 Materials and methods	24
4.3.1 Study site and species	24
4.3.2 Background natural history.....	25
4.3.3 Experimental design.....	27
4.4 Results	28
4.4.1 Experiment 1: Do bees standing on the entrance tube act as guards?	28
4.4.2 Experiment 2: Testing the reaction to volatile odours other than from <i>L. limao</i>	29
4.5 Discussion.....	30
Chapter 5 - Honey bee guards recognise allospecific intruders via “different odours” not “harmful-intruder odours”	34

5.1 Summary	34
5.2 Introduction	35
5.3 Materials and methods	36
5.3.1 Study site and environmental conditions	36
5.3.2 Set up of hives to observe guarding behaviour	37
5.3.3 Scoring of acceptance and rejection by guards	37
5.3.4 Introducing intruders to guards	38
5.3.5 Experiment 1. Comparison of treatment of 10 species of intruders and reference insects	39
5.3.6 Experiment 2. Additional study of the treatment of intruders by guards	40
5.3.7 Statistical analyses	40
5.4 Results	41
5.4.1 Experiment 1	41
5.4.2 Experiment 2	44
5.5 Discussion.....	45
Chapter 6 - The natural history of nest defence in a stingless bee, <i>Tetragonisca angustula</i> (Latreille) (Hymenoptera: Apidae), with two distinct types of entrance guards	49
6.1 Statement of contribution	49
6.2 Summary	50
6.3 Introduction	51
6.4 Methods	52
6.4.1 Study site and species	52
6.4.2 Numbers of hovering and standing guards.....	53
6.4.3 Guarding duration.....	53
6.4.4 Individual observations	54
6.4.5 Guard reactions to human breath	54
6.4.6 Nest entrance closing.....	55
6.4.7 Statistical Analysis	55
6.5 Results	55
6.5.1 Numbers of hovering and standing guards.....	55
6.5.2 Total guarding time	57
6.5.3 Individually marked bees	58
6.5.4 Individual observations	59
6.5.5 Guard reactions to breath.....	59
6.5.6 Geometry of entrance tubes.....	59
6.5.7 Opening and closing the entrance tube.....	60
6.6 Discussion.....	61
6.6.1 Guarding duration.....	62

6.6.2 Guard numbers	63
6.6.3 Reactions to breath.....	64
6.6.4 Nest entrance closure	65
Chapter 7 - Killing and replacing queen-laid eggs: measuring the cost of worker policing in the honey bee	66
7.1 Summary	66
7.2 Introduction	67
7.3 Material and Methods	69
7.3.1 Study organisms, sites and basic methods	69
7.3.2 Experiment 1: Survival of queen-laid eggs.....	71
7.3.3 Experiment 2: Replacement of eggs in empty worker and drone cells by the queen.....	72
7.3.4 Experiment 3: Replacement of eggs at different time periods after egg removal	72
7.3.5 Statistical analysis	72
7.4 Results	73
7.4.1 Experiment 1: Survival of queen-laid eggs.....	73
7.4.2 Experiment 1: Survival of worker-laid eggs	74
7.4.3 Experiment 1: Unusual death rate of eggs in worker cells of one colony	75
7.4.4 Experiment 2: Replacement of eggs in empty worker and drone cells by the queen.....	75
7.4.5 Experiment 3: Replacement of eggs at different time periods after egg removal	76
7.5 Discussion.....	77
Chapter 8 - Factors influencing virgin queen survival duration and choice in the stingless bee <i>Melipona quadrifasciata</i>	82
8.1 Summary	82
8.2 Introduction	84
8.3 Methods and Material	87
8.3.1 Study site, colonies and environmental conditions	87
8.3.2 Introduction and observation of virgin queens	88
8.3.3 Descriptive data	89
8.3.4 Statistical analysis	90
8.4 Results	91
8.4.1 Queen execution	91
8.4.2 Descriptive data	95
8.4.3 Queen choice	96
8.5 Discussion.....	99
Chapter 9 - Conclusion.....	104
References	109

Chapter 1 - Introduction

1.1 Background

This thesis covers two fundamentally different areas of conflict in social insect societies: Intra-colonial conflict, characterised by conflict over reproduction, and intercolonial conflict, characterised by the theft of resources such as honey between colonies. However, at a deeper level, both conflicts and their outcomes affect the inclusive fitness of individuals within colonies. In other words, robbing honey from conspecific colonies reduces the reproductive output and survival of the victim colony and consequently reduces the inclusive fitness of its colony members whilst it has the opposite effect on the robbing colony and its individuals. In intracolony conflict, the conflict is essentially over who reproduces and which males and queens are reared. Individuals or groups of individuals can enhance their inclusive fitness directly by developing into a queen, and as workers, directly by raising their own sons or indirectly by helping to raise more closely rather than less closely related sons.

But let me introduce this thesis somewhat more gently: Insect societies are well known for cooperation, such as in their highly coordinated foraging systems (von Frisch, 1965; Seeley, 1997) and in the altruism of workers (Bourke and Franks, 1995; Boomsma and Franks, 2006; Wenseleers and Ratnieks, 2006b; Bourke, 2011). However, their societies are also subject to considerable potential for conflict over reproduction (Ratnieks et al., 2006). Conflict arises because the members of an insect colony are a family, with relatedness among individuals of approximately 0.5, not a clone of genetically-identical individuals with identical interests, as occurs for example in the cells of a human body. This leads to conflict among individuals over reproduction, including conflict over egg laying ("male parentage") and whether a female larva develops into a queen or a worker ("female caste fate"). Thus, even though workers have a common interest in enhancing colony-level foraging efficiency, when it comes to reproduction there can be conflict among individuals, including among the workers and between the work-

ers and the queen. During my thesis we carried out experiments on honey bees and stingless bees to investigate how queens and workers directly influence the outcome of these two conflicts.

As in all organisms, every individual in an insect society should be designed by natural selection to enhance its inclusive fitness. Inclusive fitness depends both on direct reproduction (sons and daughters) and indirect reproduction via the rearing of relatives (particularly brothers and sisters, and potentially other kin). Natural selection causes individuals to maximise their inclusive fitness rather than their direct fitness (Hamilton, 1964a, b). For example, workers may increase their inclusive fitness directly, via the laying of male eggs, and female larvae may increase their inclusive fitness by developing into queens instead of workers. Individuals may, however, also enhance their inclusive fitness indirectly, such as when workers cause a female biased sex-allocation ratio by preferentially rearing sister queens versus brother males. This is because in haplodiploid systems workers are more closely related to female larvae, especially when the colony is headed by a single mother queen who is mated with one or a few males. Workers can also increase their inclusive fitness indirectly by killing eggs laid by other workers, especially when the mother queen is mated to multiple males (Ratnieks, 1988).

This thesis investigated two important areas of reproductive conflict in insect societies by carrying out experimental studies on the honey bee *Apis mellifera* and the stingless bee *Melipona quadrifasciata*. Using honey bees my thesis investigated conflict over male production, in this case from the workers' point of view. Using stingless bees this thesis investigated behavioural aspects of caste fate in *Melipona*. Thus, two projects in this thesis focused on strategies used by individuals to enhance their direct or indirect reproduction. Directly, via becoming a queen or indirectly, by preventing or policing other workers from rearing males.

Conflicts within insect societies can be subdivided into 5 areas (Ratnieks et al., 2006; Bourke, 2011): 1. Conflict over male parentage, 2. Conflict over female caste fate, 3. Conflict over female parentage (i.e. nepotism), 4. Conflict over reproductive dominance and 5. Conflict

over sex ratio. However, as I will not study the 3rd, 4th and 5th conflicts, below I describe only the first two:

1.2 Conflict over male parentage

In most species of eusocial Hymenoptera workers cannot mate but retain ovaries and can lay unfertilised eggs. Due to haplodiploidy these eggs are male and can give rise to fully viable males if reared. This trait presumably has not been lost in the course of evolution as workers' sons are reared in queenless colonies and, in many species, also in colonies with a queen. For example, a honey bee colony that has lost its queen and failed to rear a replacement queen produces hundreds or thousands of drones before the colony dies out. We think honey bee workers have not evolved complete sterility because of the latter reason and because a small proportion of workers' sons is reared in queenright colonies, i.e. in colonies with a queen. This ensures that genes for non-sterile workers are passed on to the next generation. Only in a few genera of stingless bees, ants and higher termites (*Termitidae*) are workers fully sterile. In queenright honey bee colonies few workers sons' are reared (Visscher, 1989) as "worker policing" is very effective, killing *ca.* 98% of eggs laid by workers in queenright colonies (Ratnieks and Visscher, 1989; Ratnieks, 1990b, 1993) and also serving as a disincentive for workers to activate their ovaries (Wenseleers and Ratnieks, 2006b). Generally, the percentage of workers that lay eggs in a queenright colony depends on the relatedness among the workers and the effectiveness of policing: Under enforced altruism more effective policing is expected to lead to a smaller proportion of individuals laying eggs, which is the case (Wenseleers and Ratnieks, 2006b). Under voluntary altruism, one would expect more altruism at higher relatedness between colony members, as occurs in queenless colonies in which worker policing is not possible as there are no queen's sons to favour (Wenseleers and Ratnieks, 2006b). However, as altruism is often enforced the opposite trend with relatedness is seen in queenright colonies (Wenseleers and Ratnieks, 2006b), given that worker policing is favoured by low relatedness

due to the mother queen mating with more than 2 males. Generally, a worker is related to her sons by 0.5, to her brothers (sons of her mother queen) by 0.25, and her nephews (sons of sister workers) by 0.375 and 0.125 for full sisters and half sisters, respectively. In addition, the mother queen is twice as related to her sons (0.5) than to workers' sons (grandsons, 0.25) (Ratnieks and Reeve, 1992). Thus, there is a great potential for conflict over male production because each female can lay male eggs, and because each female is more related to her own sons than to any other female's sons. Workers are also more related to their mother queen's sons than to the sons of their sisters, when queens are mated to several males because most workers are now half sisters. Plus, a queen is more related to sons than to grandsons (0.25). Combining these factors, workers have a strong incentive to lay eggs, but the mother queen and other workers, especially in species with multiple mated queens, have a strong incentive to prevent workers' sons from being reared.

1.3 Conflict over female caste fate

In social Hymenoptera with morphologically-distinct queens and workers, female caste fate (queen or worker) is determined during larval development. In most species a female larva can develop into either a queen or a worker depending on how she is fed. The proportion of female larvae that develops into queens depends on the same factors described above, kinship, coercion and constraint (Ratnieks et al., 2006). In bees with swarm-founded colonies, only a few queens need to be reared each year, and in many species, including honey bees and trigonine stingless bees, this is the case. However, *Melipona* stingless bees, studied in this thesis, are a clear exception to this rule. In honey bees, for example, female larvae in worker cells are said to be nutritionally coerced into becoming workers instead of queens. In *Melipona* stingless bees, however, females can self-determine whether to become a queen or worker (Bourke and Ratnieks, 1999). This is because they are reared in sealed cells containing enough food for a larva to develop into either caste, given that queens are not larger than workers

(Ratnieks and Wenseleers, 2005). Unlike in honey bees and trigonine stingless bees, in which the queens are larger, the adult workers have no control over the developing larvae through controlling the amount of food given to each female larva (Ratnieks et al., 2006). In addition, in honey bees the quality of the food varies. All female honey bee larvae are fed royal jelly, a food extremely rich in protein, up to the age of three days. Subsequently, only queen larvae are fed royal jelly until the cell is capped. Larvae in worker cells receive the "common food", a mixture of pollen and honey. Interestingly, the difference in the diet activates different sets of genes in a female larva causing larval development into one of the two different female castes, even though each is the same genetically (Evans and Wheeler, 1999).

Thus, unlike honey bees, immature females in *Melipona* stingless bees can self-determine their caste, and their selfish interests result in an enormous excess of queens most of which are killed after emerging from their cells. In *Melipona* relatedness is said to be the only factor affecting the ratio of individuals developing into queens versus workers (Peters et al., 1999; Ratnieks, 2001; Wenseleers et al., 2004; Queller, 2006). Differences in body weight between workers and queens occur in stingless bee genera other than *Melipona*, i.e. in the Trigonini, and in some genera female larvae evade nutritional control by developing into dwarf queens inside worker cells (Ratnieks and Wenseleers, 2005). In the latter case, a female developing in a smaller, worker, cell can develop into a dwarf queen weighing the same as workers (Wenseleers et al., 2005b) but not into normal queens that weigh more and are reared in larger cells. However, both types of queens are able to successfully head a colony (Ratnieks et al., 2006).

1.4 Nestmate recognition

The study of nestmate recognition combines chemical ecology, behavioural ecology and neurobiology. In essence, the key question is how individuals differentiate colony members from non-colony members (Blomquist and Bagnères, 2010). In chemical ecology the key question is

to determine which chemicals provide the necessary information and how they are acquired. In neurobiology one major question is how the information is processed and where in the nervous system the actual decision, accept or reject, is made. Behavioural ecology, the research approach used in this thesis, investigates the behavioural mechanisms *in situ*. Previous research has shown that recognition cues of nestmates and conspecific non-nestmates can overlap (Couvillon et al., 2009), but that this is not the case for allospecifics, such as wasps (Couvillon et al., 2009), and that the acceptance threshold can be adjusted to long term (Downs and Ratnieks, 2000) and short term (Couvillon et al., 2008a) changes in environment. This supports the acceptance-threshold model of Reeve which, roughly spoken, states that the acceptance-threshold is flexible and that it depends on the rate of interaction with desirable and undesirable individuals, and the fitness consequence for a guard of accepting or rejecting a desirable or undesirable individual (Reeve, 1989). Furthermore, research has demonstrated that nestmate recognition in honey bees is not confounded by additional external odours such as floral odour chemicals (Downs et al., 2000), nor by feeding colonies with strong smelling honey previously extracted from different colonies (Downs et al., 2001) and that an individual's colony odours are acquired from the colony environment, whether this is its own colony or a foster colony (Downs and Ratnieks, 1999). This is in agreement with the blank-slate hypothesis which states that newly emerged bees from unrelated colonies are accepted due to the lack of acquired chemical cues (Breed et al., 2004). These odours are acquired from the hydrocarbons bound in the wax combs of a colony (D'Ettorre et al., 2006). In a unidirectional experiment it later turned out that non-nestmates were more likely to be accepted if a colony contained combs from the non-nestmate colony, i.e. the guards' template is affected by the colony's Gestalt odour. However, workers from the comb-receiver hive were not more likely to be accepted by guards from the comb-donor hive, i.e. the transferring of the guards colony's hydrocarbons did not increase the acceptance of non-nestmates (Couvillon et al., 2007). In most social insect species intruders are recognised by standing guards at the entrance (Butler and

Free, 1952). Previous research has suggested that in one species, the stingless bee *T. angustula*, this is done by hovering guards (Bowden et al., 1994). An additional “feature” of social insects are kairomones which are volatile pheromones initially used by an attacking species in order to chemically mark a victim colony (Ono et al., 1995) or chemically confuse it (Wittmann, 1985; Wittmann et al., 1990) but which also alert the victim species as well. In this thesis we aimed to find out why wasps were always categorically rejected and no recognition errors were made (Couvillon et al., 2009). In principal honey bee guards could have detected harmful insects specifically by their odour which would have been a more focused defence. Since we were surprised about the fact that *T. angustula* should only possess hovering guards and that these guards were unable to detect conspecific non-nestmates (Bowden et al., 1994) we conducted nestmate recognition experiments with the bees around the entrance. Since this species is relatively unexplored this lead to a follow-up study incorporated in this thesis but many more possible studies have arisen from studying nestmate recognition in *T. angustula*.

Chapter 2 - How the thesis evolved

2.1 Time before the beginning of the PhD

In August 2005, right after my Bachelor's degree at the Karl-Franzens-Universität Graz, I came to the University of Sheffield for the first time. Initially, my aim was to spend one semester in Prof. F. L. W. Ratnieks's lab to see how honey bee research is done abroad and to improve my English which was already becoming a bit rusty after my Matura (equivalent to the British A-level taken at secondary school). For this visit I managed to get funding from the K.-F.-Universität Graz via a "Fachspezifischer Kurs", i.e. a "course specific to one subject". During this time my supervisor, Prof. F. L. W. Ratnieks, and I started several projects both in England and at our first field trip to Brazil in January and February 2006. In Sheffield I conducted my first project on nestmate recognition in honey bees, "Honey bee guards recognise allospecific intruders via "different odours" not "harmful-intruder odours"', and helped PhD student M. Couvillon by supervising another two undergraduates for a second year of experiments, collecting and organising data, and preparing some graphs. Results were published in the study "Nest-mate recognition template of guard honeybees (*Apis mellifera*) is modified by wax comb transfer". Our first field trip to Brazil was extremely successful in terms of data collection, since it led to four new projects; two of the studies have been published to date, "Standing and hovering guards of the stingless bee *Tetragonisca angustula* complement each other in entrance guarding and intruder recognition" and "The natural history of nest defence in a stingless bee, *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae), with two distinct types of entrance guards". The finding that *T. angustula* had two different types of entrance guards that work together was especially fascinating to me. When studying *T. angustula* at its nest entrances I had the impression that not only their way to discriminate intruders from nestmates but also the way they regulated the traffic at the nest entrance is highly sophisticated. And indeed, it is regulated in several ways and will eventually be published as "Nest entrance traffic organisa-

tion in *Tetragonisca angustula*". To get the most out of this field trip I helped Prof. T. Wenseleers marking and observing social spiders at night and he kindly offered me co-authorship. This study is about to be published at the time this thesis has been submitted. Its provisional title is "Bourgeois behaviour and freeloading in the colonial orb-web spider *Parawixia bistriata* (Araneae, Araneidae)".

By the end of the semester abroad Prof. F. L. W. Ratnieks offered me the opportunity to take a PhD with him. Obviously, I was highly surprised and delighted since to directly start a PhD after a Bachelor's degree is not possible in Austria, where I originally planned to do my Magister and Doktor. Consequently, the time until the official beginning of the PhD, 1.10.2006, I spent in applying for a PhD at the University of Sheffield, in writing up the projects we started and in doing some preliminary experiments in Austria for the study "Caste fate in honey bees: Evidence for a new policing system?" For one month, however, I went back to England during the summer and took part at one of PhD student J. A. Perez-Sato's studies, "Direct introduction of mated and virgin queens into queenless hives using smoke: a method that gives almost 100% acceptance". In the remainder of this summer I kept on working on my very first article "Predator-prey interaction between drones of *A. m. carnica* and insectivorous birds" which Peter Biedermann and I conducted during our undergraduate studies and that was finally published during this PhD.

The reason why the sections above are relevant to this thesis is because the two nest-mate recognition studies where I am first author have been included into this thesis. Since "research is like a pipeline where one puts something in at one end and eventually it appears at the other" (F. L. W. Ratnieks), we decided it is wiser to first finish the projects we started and then move on to the projects we started during the PhD rather than trying to remember what we have done several years ago once the PhD is finished.

2.2 Year one of the PhD (October 2006 – September 2007)

In my first year at the University of Sheffield I took part at another nestmate recognition study, this time led by Dr. H. Helanterä, which shall be published by the time this thesis is submitted: “Acceptance by honey bee guards of non-nestmates is not increased by treatment with nestmate odours”. Our second trip to Brazil was quite successful too but this time I focused on one study only: “Factors influencing virgin queen survival duration and choice in the stingless bee *Melipona quadrifasciata*”. This study will be submitted once this thesis is completed. Together with the help of PhD student C. Menezes and PhD student D. Alves we accumulated a large data set on the killing and election of virgin queens in *Melipona quadrifasciata*. During the summer I finally managed to successfully conduct the experiments for a study that will be written up soon after submitting this thesis. A provisional title for this study is “Caste fate in honey bees: Evidence for a new policing system?” One challenge of this study was to rear control queen cells in queenright colonies during the swarming season without letting the bees swarm. The other challenge was to transfer larvae first from worker cells into queens cells and then from queen cells, sizing them on a graph paper and taking photos, back into either worker or new control queen cells. In this summer I also tried to conduct the experiments on queen policing and learnt that it was already too late in the season to conduct such experiments in observation hives.

Training that I received

In my first year as a PhD student I have been trained how to communicate science to the media and how to write a literature review and project design. In numerous discussions with Prof. F. L. W. Ratnieks I learnt how to differentiate between relevant and irrelevant questions in science, how to plan future projects building on a framework of existing articles, and, most importantly since my supervisor is a great believer in graphs made in PowerPoint, how to pre-

pare graphs solely in this program. By the end of my PhD I think I managed to convince him that R graphs are a good alternative.

2.3 Year two of the PhD (October 2007 – September 2008)

After one-and-a-half years as a PhD student in Sheffield, my supervisor's research group relocated to the University of Sussex at Brighton. Since I have established a couple of good friends in Sheffield by that time, however, and I personally like Sheffield, I was not too happy about this move. During this winter we did not go to Brazil which was probably a good thing since we had too many articles to write up anyway. However, during spring 2008 I managed to get data probably for the most challenging project I did so far: "Lack of queen policing in honey bees". In the summer I managed to get the first half of the data for a study which is about to be submitted to *The American Naturalist*: "Killing and replacing queen-laid eggs: measuring the cost of worker policing in the honey bee".

Training that I received

During an intensive R course lasting for one-and-a-half weeks Prof. Beckerman and several of his colleagues attempted to teach us R. In my opinion the lecturers slightly overshot by trying to cover everything R is capable of calculating, from basic R coding to analysing gene sequences using the R package BLAST. Alas, the result was quite frustrating for most of us and I decided to stick to SPSS, which, however, wasn't ideal either. During spring and summer I was improving methods for the study of queen policing.

2.4 Year three of the PhD (October 2008 – September 2009)

In this season I finally managed to finish data collection for the project on queen policing and the one on worker policing mentioned above. The challenge of the queen policing project was

to prove that something did not happen, i.e. that the queen did not police worker laid eggs. Finding a method that prevented the workers from killing the eggs before the queen checked them in a natural way required several months and a lot of patience. Having completed these two projects I tried to get data on a project that failed until now but will be repeated after the PhD: Its provisional title is “Do workers retain developed eggs to counteract worker policing?”.

Training that I received

Since part of the training as a PhD is to develop teaching skills I attended a course where I was taught how to teach undergraduates. Before that time I was only supervising third year projects of undergraduates, twice in Sheffield and once in Sussex. One approach to find out whether egg laying worker bees deliberately postpone egg-laying would have been to compare their developmental stage at the time an egg was laid with reference eggs. Thus, I attended some lectures in microscopy and also received hands on training from Mr. M. Schofield and Dr. R. Phillips. Training specific to the Axio Imager I received from Ing. A. Hirsch from Zeiss AG.

2.5 Year four of the PhD (October 2009 – September 2010)

In autumn I took part at my last nestmate recognition study so far which has been published recently: “Alarm pheromones do not mediate rapid shifts in honey bee guard acceptance threshold”. In the following spring and summer no additional field work was conducted. Instead, I focused on data analysis, data visualisation and writing manuscripts.

Training that I received

Having received frustrating criticism of referees significantly slowing down the publication process I realised that one way of avoiding similar irritations in the future is to perfect forthcoming manuscripts. To do so I decided to bite the bullet and learn R. Dr. Christoph Grüter,

Sam Jones, MSc and Dr. Francisca Segers spent a lot of time in teaching me how to use R and how to perform Generalised Linear Mixed Effects Models. Additionally, by reading the marvelous book on data visualisation, “ggplot2: Elegant graphics for data analysis” (Wickham, 2009), I taught myself how to also plot data in R. It took me a couple of months but in my view it was worth it. I then joined the ggplot2 Google group where I received a lot of help and also learnt a lot from its members.

2.6 Year five of the PhD (October 2010 – April 2011)

No additional data were collected in the final period of the PhD. Instead, I wrote up the final two manuscripts for publications, “Killing and replacing queen-laid eggs: measuring the cost of worker policing in the honey bee” and “Factors influencing virgin queen survival duration and choice in the stingless bee *Melipona quadrifasciata*”.

Training that I received

Even though it was my final year I still spent some time on training. Dr. H. Wickham, the creator of the R packages ggplot2, plyr, reshape and others was coming to London to give an excellent two-day course on ggplot2: “Data Visualisation in R: Harnessing the power of ggplot2 to produce elegant data graphics”. This course was organised by Mango Solutions and funded by my generous parents since neither the University of Sussex nor the ÖAW (Austrian Academy of Sciences) would have funded it. At the University of Sussex I attended a course on measuring research impact using bibliometric tools and a course on EndNote. How to use EndNote in practice, however, I mainly learnt from Dr. J. van Zweden.

2.7 Projects conducted at LASI which I plan to publish following the submission of this thesis

Kärcher MH, Wenseleers T, Ratnieks FLW: Caste fate in honey bees: Evidence for a new policing system?

Kärcher MH, Ratnieks FLW: Lack of queen policing in honey bees.

Kärcher MH, Robinson EJH, Ratnieks FLW: Nest entrance traffic organisation in *Tetragonisca angustula*.

Chapter 3 - General methods and study species

This thesis is purely based on experimental studies testing ultimate and proximate mechanisms of the behavioural ecology, behaviour and evolution of honey bees and stingless bees. Studies on honey bees were conducted in England and Austria using *A. m. mellifera* and *A. m. carnica*, respectively. Spring or summer was the ideal time to study worker policing, given that this is the main time of drone rearing. Autumn proved to be a good time for nestmate recognition studies, given that natural robbing by conspecifics increases when nectar becomes scarce (Downs and Ratnieks, 2000) and colonies are still near their peak in terms of the number of individuals. In three winters we went on field trips to Brazil in order to study *T. angustula* and *M. quadrifasciata*. For the studies on honey bees and for one study on stingless bees wooden hives, as used for beekeeping, containing colonies of natural size were used. For one study on stingless bees observation hives were used. Nestmate recognition was studied at the colony entrances of honey bee and stingless bee hives. For the other two studies bees were observed within their colony, through a glass lid in *Melipona* and via the presence or absence of eggs in cells of combs in honey bees, which were observed by opening the hive to briefly remove the study combs. The advantage, or disadvantage depending on how the statistical analysis is performed, of social insects is that colonies usually consist of a large number of individuals. Large sample sizes can be accumulated, provided the method is relatively simple and not at an extreme limit of feasibility. At the end of this chapter, I devote a section to the statistics used in this thesis. Methods specific to studies presented in this thesis are described in detail in the relevant research chapters. Here I describe some basic methods and some of the key features of the three study species.

3.1 *Apis mellifera*

Honey bees, one of the most studied species by Biologists, provide a whole range of reasons why it was a perfect species also for this thesis. Honey bees are native to Europe which is conducive for field work, they consist of highly organised complex eusocial colonies endlessly providing intriguing questions, and, since they have been studied for centuries, research can build up on a solid basis of literature. Besides, since humans started to keep them for honey production already thousands of years ago, sophisticated bee keeping techniques have evolved which can be used for experimental manipulations. For example, a simple queen excluder is one of the most important “tools” to study worker policing. A queen excluder is a grating made of metal or plastic with holes no larger than a worker’s thorax but smaller than a queen’s or a drone’s thorax. A grating like this can be inserted between two hive boxes keeping the queen and the drones in one box whilst allowing the workers to access both boxes. For the nestmate recognition studies we only needed some bee hives including entrance platforms, some plastic tubes, forceps and one isolated ice box.



Figure 1: Introducing a worker honey bee, on the left, to honey bee entrance guards, such as the one on the right. For observations introduced bees were not held by forceps, as in this picture on the left. I took this photo at one of my bee hives in Austria, as betrayed by the different subspecies, *Apis mellifera carnica*.

Once a worker honey bee has been placed on an entrance platform, she is usually immediately inspected by one or several guards (Figure 1). In terms of intracolony conflicts, honey bees are an interesting species to study since unlike most other bees they have evolved eusociality, a system that requires various mechanisms to prevent anarchy. In terms of intercolony conflicts honey bees are ideal since the recognition of individuals can be studied at a well-defined place, the hive entrance, connecting the colony to its environment.

3.2 *Tetragonisca angustula*

Contrary to honey bees stingless bees are neither native to Europe, requiring field trips to countries such as Brazil, nor is there a solid basis of literature. In part, this is because they have not been studied for a comparable time; in part, however, because they contain an overwhelming number of species, all of which are fascinating and many of which have been studied at the same time. This has caused some confusion amongst researchers and decelerated progress in research. However, stingless bees also have some good reasons why it makes sense to study them. They provide good opportunities for comparative studies amongst stingless bee species or eusocial hymenoptera in general, and, unlike honey bees, they provide a whole series of bizarre traits evolved under extreme selection in the tropics. Working with gentle honey bees is unproblematic, as is working with pure bred *A. m. carnica* colonies. This contrasts with studying nervous, aggressive African hybrids. Another obvious advantage of studying stingless bees is that they do not sting. *T. angustula* does not even bite, or only rarely, which is the normal method stingless bees use to defend their colonies. *T. angustula*, also called Jataí in Brazil, is one of the most common Neotropical stingless bee species and is often kept by people for small scale honey production. This rather small, fragile species was also kept in a large number of colonies at our field site in Brazil, Fazenda Aretuzina. In gaps of some of the walls of the buildings natural colonies settled down as well, building wax made entrance tubes in the same way as the colonies kept in wooden hives where we studied their nestmate recognition.

One example of a bizarre feature of stingless bees is the existence of two distinct types of entrance guards which, to our knowledge, only occur in *T. angustula* (Kärcher and Ratnieks, 2009). This opens an entirely new aspect to study the mechanism of nestmate recognition and colony defence. This species also seems to be ideal to study nestmate recognition in general since contrary to honey bees, colonies need little care from humans, possess a sufficiently large number of individuals (van Veen and Sommeijer, 2000), and *T. angustula* appears to be an extremely precise species in nestmate recognition (Kärcher and Ratnieks, 2009). For the two studies in this thesis where *T. angustula* was studied, regular wooden hives were used and observations mostly focused on the nest entrances or their vicinity.

3.3 *Melipona quadrifasciata*

In order to study the killing or choice of virgin queens in queenright or queenless colonies of this *Melipona* species we used observation hives with horizontal combs. This is because most stingless bee species, such as *Melipona*, build horizontal combs with cells facing upwards. The photo below depicts such an observation hive (Figure 2). Tapes connecting the removable glass slides on top of the hive were necessary to prevent phorid flies from entering the hive. The glass slide on the right has two holes covered with additional glass slides. These holes allowed the introduction of individuals. In the centre and bottom left of the hive you can see two artificially separated layers of brood. One or two layers of brood might have been kept in a separate incubator at the time this photo was taken. The brownish, undulated, flat material is called “involucrum” containing wax and eventually covering the entire brood area if not removed by the observer. In the upper half of the picture bees arranged their storage pots containing honey or pollen. In the top right of it we placed a trap for phorid flies which occasionally managed to enter during hive management. Somewhat underneath we placed a pollen feeder since colony manipulations weakened the colonies. In the bottom left and bottom right corner rubbish piles can be seen and in the top and centre of the colony bees built the exit

area made of involucrum connected to the plastic hose leading through the wall to outside the house. Bees covered entire glass slides with a mixture of soil and propolis within a day as a defence against phorid flies. For our observations, however, glass slides had to be cleaned by means of water, knives and razor blades prior to observations.



Figure 2: A typical observation hive to study *Melipona quadrifasciata*. The photo was taken facing downwards.

Queen execution has been described in several species of *Melipona*. Unfortunately, findings differ significantly and it cannot be disentangled whether differences are due to different species, different observers or different methods. Thus, in our opinion, it would be highly desirable if stingless bee researchers could agree to study only a few study species, in this case only one *Melipona* species, rather than studying the basics repeatedly in every species. Once a study species has been studied in detail comparative studies using other species might be conducted. We feel that research would progress faster and more efficiently in this way. *Melipona scutellaris* seems to be an ideal species to study *Melipona* in general since, contrary to *M. quadrifasciata*, it has a much lesser tendency to cover glass slides with soil or resin.

3.4 From classical to modern statistics in social insect research

During my PhD I realised that statistics in social insect research is slowly changing, which the reader will notice if he compares chapter 4, 5 and 6 with chapter 7 and 8. In part this is because social insects can sometimes go beyond the current availability of statistically correct methods; in part this is also due to my learning curve as a PhD student.

Suppose one takes a sample of 1,000 bees from 3 colonies then, using classical statistics, the sample size would be 3,000. However, especially in extreme cases such as when studying social insects, Mixed Effects Models would be more appropriate eliminating two key statistical problems that have been largely ignored in social insect research until recently. Mixed Effects Models correct for pseudoreplication and calculate an “effective sample size” which is much lower thereby making p-values less significant. Pseudoreplication exists since data points of one colony are not independent. (I once run the same model as a GLM and as a GLMM and the difference in p-values was shocking.) However, Mixed Effects Models also remove unwanted variation of the data thereby making p-values more significant. In the simplest example, as for the 3 colonies above, one might not be interested in e.g. whether colonies differ in aggression but whether nestmates are more likely to get accepted than non-nestmates. In order to do so colony would be classified as a “random effect” and variation caused by the different level of aggression of different colonies would be removed. This significantly helps finding existing differences that might otherwise stay undetected due to general variation in the data. Additionally, more than one random effect can be included in one model, say single cells of several colonies where studied repeatedly then colony and cell can both be classified as random effects. GLMMs were applied in the latter two research chapters of this thesis since I was not aware of this statistical method at the beginning of my PhD. However, I made an extensive investigation how to analyse data of chapter five, “Honey bee guards recognise allospecific intruders via “different odours” not “harmful-intruder odours””, in the best possible way when this study was about to be published. As mentioned in (van Zweden and

d'Ettorre, 2010) the acceptance or rejection of an individual often is a graded response, i.e. ordinal data, rather than a clear yes or no result, i.e. binomial data. Since a GLMM has not been developed for ordinal data at the time this thesis was written we had three options to solve the statistical dilemma in the best possible way: A) one could multiply the time spent for various levels of acceptance or rejection, respectively and calculate one metric value of aggression. This has been done by several authors, however, is statistically not correct since there is no justification for turning ordinal into metric data. In this case a GLMM could be applied. B) One could ignore that the response of guard bees was gradual and simplify the data into binomial data. The disadvantage of this method is that a lot of biological information simply gets lost. In this case a GLMM could be conducted as well. C) One could analyse these ordinal data using non-parametric tests such as the Kruskal-Wallis and Mann-Whitney U test forgoing a GLMM.

Chapter 4 - Standing and hovering guards of the stingless bee *Tetragonisca angustula* complement each other in entrance guarding and intruder recognition

Martin H. Kärcher and Francis L.W. Ratnieks

4.1 Summary

Previous research has shown that colonies of the stingless bee *Tetragonisca angustula* have hovering guards that can detect allospecific intruder bees with volatile odours or different body colour, but conspecific intruders were not detected. Here we show that *T. angustula* colony entrances are also defended by guards standing on the entrance tube. These standing guards made very few errors in recognition, accepting 100% of the nestmates and rejecting 92% of the conspecific non-nestmates presented to them at the nest entrance. In addition, 87% of the nestmates contaminated with odours from the stingless bee *Scaptotrigona bipunctata* were rejected. Standing guards also reacted to volatile odours from the stingless bees *S. bipunctata* and *Melipona rufiventris* by switching to hovering. The defence reaction, i.e. the increase in the number of hovering guards, was not, however, comparable to the reaction previously reported to citral, the propaganda chemical used by the obligate robber bee *Lestrimelitta limao* when attacking other bee colonies.

4.2 Introduction

Insect colonies are usually defended by workers who stand at the nest entrance and exclude both conspecific and allospecific intruders (Butler and Free, 1952; Wittmann, 1985; Sherman et al., 1997). Honey bee (*Apis mellifera*) hive entrances, for example, have approximately one to ten guards who patrol the entrance platform (Downs and Ratnieks, 2000). In social bees, guarding is not, however, only carried out by standing guards. Nests of the Central and South American stingless bee, *Tetragonisca angustula*, have guards that hover near the nest entrance tube (Wittmann, 1985).

Bowden *et al.* (1994) showed that hovering *T. angustula* guards attacked allospecific intruders, which they recognised by both body colour and volatile odours. In terms of visual detection, 70 times as many *T. angustula* hovering guards attacked dark coloured intruders (workers of the stingless bees *Scaptotrigona mexicana* and *Trigona corvina*) as yellow intruders (workers of the stingless bee *Tetragona dorsalis zieglei*) similar in colour to *T. angustula* workers. The role of volatile odours in recognition was shown in two bioassays. Firstly, twice as many guards attacked black or yellow polystyrene models treated with cephalic odours of the stingless bee *S. mexicana* compared to controls (Bowden et al., 1994). Secondly, Wittmann (1985) and Wittmann *et al.* (1990) showed that *T. angustula* guards reacted strongly to citral, the volatile lemon odoured propaganda chemical released by workers of the obligate robber stingless bee *Lestrimelitta limao* (Sakagami et al., 1993), by rushing out from the entrance in large numbers (Wittmann et al., 1990).

These studies provide important results, but also lead to further questions. Bowden *et al.*'s (1994) results lead to a puzzle. Unlike guards of the honey bee *Apis mellifera* (Downs and Ratnieks, 1999) and the stingless bees *Trigona* (*Tetragonula*) *minangkabau* (Suka and Inoue, 1993) and *Frieseomelitta varia* (Couvillon and Ratnieks, 2008), *T. angustula* hovering guards treated non-nestmate conspecifics the same as nestmates. On average, only 0.6 and 0.4 hovering guards responded within 3 minutes to conspecific nestmates and non-nestmates,

respectively (Mann-Whitney U test, $p = 0.88$), that were presented to them suspended on threads (Bowden et al., 1994). Given this surprising result, Bowden *et al.* (1994) suggested that non-nestmate conspecifics were detected within the colony. Wittmann *et al.* (1990) interpreted their results as evidence that the odours of *L. limao* acted as a kairomone, that is, a pheromone of one species that also triggers a specific response in another species (Ruther et al., 2002). They did not, however, use any controls such as volatile odours of other stingless bees to determine whether the guarding reaction to *L. limao* was greater than to odours of other species of stingless bee.

Our study follows up on this previous work. More precisely, we wanted to test a) whether *T. angustula* detects conspecific intruders via standing guards and b) whether this species reacts to volatile odours of other stingless bee species in the same way as to citral. We show that non-nestmate conspecifics are, in fact, detected and rejected by standing guards at the nest entrance, not inside the colony as suggested by Bowden *et al.* (1994). We also found that the presence of volatile odours of other stingless bee species leads to some of the standing guards switching to hovering, which is not comparable with the reaction to citral, however.

4.3 Materials and methods

4.3.1 Study site and species

Data were collected from 11 January to 14 February 2006 at Fazenda Aretuzina, formerly a coffee farm and now a research station owned by Prof. Paulo Nogueira-Neto. It is located in an area of sugar cane fields and dry, secondary forests near the town of São Simão, São Paulo State, Brazil. During the study period weather conditions were hot with temperatures exceeding 30°C on most days with occasional heavy rain. Flowers were abundant, and colonies foraged actively in the daytime. Fifteen colonies of *T. angustula* (local name “Jataí”) housed in wooden hives (ca. 30 x 20 x 20 cm) were studied to test the reaction to odours other than that

of *L. limao* (Experiment 1) and to obtain basic natural history information. Five of these colonies were used to obtain information on nestmate recognition (Experiment 2). Hives were held 1-2m above ground on metal stands or attached to buildings.

4.3.2 Background natural history

The bees in each colony had constructed a cylindrical, more or less horizontal, wax entrance tube approximately 3cm long and 1cm wide with a circular opening at the tip ($d \approx 6\text{mm}$, Couvillon et al., 2008b). The entrance tube was built onto the wider entrance hole drilled into the side wall of the wooden hive. The entrances of natural colonies nesting nearby in wall cavities were the same in appearance as those of colonies living in hives. The tip opening at the end of the tube is large in relation to the bees, 3.9 times wider than the thorax of a worker bee (*ca.* 1.6mm, Couvillon et al., 2008b). This allows space for approximately 1-3 guards to stand inside the tip and still leave enough space for a single forager to pass (Figure 1). Outside, there is enough space for up to *ca.* 10 workers to encircle the tip, standing facing out (Figure 1 and Couvillon et al., 2008b). Returning foragers land on the rim of the entrance tube before entering (Figure 1a), providing an opportunity for the workers standing at the tip, either inside or outside, to contact them (Figure 1b). At night, the end of the entrance tube is often closed with wax, presumably to deter ants or other insects from entering. The entire entrance tube is perforated with hundreds of small holes (Figure 1a), diameter *ca.* 0.5mm, presumably to allow gas exchange.

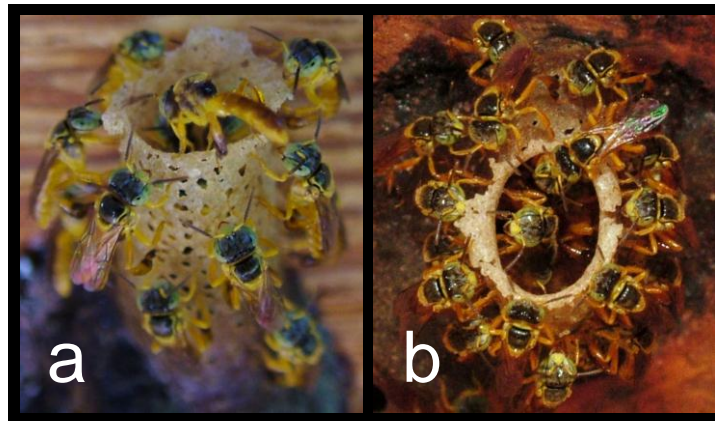


Figure 1: Wax made entrance tube with standing guards of *T. angustula* (local name Jataí). a) A forager landing on the rim of the entrance tube; numerous fine holes in the wax entrance tube and several entrance guards standing outside the entrance tube can be seen. b) Entrance guards standing inside and outside the entrance tube can be seen. Passing these guards without contacting their antennae seems unlikely.

Colonies were in good condition, actively foraging and queenright. The hive boxes were filled with horizontal brood combs and honey pots. The brood chamber was approximately spherical and covered by an involucrum ($14.0 \pm 2.0 \times 12.4 \pm 1.9 \times 15.0 \pm 3.4$ cm; mean \pm SD; $n = 15$ colonies). Mature colonies of *T. angustula* in Costa Rica were estimated to have approximately 10,000 workers (van Veen and Sommeijer, 2000).

Standing guards were defined as workers walking or standing on the outside of the entrance tube without flying away. Hovering guards were workers that remained hovering near the hive, without leaving the area or entering the hive as do foragers (Wittmann, 1985). Guards usually hovered in three locations. A main group of “front hoverers” was always present during foraging hours, approximately 15cm in front of the entrance. Two smaller groups of “side hoverers” were often, but not always, present approximately 20-50cm to the left and right of the entrance. Inspection of hovering bees showed that all were workers, in agreement with Wittmann (1985) ($n = 3$ front hoverers and 3 side hoverers from each of 5 colonies). In total, there were more standing (72%) than hovering (28%) guards. The number of guards varied across the day, and was highest between 15:40 and 16:00 h (front hoverers $5.0 \pm$

3.8 (mean \pm SD, $n = 3$ days \times 15 colonies); side hoverers, both sides combined, 2.9 ± 2.8 ; standing guards 13.8 ± 6.1).

4.3.3 Experimental design

Experiment 1: Do bees standing on the entrance tube act as guards?

We placed conspecific non-nestmates, untreated nestmates, and nestmates to which, via touching, odour of crushed *Scaptotrigona bipunctata* was applied between workers standing on the outside of the entrance tube. Each of the five discriminator hives received 10 untreated nestmates, 10 untreated non-nestmates from one of the other hives, and 10 nestmates with odour from crushed *S. bipunctata*. Due to a period of very heavy rain at the end of the study period we were unable to offer two of the discriminator colonies nestmates with odour from crushed *S. bipunctata*. The introduced bees were returning foragers that had been caught with an aspirator at the entrance, chilled in a freezer for about three minutes, put separately in Eppendorf tubes, kept chilled in a polystyrene box containing ice and then, after warming up outside, presented one at a time with fine clean forceps onto the outside of the entrance tube among the “standing guards”. After chilling and re-warming, the introduced bees walked actively but did not fly away, similar to honey bees (Downs and Ratnieks, 2000). An introduced bee was classified as “rejected” if it was bitten or dragged by one or a few standing guards. Almost always in a rejection, the guard or guards clamped their mandibles onto the other bee and, entangled together, both fell off the entrance tube.

We transferred odour from crushed *S. bipunctata* onto nestmates by touching them in order to investigate whether standing guards react to volatile odours, as do hovering guards (Bowden et al., 1994). *S. bipunctata* is not an obligate robber, as is *L. limao*. Consequently, *T. angustula* would presumably not have evolved to use odours from this bee as a kairomone. Furthermore, *S. bipunctata* releases a strong odour, especially if crushed which is why we chose this species as an odour source.

Experiment 2: Testing the reaction to volatile odours other than from L. limao

Wittmann *et al.* (1990) found that citral, which consists of the two stereo-isomers geranial and neral (Blum, 1966; Blum *et al.*, 1970), is used by *Lestrimelitta limao* to confuse a victim colony (Sakagami *et al.*, 1993) and acts as a kairomone¹ in *T. angustula*. Thus, we tested whether the odour of stingless bee workers other than *L. limao* also leads to a noticeable characteristic reaction in which many workers rush out of the entrance in order to collectively attack an allospecific intruder close to their nest entrance. To determine how guards react to the odours of stingless bees other than *L. limao*, we crushed workers of *S. bipunctata* or *Melipona rufiventris* and put them into a small plastic bowl below the entrance similar to Wittmann (1990). Crushing was necessary to release the contents of the glands. In this way the odour could easily reach the entrance guards. To quantify this behaviour we counted the number of *T. angustula* workers at the entrance before and after offering the odour.

4.4 Results

4.4.1 Experiment 1: Do bees standing on the entrance tube act as guards?

Standing guards never rejected nestmates (0%, $n = 50$), but they rejected almost all the nestmates that had been treated with volatile odours from *S. bipunctata* (87%, $n = 30$) and untreated conspecific non-nestmates (92%, $n = 50$) (Figure 2). Both had a significantly greater chance of being rejected than control nestmates, but were not significantly different from each other (two-tailed Fisher's Exact Test; $p < 0.05$; with Bonferroni adjustment). Nestmates with *S. bipunctata* odour were attacked by an average of 1.46 (SD = 0.76, $n = 46$) standing guards and conspecific non-nestmates by 1.78 guards (SD = 0.76, $n = 26$) (Figure 2). These were

¹ A kairomone is a pheromone of one species that also triggers a specific response in another species (Ruther *et al.*, 2002). For example, this can be a volatile pheromone initially used by an attacking species in order to chemically mark a victim colony (Ono *et al.*, 1995) or chemically confuse it (Wittmann, 1985; Wittmann *et al.*, 1990) but which also directly alerts the victim species as well.

both significantly greater than zero, but not significantly different from each other (Mann-Whitney U test; $p < 0.05$; with Bonferroni adjustment).

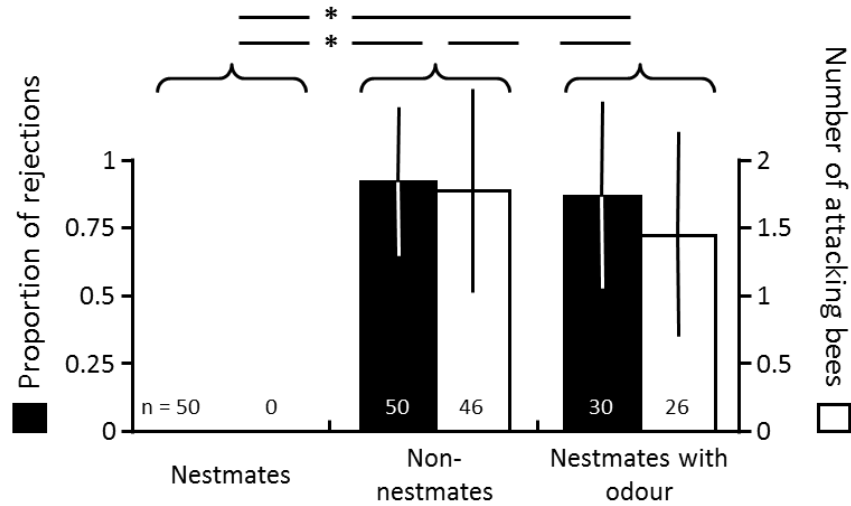


Figure 2: Rejection of conspecifics by standing guards. “Odour” originates from crushed *S. bipunctata* individuals. Asterisks indicate significant differences ($p < 0.05$; with Bonferroni adjustment). Error bars represent standard deviations.

4.4.2 Experiment 2: Testing the reaction to volatile odours other than from *L. limao*

Guards reacted to the odours of crushed bees from both *S. bipunctata* and *M. rufiventris*. The decrease in the number of standing guards at the entrance tube that started to hover, is highly significant for both *S. bipunctata* (dropping by 58% from 10.7 to 4.5, $p < 0.0005$; $n = 15$) and *M. rufiventris* (dropping by 61% from 10.9 to 4.2, $p < 0.0005$; $n = 15$) (two-tailed Mann-Whitney U test with exact significance) (Figure 3). This decrease in the number of standing guards was consistent, as a decrease occurred in all 30 trials. However, at least one standing guard always remained on the entrance tube. In addition, we never saw additional bees rushing out of the colony via the entrance tube in response to the odours presented.

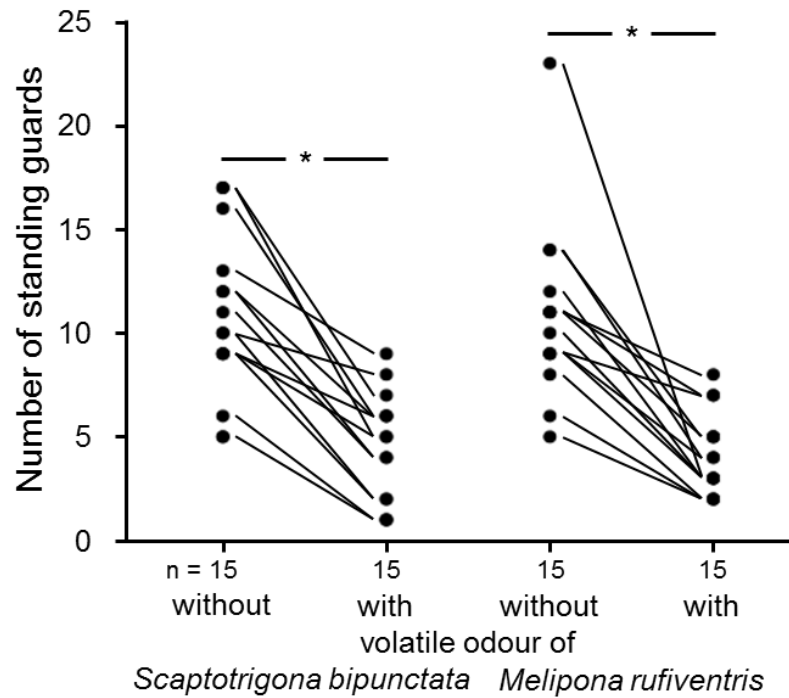


Figure 3: Decrease of the number of standing guards at the entrance tube caused by volatile odour from two allospecific species of stingless bees. The asterisks indicate significant differences at $p < 0.05$. The same 15 colonies were used for each of the two odours presented in two separate trials. Data points are overlapping. Lines are connecting measurements of the same colonies.

4.5 Discussion

Our results clearly show that *T. angustula* workers standing on the entrance tube act as guards and are excellent at discriminating nestmates from non-nestmates. None of the nestmates were rejected compared to 92% of the conspecific non-nestmates and 87% of the nestmates treated with odour from *S. bipunctata* (Figure 2). The ability of guard *T. angustula* to discriminate nestmates versus conspecific non-nestmates, 0% vs. 92% rejection, is greater than has been seen in honey bees or other stingless bees. In honey bees, the greatest difference in rejection seen by Downs and Ratnieks (2000) was 55% (20% nestmates and 75% non-nestmates) and 63% and 65% between related and unrelated nestmates and non-nestmates, respectively (Downs and Ratnieks, 1999). In the stingless bee *Frieseomelitta varia* the

difference was 62%, with 11% rejection of nestmates versus 73% for conspecific non-nestmates (Couvillon and Ratnieks, 2008).

Hovering and standing guards did react to volatile odours from crushed workers of *S. bipunctata* and *M. rufiventris*, but not in the same way as they are reported to react to citral used by the obligate robber species *L. limao* (Wittmann et al., 1990). There was no mass exodus of bees from the colony. What did happen was that about half of the standing guards started to hover and fly around, presumably searching for the enemy (Figure 3). This shows that volatile allospecific odours from species that are not obligate robbers do not lead to a reaction comparable to citral. This strengthens the conclusion that citral does act as a *L. limao* kairomone for *T. angustula* (Wittmann et al., 1990).

Given that Bowden *et al.* (1994) have shown that hovering guards primarily use colour as a recognition cue, guarding in *T. angustula* paints an intriguing picture. Guarding is clearly split into two tasks, hovering and standing. The hovering guards can detect allospecific intruders visually if they have a different body colour (i.e., if they are not yellow) or if they have strong volatile odour. Standing guards can also detect volatile odours, but additionally detect non-volatile odours via direct physical contact by antennation. In particular, it seems that non-volatile odours provide the information necessary to discriminate conspecific non-nestmates from nestmates. Thus, hovering guards significantly improve the defence system by detecting allospecific intruders more quickly and at a greater distance before they arrive at the nest entrance. However, they are insufficient on their own since they are unable to detect conspecific non-nestmates (Bowden et al., 1994).

Our results may also indicate that guarding in *T. angustula* can rapidly adjust to circumstances, inasmuch as some standing guards started hovering when they detected volatile odours of allospecific stingless bees. In theory, these volatile odours could be the same as the ones of crushed nestmates which we did not test. Even if this was the case, however, they still react to volatile cues of allospecific stingless bees as well. This suggests that there

may be task switching but not division of labour in standing versus hovering guarding tasks (Ratnieks and Anderson, 1999). A far stronger reaction, including rapid recruitment of bees from inside the colony, can happen during an attack by *L. limao*, and might be vital for the colony (Wittmann, 1985). In the presence of odour of crushed *S. bipunctata*, 58% (n = 15 colonies) of the standing guards started to hover, i.e. were on alert against robber bees, 61% did so when offered odour from crushed *M. rufiventris* (n = 15 colonies) (Figure 3). However, this reaction was not comparable to the reaction to *L. limao* described by Wittmann (1985) and Wittmann *et al.* (1990). In particular, no additional defenders were recruited from inside the nest and interestingly, not all of the standing guards started hovering. When only one or two standing guards remained, these would never start to hover even if they were directly presented with the odour of crushed allospecific stingless bees (Figure 3; personal observations). Whilst hovering guards use only volatile odours and colour (Bowden *et al.*, 1994), our results show that standing guards can use both non-volatile (Figure 2) and volatile (Figure 2 and Figure 3) cues. Our results also indicate that non-volatile chemical cues are sufficient to detect conspecific non-nestmates and that they are detected outside the nest and not inside, as assumed by Bowden *et al.* (1994).

T. angustula is a very fragile bee that builds very vulnerable nest entrances where other stingless bee species could easily enter. Additionally, it stores large amounts of honey, and is used for beekeeping in Brazil. The level of rejection of conspecific non-nestmates is much higher compared to European honey bees (Downs and Ratnieks, 1999, 2000). It is possible that there is “a higher tendency to steal” in some environments compared to others. This might be due to differences in length of the foraging seasons and lengths of periods where nectar and pollen is abundant, or fluctuations in the amount that is available. Alternatively, simply the fact that most stingless bee species such as *T. angustula* possess a narrow entrance tube might facilitate and improve nestmate recognition compared to European honey bees. Apart from the width of the entrance one key point might be that the entrance tube is made of

wax. This may provide a convenient referent to the guards, enabling them to discriminate more accurately. In the honey bee, comb waxes are used in nestmate recognition including the guard template (Breed, 1998; D'Ettorre et al., 2006; Couvillon et al., 2007).

Further study is needed to determine whether standing guards can detect intruders by visual cues as well (by black and yellow models carrying hydrocarbons of nestmates), and how the two groups interact. This would close the last gap and give a full picture of which recognition cues and modalities are used by hovering and standing guards, respectively. Further study is also needed to determine whether actual division of labour occurs (by marking standing and/or hovering guards with colours), or whether the individual guards continuously switch between the two tasks. Preliminary data suggest that without offering volatile odour they do not or if, then very rarely, as within five minutes out of 4.78 ± 3.62 hovering guards (mean \pm SD; $n = 15$ colonies twice studied) only 0.13 (mean) guards landed between the guards or entered the hive and only 0.067 (mean) left the hive to start hovering.

Our study provides the first example of behavioural specialisation between two groups of entrance guards which, if need be, can even complement each other. Because of this feature and because of the fact that their standing guards are the best ever recorded in nestmate recognition in social bees so far, we think that *T. angustula* is the perfect study species for future nestmate recognition studies.

Chapter 5 - Honey bee guards recognise allospecific intruders via “different odours” not “harmful-intruder odours”

Martin H. Kärcher and Francis L.W. Ratnieks

5.1 Summary

Honey bee guards, *Apis mellifera*, recognise intruders primarily by odour. This study tested two competing hypotheses underlying the rejection of allospecific intruders: “different odour”, whereby intruders are recognised by guards because their odours do not match those of honey bees in general, versus the “harmful-intruder odour”, whereby guards specifically recognise and reject intruders of harmful species. We presented 8 species of harmless arthropod and 3 harmful insects (worker common wasps, *Vespula vulgaris*, adult wax moths, *Galleria mellonella*, and wood ants, *Formica lugubris*) to entrance guards. Guards rejected all but woodlice (*Porcellio scaber*) more than non-nestmate bees and at rates similar to or greater than common wasps. This is not as predicted by the harmful-intruder odour hypothesis, and thereby strengthens the different odour hypothesis. Woodlice were accepted at rates similar to non-nestmate bees, possibly because they have few cuticular hydrocarbons and may smell mainly of their environment.

5.2 Introduction

Recognition is important in many biological contexts, from the immune system to mate choice. Insect societies have guards whose purpose is to recognise and deter intruders. In the honey bee, *Apis mellifera*, entrance guards (Butler and Free, 1952; Free, 1977) deter both conspecific robber bees from other honey bee colonies (Ribbands, 1953; Seeley, 1985) and animals of many other species ranging from insects to mammals (Morse and Nowogrodzki, 1990).

A key step in defence is intruder recognition. This can be difficult. Guard honey bees frequently make errors in discriminating conspecific intruders from nestmates (Breed, 1998; Downs and Ratnieks, 2000; Downs et al., 2001). This is probably because the cues used to discriminate nestmate bees from non-nestmate bees are overlapping (Getz, 1981; Reeve, 1989; Sherman et al., 1997; Breed, 1998; Downs and Ratnieks, 2000). At least one allospecific intruder, such as the death's head hawkmoth *Acherontia atropos* (Moritz et al., 1991), gains entry by chemical mimicry. By contrast, some allospecific intruders are easily recognised. For example, 100% of worker common wasps, *Vespula vulgaris*, presented to guards were rejected (Wood and Ratnieks, 2004; Couvillon et al., 2009).

Common wasps and honey bees are sympatric in Britain and Europe generally. Could the unerring ability of honey bee guards to recognise common wasps be a specific adaptive response (Wood and Ratnieks, 2004), given that common wasps can be a major honey bee predator (Morse and Nowogrodzki, 1990) and can even kill small-population colonies of *A. mellifera*? A precedent for this, the "harmful-intruder odour" hypothesis, whereby guards specifically recognise and reject intruders of harmful species, is shown by the relationship between the Asian honey bee *Apis cerana* and the giant hornet, *Vespa mandarinia*, which are sympatric in parts of Asia including Japan. Workers of *A. cerana* detect the giant hornet's aggregation pheromone and respond by initiating a specialised group defence in which the invading hornet is entrapped in a ball of worker bees and killed by the heat they generate (Ono et al., 1995; Oldroyd and Wongsiri, 2006). Workers of *A. mellifera*, which was relatively recently

introduced into Japan for beekeeping, do not respond to the giant hornet's pheromone (Ono et al., 1995). Alternatively, guard honey bees may simply reject common wasps because they have odour distributions that are distinct from those of *Apis mellifera*, the “different odour” hypothesis, and so can easily be recognised as intruders even if they are not specifically recognised as being a dangerous predator.

The aim of this study was to test between these two competing hypotheses for intruder recognition: “different odour” versus “harmful-intruder odour” hypothesis. We did this by introducing 8 species of harmless arthropods and 2 harmful insects (adult wax moths, *Galleria mellonella*, and wood ants, *Formica lugubris*) (Morse and Nowogrodzki, 1990) to hive entrances and comparing their treatment by guards to the treatment of three types of reference insect: worker common wasps, *Vespula vulgaris*, and nestmate and non-nestmate worker honey bees. Using the “harmful-intruder odour” system we predict that guards only attack harmful intruders, whilst using the “different odour” system we predict that they attack every intruder. Our results support the different odour hypothesis.

5.3 Materials and methods

5.3.1 Study site and environmental conditions

Experiments were carried out in the Fulwood apiary, University of Sheffield, using 4 discriminator hives to quantify the treatment of intruders by guards. Two other hives were sources of non-nestmate worker bees. The honey bees used were of mixed background, but predominantly the native northern European subspecies *Apis mellifera mellifera* (“black bees”). Data were collected on days when colonies were actively foraging from 23 August to 20 September 2005 (Experiment 1) and 21 to 24 September 2005 (Experiment 2).

5.3.2 Set up of hives to observe guarding behaviour

Each hive was housed in a single Langstroth deep hive body with a circular entrance hole, diameter 4.5cm. Immediately below each entrance hole was a rectangular wooden platform 10cm long x 16.5cm wide with 4.5cm high wooden walls on two sides. Intruders were placed onto the platform where they were contacted by the colony's guards.

5.3.3 Scoring of acceptance and rejection by guards

The species of intruders studied are listed in Table 1. Interactions between guards and intruders were scored on a 5-level scale, where A1 and A2 were two levels of acceptance and R1, R2 and R3 were three levels of rejection. This expanded on the standard introduction bioassay devised by Downs and Ratnieks (2000) and used subsequently in several studies (Downs et al., 2001; Wood and Ratnieks, 2004; Couvillon et al., 2009), which has only two levels: accept or reject. We used a 5-level scale to make it possible to distinguish more precisely between the treatment of different types of intruders by guards.

- | | |
|---------------------------------------|---|
| <i>A1 (Accepted immediately)</i> | Completely ignored, or if examined by a guard only licked or engaged in trophallaxis; not bitten. |
| <i>A2 (Accepted slowly)</i> | Examined and bitten/tugged by one or several guards for part of the 3-minute observation period. Then either left alone and allowed to enter the nest or to remain on the entrance platform without further biting/tugging. |
| <i>R1 (Rejected at low level)</i> | Bitten/tugged by guards throughout the observation period. Intruder did not enter nest. |
| <i>R2 (Rejected at high level)</i> | As for R1 but guards also stung or attempted to sting the intruder. |
| <i>R3 (Rejected at highest level)</i> | As for R2 but guards also dragged the intruder from the entrance platform or flew off with it. |

Common name	Scientific name	Source	Body length (mm, ca.)	Test days	Treatment
<i>Reference insects</i>					
Worker bee (nestmate)	<i>Apis mellifera</i>	lab colonies	15	all	chilling
Worker bee (non-nestmate)	<i>Apis mellifera</i>	lab colonies	15	all	chilling
Worker common wasp	<i>Vespula vulgaris</i>	wild	13	all	chilling
<i>Test species</i>					
Woodlouse	<i>Porcellio scaber</i>	wild	11	1, 2	chilling
Ladybird beetle	<i>Coccinella septempunctata</i>	wild	7	10	chilling
Mealworm beetle	<i>Tenebrio molitor</i>	lab colony	15	1, 2	chilling
Cricket	<i>Gryllus campestris</i>	purchased	13	3, 4	chilling
Locust	<i>Schistocerca gregaria</i>	purchased	18	6	chilling
Worker wood ant	<i>Formica lugubris</i>	wild	5	5	chilling (+CO ₂)
Earwig	<i>Forficula auricularia</i>	wild	16	7	ether
Fly	<i>Calliphora vomitoria</i>	purchased	10	8	chilling
Flour moth	<i>Plodia interpunctella</i>	lab colony	7	3, 4	killed by freezing
Wax moth	<i>Galleria mellonella</i>	purchased	15	9	chilling

Table 1: Experiment 1. Reference insects and test species offered to the entrance guards. All were adults except the crickets and locusts which were nymphs. Some were obtained from lab colonies maintained at the University of Sheffield, some purchased, and some obtained wild in the local area. The test days are the 10 study days in August and September 2005 as shown in Figure 1.

5.3.4 Introducing intruders to guards

The normal guarding bioassay of Downs and Ratnieks (2000) introduces forager worker bees that have been collected as they return to their hive. These bees are first placed in a tube, then chilled in an ice chest, and then allowed to warm up and become active just before introduction. Chilling reduces the likelihood that the introduced bee will fly away. Chilling also works with common wasps (Wood and Ratnieks, 2004; Couvillon et al., 2009), but did not work for all intruder species used in this study. In fact, no single method was suitable for preparing all species for introduction. Earwigs and flour moths remained active when chilled. We anaesthetised earwigs by placing them in a vial with diethyl ether vapour and then allowed them to recover sufficiently to be active at introduction. No ether was present at the time of introduction since earwigs were not contaminated directly with ether but only exposed to ether vapour, and then exposed to fresh air in which ether rapidly evaporates. However, ether did not work on the flour moths because they recovered quickly and flew away. As a result it was necessary to kill

them first by freezing. Freezing affects acceptance or rejection of honey bee workers (Downs and Ratnieks, 2000) but does not significantly affect acceptance or rejection in ants (Nowbahari et al., 1990). Flour moths were the only species not alive and active at introduction. Wood ant workers readily release formic acid. This will kill the ant if it is confined in a tube. To prevent this, ants were first anaesthetised with carbon dioxide gas before being separately chilled in Eppendorf tubes without additional CO₂. Thus, they were classified as chilled like most other species but pre-treated with CO₂. We used chilling as the general method rather than CO₂ since chilling has worked well in previous studies and is easy to use in the field. In addition, CO₂ does not work on all species. Flour moths, for example, recovered very rapidly when exposed to fresh air.

5.3.5 Experiment 1. Comparison of treatment of 10 species of intruders and reference insects

We introduced individuals one at a time and observed how they were treated for 3 minutes. On each study day we introduced 3 nestmate forager worker bees, 3 non-nestmate forager worker bees from each of the 2 source hives, 1 forager worker common wasp, and 6 intruder individuals into each of the 4 discriminator hives. These different types were introduced in random order with all 4 discriminator hives receiving the same type on a particular round of introductions. For example, 4 nestmate bees were introduced one each per discriminator hive, then 4 non-nestmate bees, then 4 allospecific intruders etc. In total, we observed the interactions of guards with 24 individuals of each of the 10 intruder species, and with 40 common wasps, 240 non-nestmate workers and 120 nestmate workers as reference insects.

Because some intruders were only available at certain times, it was not practical to introduce all 10 species on every study day. Therefore, on any particular study day only 1 or 2 intruder species were introduced (Table 1). All 3 types of reference insects were introduced on each study day.

5.3.6 Experiment 2. Additional study of the treatment of intruders by guards

During experiment 1 we observed that worker wasps, which fight back and can sting, appeared to be treated differently to more defenceless intruders. We therefore decided to compare the treatment of male wasps, which lack stings but are approximately the same size as workers, with worker wasps. Introductions were videotaped with a digital video camcorder (Sony, 640 x 480 pixels, 25 frames per second). We also made further study of the woodlice because they were the only intruder often accepted by guards. For comparison, we also videotaped the treatment of mealworm beetles, and nestmate and non-nestmate forager worker bees.

Ten individuals of each type were introduced, 5 each into 2 discriminator hives. From the videotape we determined treatment every 5 seconds for 90 seconds or until the intruder departed the entrance platform or entered the nest entrance. From these data we determined the time since introduction at which a particular level of rejection occurred for the first time, the highest level of rejection, and the total number of different levels of rejection. We used the same categories as in experiment 1 except that we combined the two acceptance categories (A1, A2).

5.3.7 Statistical analyses

In each experiment we first tested whether there was at least one significant difference among the different types of arthropods introduced using the Kruskal-Wallis test. We then made pairwise comparisons using Mann-Whitney U tests. To have a 95% significance level despite multiple comparisons we made a Bonferroni correction as follows: in experiment 1 we corrected α ($=0.05$) to α ($=0.0015$) (33 comparisons), in experiment 2 we corrected α ($=0.05$) to α ($=0.01$) (5 comparisons). In this study we used the most conservative application of Bonferroni correction, which means that α was obtained by dividing α by the number of comparisons.

5.4 Results

5.4.1 Experiment 1

As expected, the three reference insects were treated significantly differently (Figure 1, Table 2), with nestmate worker honey bees being accepted most and worker common wasps least. For example, all (100%, $n = 120$) nestmate bees, 63% ($n = 240$) non-nestmate bees, but only 5% ($n = 40$) wasps were accepted at Level A1. Although not all wasps were rejected, the proportion of wasps rejected (95%, Levels R1 - 3) was very high, similar to the previous studies which both reported 100% rejection (Wood and Ratnieks, 2004; Couvillon et al., 2009). The proportion of wasps evicted from the entrance, 35% (Level R3), was lower than in the previous study, 74% (Wood and Ratnieks, 2004).

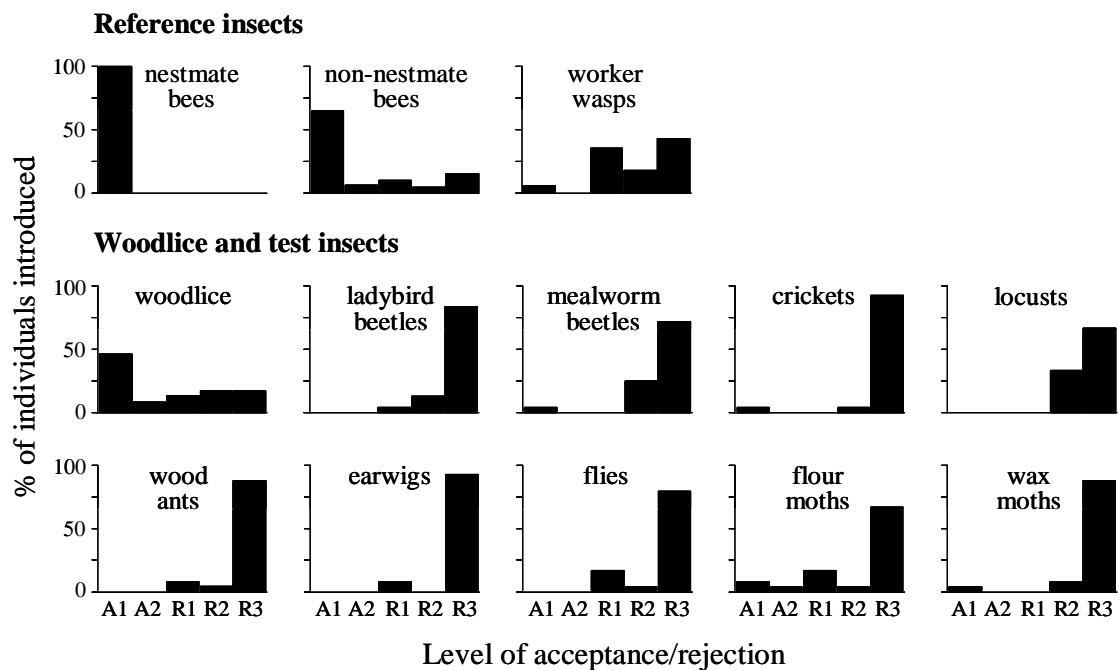


Figure 1: Experiment 1. Treatment of reference and test insects by honey bee entrance guards on a 5 level ordinal scale from acceptance to rejection. Levels A1 and A2 are decreasing levels of acceptance, and levels R1 to R3 increasing levels of rejection. Comparing the per cent of wasps or test insects introduced being accepted or rejected at various levels shows that wasps did not receive more but rather less aggression than test insects. Note also the similarity between the acceptance/rejection of non-nestmate bees and woodlice.

Test arthropods		wood-louse	ladybird beetle	mealworm beetle	cricket	locust	wood ant	earwig	fly	flour moth	wax moth
Reference insects											
* [nestmate worker bee	*	*	*	*	*	*	*	*	*	*
	* [non-nestmate worker bee	n	*	*	*	*	*	*	*	*	*
	* [worker wasp	*	*	n	*	n	*	*	n	n	*

Table 2: Experiment 1. Significant differences in the data in Figure 1. Levels of acceptance and rejection of the test arthropods versus reference insects (Figure 1) using Mann-Whitney U tests with Bonferroni adjustment (* indicates a significant difference, $p < 0.05$; n indicates non-significant, $p \geq 0.05$.)

As all nestmate bees were accepted, there was no trend in how they were treated across the study days. The other two reference insects showed small but significant changes during the same study period, but these were in opposite directions. Wasps were treated slightly less aggressively (Spearman Rank Correlation test: level = -0.3292, $p = 0.0385$, $n = 40$) and non-nestmate bees slightly more aggressively across the study (Spearman Rank Correlation test: level = 0.2425, $p = 0.0002$, $n = 240$) (Figure 2).

The treatment of the 10 intruder species, from clear acceptance (A1) to clear rejection (R3), was displayed on a 5 level scale (Figure 1). All were rejected more than nestmate worker bees, and all except the woodlice were rejected more than non-nestmate worker bees. The proportions accepted (levels A1 and A2) were 0% (ladybird, wood ant, locust, earwig, fly), 4% (mealworm, cricket, wax moth), 13% (flour moth), and 54% (woodlice). The distribution of treatment levels towards woodlice is similar to non-nestmate honey bees (Figure 1, Table 2).

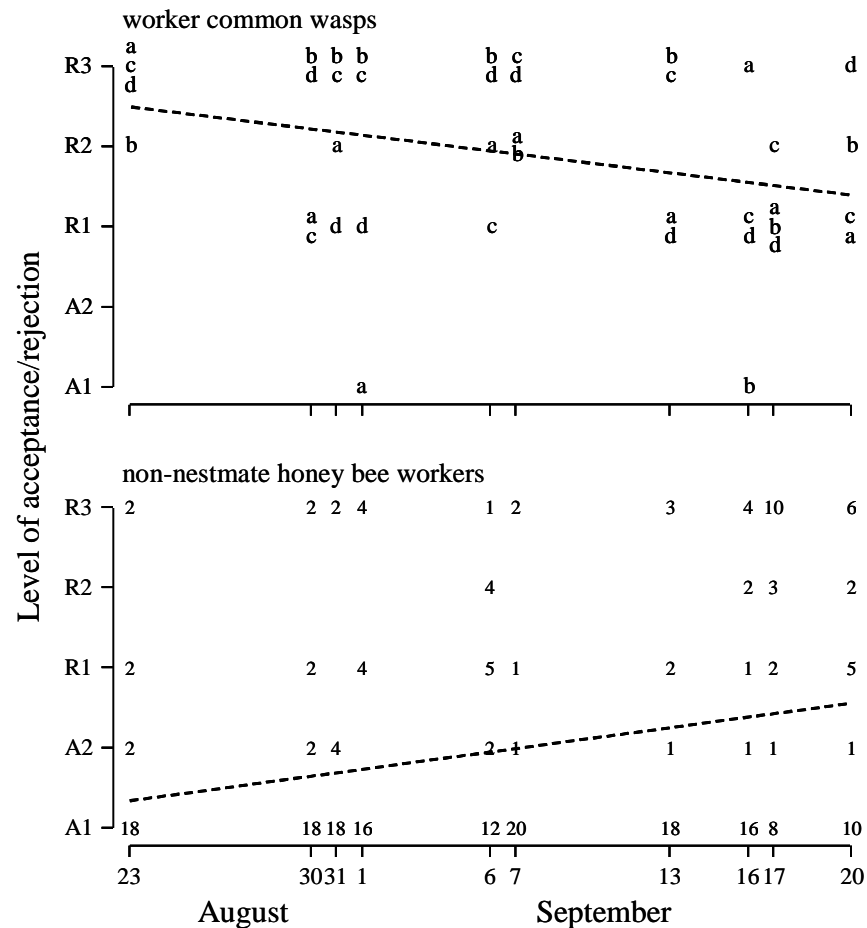


Figure 2: Experiment 1. Treatment of reference insects by guards over the 10 study days pooled across the 4 discriminator colonies. There was a negative correlation between study day and level of acceptance/rejection for worker wasps, and a positive correlation for non-nestmate honey bees. All nestmate honey bees were accepted at level A1 (data not shown; n = 12 per day). The letters a-d represent the treatment of the one wasp per day introduced into each of the 4 discriminator hives a-d. The numbers represent the number of individuals pooled across the 4 discriminator hives. Dotted lines represent regression lines.

Worker common wasps were almost always rejected (95%) similar to the 9 intruder insect species. However, the distributions of the treatment levels (Figure 1) show, if anything, lower level rejection of worker wasps than harmless insects. For example, 35% of the wasps received the lowest level of aggression (R1), more than crickets, locusts, mealworms and wax moths (0%), ladybirds (4%), wood ants and earwigs (8%), flies and flour moths (17%). Pairwise comparisons showed that there were significant differences between the treatment of wasps and

all harmless species except mealworms, locusts, flies and flour moths (Table 2). Inspection of Figure 1 suggests that a component of this difference is the rejection of wasps at the lower level (R1) than other intruders.

5.4.2 Experiment 2

Worker wasps were rejected more rapidly than non-nestmate honey bees ($p = 0.007$). All wasps ($n = 10/10$) were rejected within 5 seconds, whereas fewer 8/10 non-nestmate worker bees were rejected (median time to rejection 12.5s). Male wasps, mealworm beetles and woodlice were not rejected significantly earlier or later than non-nestmate bees. There was no significant difference in the time to rejection of worker versus male wasps (Figure 3). More worker wasps (9/10) were, however, evicted from the entrance than were male wasps (3/10) (Figure 3). The highest level of rejection received by worker wasps was significantly greater than for male wasps (Kruskal-Wallis test: $p = 0.008$, $n = 10$ for worker and male wasps). A higher proportion of worker wasps were rejected at the highest level, R3, in experiment 2 than in experiment 1. This may be because in Experiment 1 each discriminator colony received only one introduced worker wasp per day, but in Experiment 2 each received 5 worker and 5 male wasps on the same day.

As in Experiment 1, woodlice were treated in a similar way to non-nestmate honey bees and mealworm beetles were highly rejected. In terms of the highest level of rejection received, mealworms were not significantly different to worker wasps, and woodlice were not significantly different from non-nestmate honey bees.

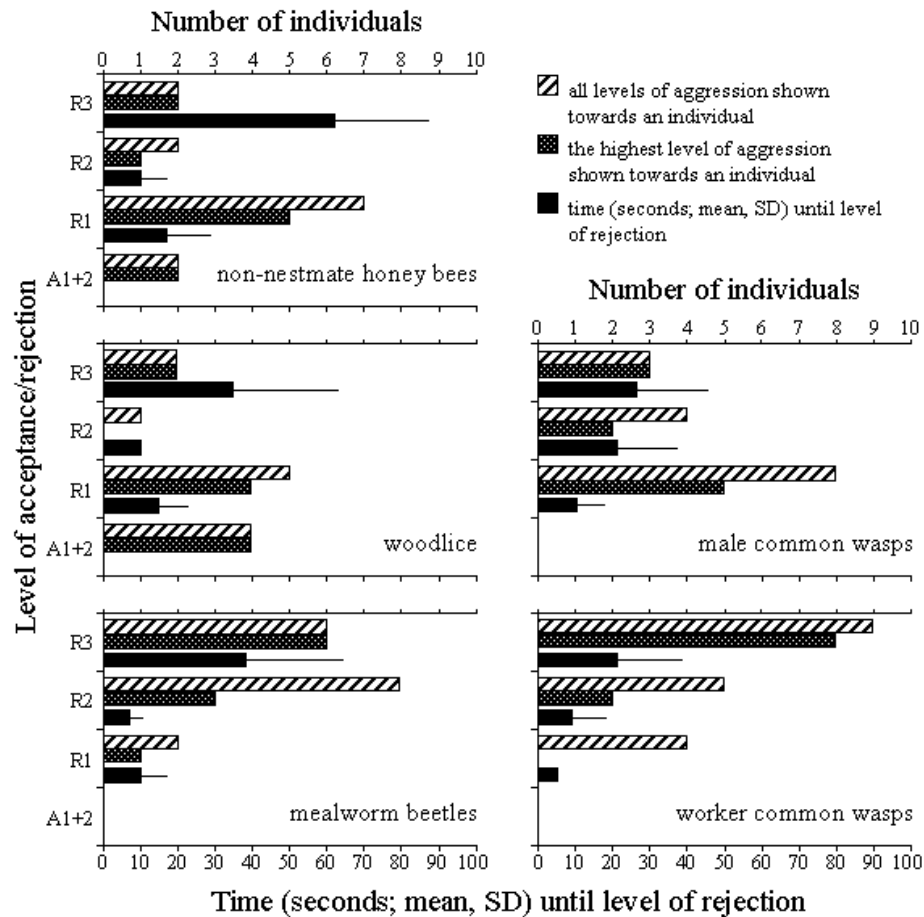


Figure 3: Experiment 2. Time in seconds before a specific level of aggression by guards was shown for the first time to an introduced individual (Mean; SD), total number of individuals that received a specific level of aggression, and number of individuals that received a specific level of aggression as their highest one. Note the different treatment of male and female common wasps and the similar treatment of non-nestmate workers and woodlice, as well as mealworm beetles and worker common wasps.

5.5 Discussion

The results clearly show that guards rejected all 7 harmless allospecific insects tested as readily as worker common wasps (*Vespula vulgaris*), wax moths (*Galleria mellonella*) and wood ants (*Formica lugubris*) which are harmful allospecific intruders (Morse and Nowogrodzki, 1990) that are sympatric with *Apis mellifera*. If anything, harmless intruder insects were rejected at a higher level than wasps. Although we cannot exclude the possibility that these three harmful

insects were rejected using a different recognition mechanism (i.e., harmful-intruder odour) than the harmless insects (i.e., different odour), this is not likely. Overall, the results support the different odour hypothesis and weaken the harmful-intruder odour hypothesis.

Rejecting insect intruders that have odours different from honey bees should be a simple and effective mechanism of excluding allospecific intruders. Given that insect cuticles typically have many different chemicals and that there is considerable interspecific variation (Lockey, 1988; Singer, 1998) this mechanism should allow most species of allospecific intruders to be recognised. One exception would be species, such as the death's head hawk moth (Moritz et al., 1991), that produce chemicals (two unsaturated and two saturated fatty acids) which are also found on a honey bee's cuticle and are probably highly weighted in recognition (Sherman et al., 1997).

If detecting odours that differ from those normally found on honey bees is sufficient to recognise an intruder, why has *Apis cerana* evolved the ability to recognise the aggregation pheromone of the giant hornet (Ono et al., 1995)? One possibility is that a highly specialised defence, requiring the coordination of many workers, is used against this predator. By contrast, a specialised defence is not necessary against common wasps. Worker common wasps are slightly smaller than worker honey bees, and can be prevented from entering the nest by normal guarding activities provided that there are sufficient guards for the size of the entrance (FLW Ratnieks, personal observations). Recognition of a specific predator also occurs in stingless bees (Meliponinae). In Brazil, workers of *Tetragonisca angustula* detect the aggregation pheromone/propaganda chemical of the obligate robber species *Lestrimelitta limao* and leave their nest in large numbers to attack the robbers (Wittmann et al., 1990).

A second possibility is that both *V. mandarinia* and *L. limao* are recognised by volatile chemicals that they release, which act as kairomones to the victim species. This suggests that the recognition of particular enemies may be facilitated when the recognition chemicals can be detected at a distance rather than by contact. A third factor is that the enemy should be

sufficiently common and harmful to be an important mortality factor, and hence select for specific recognition. As mentioned previously, common wasps can be a major predator of honey bees (Morse and Nowogrodzki, 1990) and frequently kill small colonies of *A. mellifera*, being common throughout much of the native range of this species. This indicates that being a serious mortality factor is not in itself sufficient to cause a victim species to evolve the ability to specifically recognise a particular enemy species.

Woodlice were less rejected than allospecific insects. One possible reason for this is that woodlice live in moist microenvironments and have an exoskeleton that is permeable to water, and hence have fewer waterproofing chemicals in the cuticle, especially hydrocarbons, than do insects (Warburg, 1993). If this is the case it would be further evidence for the importance of hydrocarbons in nestmate recognition (Breed et al., 1995; Sherman et al., 1997; Breed, 1998; Singer, 1998) and in keeping with the ease with which newly emerged “blank slate” worker honey bees are accepted (Breed et al., 2004). Alternatively or additionally, woodlice may smell mainly of their typical environment, which may act as chemical camouflage. Another possible factor is the characteristic armoured and all-covering dorsal surface of the woodlice, which makes them hard for the guards to grasp with their mandibles. We observed that even when a guard was aware of a woodlouse on the entrance platform, the woodlouse could still run past the guard. Further study is needed to determine why woodlice are less often rejected, and if this is due to the chemistry or morphology of their exoskeleton, or both.

Wax moths were treated in a similar way to flour moths and to the other intruder insects. Thus, our data do not suggest that they are able to evade bee defences, as does the death's head hawk moth *Acherontia atropos* (Moritz et al., 1991). We used, however, commercially available wax moths which are reared and sold as food for pet reptiles. It is possible that many generations of rearing in culture had caused changes in their physiology or behaviour that may affect their ability to enter hives.

Nestmate recognition in honey bees and social insects has been extensively studied. Over 100 years ago the eminent Victorian Lord Avebury, who was also Charles Darwin's neighbour in the village of Downe, discussed the recognition of "friend and foe" in his book "Ants, bees, and wasps" (Lubbock, 1882). Despite this, we still do not fully understand the chemical basis of nestmate recognition in the honey bee, or indeed in any species (Breed, 1998), but much progress has been made. For example, although we do not know the exact chemicals used, we do know that certain classes of compounds are more important than others (Fröhlich et al., 2001; Akino et al., 2004; Dani et al., 2005). We also have a good understanding of the main theoretical principles involved (Reeve, 1989; Getz, 1991; Sherman et al., 1997), and in the sensory abilities of individuals (Sherman et al., 1997; Ozaki et al., 2005). Our study makes a further contribution. In particular, it indicates that most intruder insects are recognised by guards simply because they have different odours to honey bees, not because they are specifically recognised as harmful.

Chapter 6 - The natural history of nest defence in a stingless bee, *Tetragonisca angustula* (Latreille) (Hymenoptera: Apidae), with two distinct types of entrance guards

Christoph Grüter, Martin H. Kärcher and Francis L. W. Ratnieks

6.1 Statement of contribution

FLWR initiated this project in January 2006 because *T. angustula* appears to be an excellent model species for future nestmate recognition studies. However, no natural history data on the behaviour at nest entrances existed. MHK conducted the experiments in 2006, did some preliminary analysis, presented the data in graphs, and wrote a first draft of the article. CG expanded this project in 2009 by adding experiments, such as investigating whether division of labour or task switching occurs between standing and hovering entrance guards. He also repeated some of the measurements of MHK and obtained identical results. CG analysed and visualised the final dataset of this study in R. CG also finished the draft in order to submit it to *Neotropical Entomology*. For publication in the final version of this thesis, MHK had to make minor amendments.

6.2 Summary

The stingless bee *Tetragonsica angustula* is the only social bee known that has two different types of nest entrance guards. As in other stingless bees and the honey bee (*Apis mellifera*) one type stands on, in or near the nest entrance. The second type, so far only known in *T. angustula*, hovers near the nest entrance. In order to gain further understanding of this unique situation we studied guarding behaviour in both types of guards. Using marked bees, we found that individual worker bees guarded for a long time, up to 20 days (5.4 ± 5.5 ; mean \pm SD), relative to their short, average *ca.* 21 day, lifespan. Relatively few, 33%, individually marked guards were seen performing both types of guarding. The others only acted as standing guards. The bees that did perform both types did so over similar periods of their life (2.83 ± 3.04 days standing; 2.43 ± 2.31 days hovering). Hovering bouts were 57 minutes long, interrupted by breaks inside the hive of a few minutes (3.3 ± 1.5 min). Standing bouts were slightly longer (74 min) and also interrupted by short breaks (7.82 ± 6.45 min).

Human breath, mimicking a vertebrate intruder, caused the guards to retreat into the nest rather than to attack the intruder. Some colonies protected themselves against intruders by closing the entrance during the night (32% and 56% of colonies during two nights). In summary, our results indicate that nest entrance guarding in *T. angustula* involves division of labour between the two types, in which most guarding individuals only act as standing guards.

6.3 Introduction

The nests of insect societies are defended by entrance guards (Butler and Free, 1952; Wilson, 1971; Roubik, 1989; Hölldobler and Wilson, 2009) who help protect the valuable resources inside, including adults, brood, food stores and nest material, and even the nest site itself. The guards admit nestmate workers but exclude intruders. In social bees, food stores can be robbed by both conspecific and allospecific intruder bees (Michener, 1974; Roubik, 1989; Morse and Nowogrodzki, 1990). For example, honey bee (*Apis mellifera*) colonies in Europe are often robbed by worker bees from other honey bee colonies (De Jong, 1990; Downs and Ratnieks, 2000). In addition, there is a small amount of robbing by bumble bees (FLW Ratnieks, personal observation). Stingless bees also rob both allospecifically and conspecifically, and in both tropical America and Africa there are obligate robber species, *Lestrimelitta limao* (Smith) and *Cleptotrigona*, respectively, that rob both honey and brood food (Roubik, 1989). Social bee nests are also subject to robbing by a wide range of other animals, from wasps to bears, and guards also defend against these intruders (Roubik, 1989; Morse and Nowogrodzki, 1990).

The guards that defend a bee nest usually stand on, near or in the nest entrance (Butler and Free, 1952; Wittmann, 1985). The stingless bee *Tetragonsica angustula* is unique in that it has two distinct types of guards (Kärcher and Ratnieks, 2009). As in the honey bee and other stingless bees there are standing guards, which in *T. angustula* are positioned on the inside and outside of the wax entrance tube. The other type, so far known only in this species, are hovering guards. Most hovering guards (“main group”) (Kärcher and Ratnieks, 2009) hover within 20 cm of the entrance (Figure 1 in Wittmann, 1985) facing the flight path in front of the entrance (Wittmann, 1985; Wittmann et al., 1990; Kelber and Zeil, 1997). A few guards hover 20-50 cm from the entrance, to the right or left (“side groups”) (Kärcher and Ratnieks, 2009).

Hovering guards attack allospecific intruders, particularly those with a volatile odour, such as workers of *L. limao* which smell strongly of citrus, or which are of a different colour than *T. angustula* workers (Wittmann et al., 1990; Bowden et al., 1994). The hovering guards

force intruders to the ground by grasping legs and wings with their mandibles (Wittmann, 1985; Bowden et al., 1994).

However, hovering guards do not detect conspecific intruders (Bowden et al., 1994). These are detected by the standing guards (Kärcher and Ratnieks, 2009). Presumably, discrimination between nestmate and non-nestmate conspecifics is relatively difficult and requires the assessment of non-volatile chemicals present on the cuticle that need contact chemoreception.

Further information is needed in order to better understand this unique form of nest defence in bees. The aim of this study was, therefore, to collect data on the natural history of nest entrance guarding in *T. angustula* and to perform simple experiments in order to find out whether guards of one type switch to the other type of guarding (task switching) or if they specialise in performing one kind of guarding (division of labour; for definitions see: Ratnieks and Anderson, 1999). We performed individual observations and estimated the duration of guarding bouts and the overall guarding time. In addition, we also investigated the response of guards to human breath, imitating the presence of a vertebrate predator, and closing of the entrance tube at night.

6.4 Methods

6.4.1 Study site and species

The study was conducted in January and February 2006 and 2009 at Fazenda Aretuzina, a farm dedicated to the study of stingless bees and the conservation of Brazilian wildlife, near the town of São Simão, São Paulo State, Brazil. The total observation time was about 60 days. At Fazenda Aretuzina, ca. 30 colonies of *T. angustula* are kept in wooden hives in the apiaries around the central farm buildings. In addition, there are ca. 10 colonies living in cavities in the walls of these buildings. *T. angustula* is a common species ranging from Veracruz, Mexico, to

Misiones, Argentina (Michener, 2007). Mature colonies in Costa Rica contained approximately 10,000 bees (van Veen and Sommeijer, 2000).

6.4.2 Numbers of hovering and standing guards

We counted the numbers of hovering and standing guards during daytime by monitoring 15 colonies every two hours from approximately 8:00 to 20:00 on 3 study days in 2006. Counting was accurate because guards of both types move very little. For statistical analysis, we averaged the data collected over the 3 days for each colony. In order to relate the guard numbers to colony size, we used the foraging activity of colonies as an indirect measure of colony size. This is based on the assumption that larger colonies have more foragers. Foraging activity was measured by counting the bees entering the colony during 60 seconds.

6.4.3 Guarding duration

In order to estimate the duration of guarding duties, we first marked guards of both types and scan-sampled nest entrances four times per day (10:00, 12:00, 14:00, 16:00). Individually marked bees were estimated to have stopped acting as guards when they had not been seen acting as guards for at least two days. To mark the bees, they were immobilised by putting them into a freezer (*ca.* -4°C) for 6 minutes. We marked bees in two different ways. First, we captured guards of both types (from 8 colonies) and group marked them using two different colours (acrylic paint) to indicate whether they were standing guards or hovering guards when collected. Additionally, we individually marked guards of both types using different combinations of dots of two colours (from 7 colonies). Individual marking with acrylic colours was challenging because the bees are small (body length is *ca.* 5 mm) (Wittmann, 1985), but was necessary to investigate possible changes in guarding behaviour. A colour mark weighed *ca.* 0.2%

of the body weight (unpublished data). We discarded bees if the markings affected their ability of guards to fly, such as when the paint also marked the wings.

6.4.4 Individual observations

We observed individually marked guards (6 hovering guards, 15 standing guards of 7 different colonies) for 60 minutes and recorded the time spent guarding, the time inside the nest, and whether guards switch from one type of guarding to the other. The colour marks were easy to recognise on both types of guards and visible from several meters.

6.4.5 Guard reactions to human breath

Vertebrate breath can set off defensive reactions in both social (DeGrandi-Hoffman et al., 1998) and non-social (Conner et al., 1985) insects. However, *T. angustula* is a rather timid and small bee. For this reason we wanted to find out whether they attack, as do for example African honey bees (DeGrandi-Hoffman et al., 1998), or retreat when exposed to breath. To quantify the reaction we counted the number of hovering and standing guards before and after exposure to human breath. We divided the hovering guards into one main group hovering close to the entrance (see introduction) and two side groups usually hovering separately on the left and right side of the hive. The application of breath was standardised by slowly breathing out through a plastic hose ($d \approx 5\text{mm}$) from a distance of approximately 20 cm (distance measured from the end of the hose). For this experiment we used 15 colonies, treating them nine times each (3 days x 3 trials per day). For statistical analyses, we averaged the data per colony.

6.4.6 Nest entrance closing

Colonies of *T. angustula* often close the tip of their entrance tube with a sheet of wax perforated with many small holes ($< 0.5\text{mm}$) during the night, presumably to prevent nocturnal enemies from entering. The holes presumably aid in gas exchange. We monitored the entrances of 25 study colonies every 30 minutes in the evening (19:30-24:00) and morning (6:30-9:30) for 2 nights in 2009, with similar average temperatures (2.2.2009: 22.2°C ; 14.2.2009: 21.9°C ; 10 measurements every 30 min from 19:30-24:00).

6.4.7 Statistical Analysis

We used both parametric and non-parametric tests to analyse our data in R 2.8 (R Development Core Team, 2008). If transformations of the dependent variable were necessary to achieve a normal distribution and to stabilise the variance we used the Box-Cox method to find the best transformation (Crawley, 2002). Descriptive statistics are given as mean \pm standard deviation. All tests are two-tailed.

6.5 Results

6.5.1 Numbers of hovering and standing guards

Guarding was most intense between 12:00 and about 18:00 (Figure 1). In this period there were about twice as many standing guards, (14.3 ± 6.46 at 14:00), as hovering guards, (6.37 ± 4.64 at 14:00). No bees were hovering at 08:00 and 20:00, so standing seems to take place for a greater proportion of the day time (Figure 1). Hovering activity seems to be most intense during late afternoon. Figure 2 shows that there is a significant positive relationship between the number of standing guards and hovering guards per colony (Linear model (LM): cube root transformed data: $n = 25$; $F_{1,23} = 12.92$; $R^2 = 0.33$, $p = 0.002$).

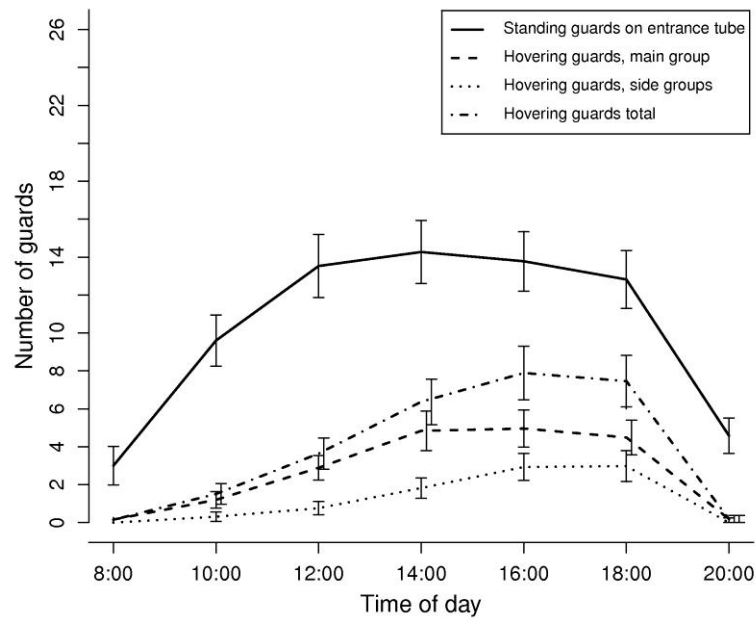


Figure 1: The number of standing and hovering guards (mean \pm SE), in both the main group and the side groups, at different times of the day ($n = 15$ colonies). The SE was only calculated for this particular graph. The number of standing guards peaks at 14:00. The total number of hovering guards, however, peaks about 2 hours later at *ca.* 16:00.

We also found a significant positive relationship between the number of hovering guards and entrance traffic (number of bees entering in 60s) (LM: cube root transformed data: $n = 25$; $F_{1,23} = 5.03$; $R^2 = 0.14$, $p = 0.035$). There was also a positive trend between number of standing guards and forager traffic, although this was not quite significant (LM: cube root transformed data: $n = 25$; $F_{1,23} = 3.4$; $R^2 = 0.09$, $p = 0.08$).

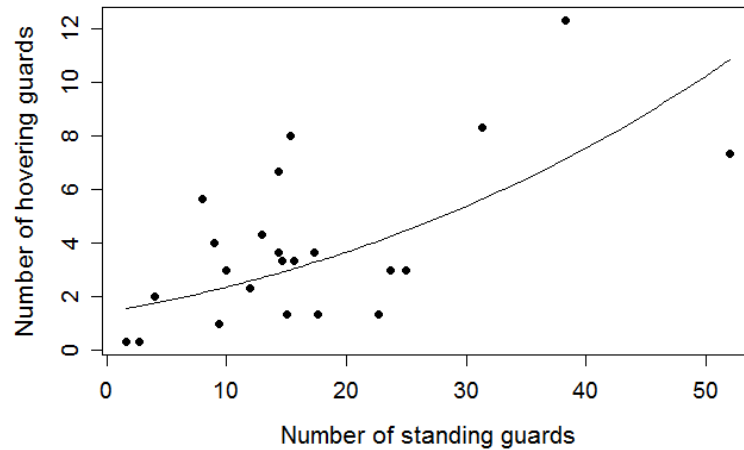


Figure 2: Relationship between the number of standing guards and the number of hovering guards. Each point represents the mean of 3 counts per colony ($n = 25$). There is a significant positive relationship between the two types of guards (LM: cube root transformed data: $n = 25$; $F_{1,23} = 12.92$; $R^2 = 0.33$, $p = 0.002$). Y-axis shows the untransformed data. The curve represents the best fit line.

6.5.2 Total guarding time

Data were collected from 46 marked bees. The number of guards and the type of guarding they did was recorded for each day following the marking procedure (Figure 3). The maximum guarding duration was 20 days (5.4 ± 5.48 ; Figure 3). The day following marking, more bees than expected were observed performing the role of standing guard. We had marked 49% as hovering guards and 51% as standing guards, then found that 91% were standing on the tube and 9% were hovering (Chi-square test: $\chi^2 = 28.5$, $df = 1$, $p < 0.001$). The same pattern can be seen during later days (Figure 3).

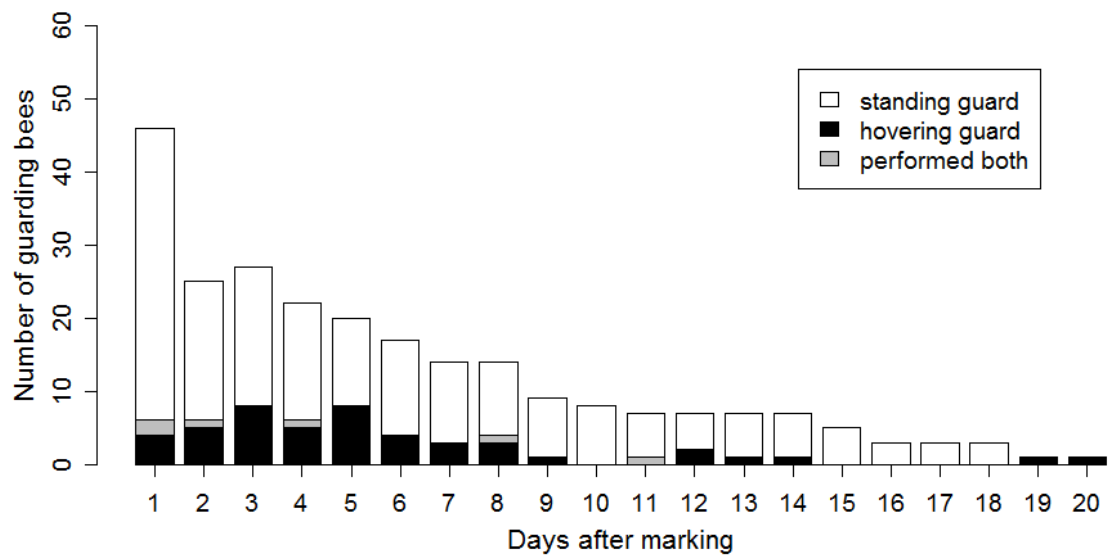


Figure 3: The number of marked bees acting as standing guards, hovering guards, or as both, after marking. For each day the figure shows the total number of marked bees still guarding. Note the clear drop of marked hovering guards from day 0 to day 1 (49% of the marked guards were hovering), the small number of guards that performed both tasks on one day, and the maximum time a bee acted as a guard.

6.5.3 Individually marked bees

Of 18 individually marked bees, subsequently 12 were seen as standing guards, 6 as both types of guards, and none of them exclusively as a hovering guard. Hence, 33% of all individually marked guards changed from one type of guarding to the other. Five of the 6 bees that changed duty during the sampling period were initially captured as hovering guards. We tested if bees initially captured as hovering guards switched from one task to the other more often than bees initially captured as standing guards. This was the case (Mann-Whitney U test: $W = 63$, $n = 8/10$, $p = 0.02$). Hence, some bees have a higher propensity to change tasks than others. The bees that changed task did so on average after 2.45 ± 2.04 days.

6.5.4 Individual observations

We observed 21 bees, each for a 60 minute period. During this time, hovering guards hovered for 56.67 ± 1.51 minutes ($n = 6$) and entered the hive 1.0 ± 0.0 times per bee. Hive stays were on average only 3.3 ± 1.5 minutes. Standing guards stood on the entrance tube for 54.2 ± 9.1 minutes, interrupted by 0.73 ± 1.09 minute hive stays per bee of 7.82 ± 6.45 minutes duration.

In 21 hours of individual observations we never observed a bee switching from one type of guarding to the other. From the duration and rate of hive stays we estimated (by dividing the mean guarding duration per 60 minutes by the number of hive stays during the observation time) that single guarding bouts (time guarding between hive stays) are about 57 minutes for hovering guards and 74 minutes for standing guards.

6.5.5 Guard reactions to breath

After breath treatment, the number of hovering guards in the main and side groups decreased from 4.7 ± 4.02 and 2.2 ± 2.62 to zero, and the number of standing guards decreased from 12.2 ± 5.16 to 1.9 ± 2.54 . These changes are all highly significant (Wilcoxon-signed rank test: hovering bees main group: $V = 120$, $p < 0.001$; side group: $V = 91$, $p = 0.002$; standing guards: $V = 120$; $p < 0.001$; $n = 15$).

6.5.6 Geometry of entrance tubes

Each colony has a cylindrical or conical wax entrance tube made by the bees that was built into the hive entrance hole. (The wooden hive boxes had a circular entrance hole 1.8 cm in diameter. Colonies had built a wax entrance tube from the inner surface of this hole.) The tubes point slightly upward ($48.8^\circ \pm 9.27^\circ$ from horizontal; $n = 25$). As a result the lower length of the tube to the entrance hole was slightly longer than the upper length ($1.93\text{cm} \pm 0.87\text{cm}$ versus

0.81 cm \pm 0.73 cm). The entrance was at the end and roughly circular (diameter 5.7 mm \pm 0.98 mm, $n = 25$; data from the colonies studied in 2009).

6.5.7 Opening and closing the entrance tube

On day 1 (2 February 2009), 14 of 25 colonies (56%) closed their nest entrance (sunrise: 06:46; sunset: 19:54 local time). On day 2 (14 February 2009), 8 of 25 (32%) colonies closed their entrance (sunrise: 06:54; sunset: 19:47 local time; Figure 4). Ten of the 11 colonies that did not close on night 1 also did not close on night 2. However, this is not significantly different from randomness (Chi-square test: $\chi^2 = 4.73$, $df = 3$, $p = 0.19$).

Nest entrances were usually closed after sunset (11 of 14 on day 1, 8 of 8 on day 2). In order to be sure that colonies do not close their entrances after midnight we checked a subsample of the colonies at 02:00 on night 1 and again found 2 out of 5 colonies open. This suggests that there is no change in entrance status after midnight. The difference between night 1 and 2 in the proportion of closed entrances is not significant (McNemar test, $n = 25$, $p = 0.07$). Colonies with greater foraging activity were more likely to close their nest entrances (Mann-Whitney U test: night 1: $W = 116$, $p = 0.035$; night 2: $W = 101.5$, $p = 0.054$; $n = 25$).

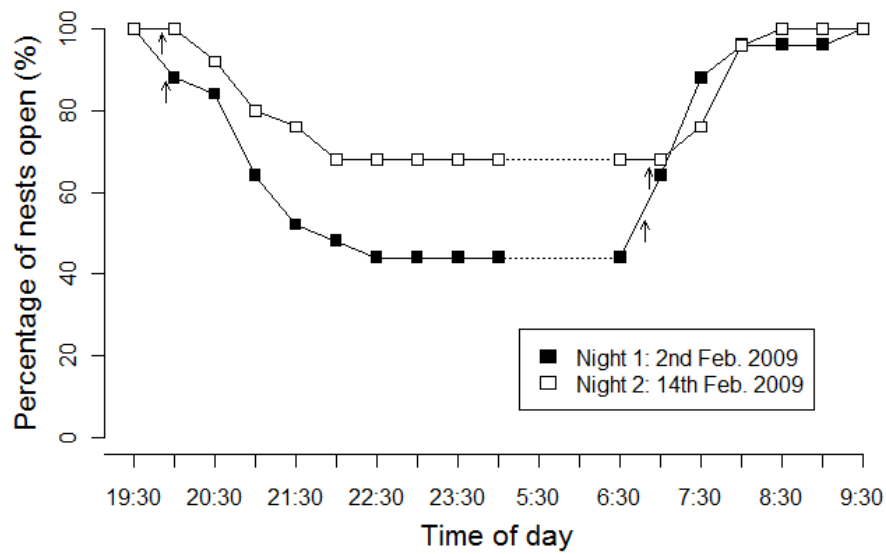


Figure 4: Proportions of nest entrance tubes that are open at different times of the day and night. The nest entrances were observed every 30 minutes from 19:30-24:00 and 06:30-09:30 during 2 different evenings and mornings. The arrows indicate the time of sunset and sunrise on both days.

6.6 Discussion

Our data give new information on guarding and nest defence in *T. angustula*. In particular, the results indicate that changing task from one type of guarding to the other is rare. The individually marked bees that did change from one type to the other (33% of all guards) did so on average every 2.65 days. About 66% only performed guarding as standing guards, while no guard was exclusively hovering. This suggests that hovering is performed by a sub-group of guards that change from standing to hovering. The low rate of changing tasks indicates that nest defence in *T. angustula* is an example of division of labour, rather than “task switching”. Frequent switching between tasks as occurs, for example, during nest construction in *Polybia occidentalis* (Olivier) when workers switch between three different tasks, water foraging, pulp foraging and building (Jeanne, 1986). Our data indicate that specialisation in performing standing guard duties is greater than for hovering guard duties.

Although changing task was rare during our observation periods, there is some indication that allospecific intruders induce standing guards to switch to hovering (Kärcher and Ratnieks, 2009). Further study is needed to explore whether standing guards indeed switch to hovering for extended time periods after exposure to cues from allospecific intruders. We speculate that division of labour between hovering and standing guards increases the overall efficiency of nest defence. Indeed, the hovering guards effectively detect allospecific intruders (Wittmann, 1985; Bowden et al., 1994), making colonies almost immune against attacks of the cleptobiotic bee *Lestrimelitta limao* (Smith) (P Nogueira-Neto, personal communication). Hovering guards might also help the colony to detect allospecific intruders faster or at greater distance from the nest. Standing guards, on the other hand, have remarkable abilities to discriminate nestmates from conspecific non-nestmates (Kärcher and Ratnieks, 2009) making fewer errors than any other social bee previously studied (*Melipona quadrifasciata*, *M. scutellaris*, *M. rufiventris* (Breed and Page, 1991); *Frieseomelitta varia* (Couvillon and Ratnieks, 2008); *Apis mellifera* (Downs and Ratnieks, 1999, 2000)).

6.6.1 Guarding duration

In our study, *T. angustula* workers guarded for up to 20 days, with a mean guarding duration of 5.41 days (median = 3). This is longer than in European or African honey bees (*Apis mellifera*). In European honey bees guarding is performed by a small proportion of the workers, and most of those that do guard do so for less than one day (Moore et al., 1987). The maximum guarding duration was around 6-7 days (Moore et al., 1987; Hunt et al., 2003). African honey bees may guard for up to 10 days (Hunt et al., 2003). In both honey bees and *T. angustula*, most guards perform guard duties for only a relatively short period of time. The long guarding time in *T. angustula* might indicate a higher degree of specialisation than in honey bees. Interestingly, Grosso and Bego (2002) found that *T. angustula* workers perform fewer tasks in their life time than other stingless bee species, which suggests a relatively high degree of task specialisation

compared to other species. The long average guarding duration is even more remarkable given that *T. angustula* workers live on average for only 20.9 days as adults, which is the lowest mean life span found in stingless bees (Grosso and Bego, 2002). Observations on individuals show that guarding bouts (time between leaving the nest and entering the nest) are about 57 minutes for hovering guards and 74 minutes for standing guards. These bouts were interrupted by hive stays of 3.3 minutes for hovering guards and 7.8 minutes for standing guards. Presumably, hovering guards need to return to the nest to refuel but it is unclear why standing guards take breaks in the nest. The fact that standing guards take breaks suggests that they have more than a refuelling function. Taken together, the results of the focal and scan samplings suggest that bees often guard for many hours per day. Some individual bees can be observed standing or hovering at the same location from 10:00 to 16:00 for several consecutive days (C Grüter, personal observation). We do not have information about the age of the guards but in other stingless bees as well as in honey bees guarding is performed towards the end of the age polyethism sequence, after completing in-hive tasks and before starting to forage (Winston, 1987; Roubik, 1989). As in other social insects, foraging in *T. angustula* seems to be the final task (Grosso and Bego, 2002). On several occasions we observed returning bees (putative foragers) with paint marks. With respect to the two different types of guarding, the results presented in Figure 3 do not suggest a particular type of temporal sequence with one type of guarding preceding the other. However, more detailed studies on age polyethism including guarding in *T. angustula* are needed.

6.6.2 Guard numbers

We found that colonies have more standing guards than hovering guards (Figure 1). This might simply be because standing on the entrance tube is energetically more affordable for colonies than hovering. On the other hand, if we assume that standing guards are more efficient against conspecific robbers (Kärcher and Ratnieks, 2009), then higher numbers of standing guards

might indicate a generally higher risk of conspecific robbing compared to allospecific robbing. There is evidence, however, that the number of hovering guards can be adjusted to the perceived risk of allospecific robbing. Standing guards perceiving odours of allospecific stingless bees often start hovering (Kärcher and Ratnieks, 2009). Hovering and standing guards have different activity peaks (Figure 1): While standing guards were most numerous from midday to late afternoon (12:00-18:00; Figure 1), hovering guards were most active during the late afternoon. It is possible that this is caused by different activity times of potential conspecific and allospecific intruders.

We also found that the forager traffic of colonies positively correlates with the number of guards. This suggests that larger colonies also have more guards.

6.6.3 Reactions to breath

Eusocial bee nests are not only threatened by other bees but also by vertebrate predators (Roubik, 1989; Kajobe and Roubik, 2006) including humans, apes (e.g. *Pan troglodytes*), bears (*Tremarctos ornatus*), mustelids (e.g. *Eira barbara*), armadillos (*Prionomys sp.*), anteaters (*Tamandua sp.*), birds (e.g. *Indicator sp.*), toads and geckos (MH Kärcher, personal observation). Consequently, the breath from vertebrate predators elicits a defensive response in some bees (DeGrandi-Hoffman et al., 1998). In *T. angustula*, breath did not cause aggressive defensive reactions. Instead, the number of guarding bees rapidly decreased as the standing guards retreated into the nest. During our experiments, guards of only one colony were occasionally aggressive against human observers that were standing close to the entrance for long periods of time. The bees of this particular colony tried to bite exposed skin. The nest cavities of *T. angustula* colonies probably provide a defensive barrier against these predators. Whether our results can be attributed to the small body size is not clear, since stingless bees of similar size can be aggressive against humans (e.g. *Plebeia pugnax*, FLW Ratnieks, personal observation).

6.6.4 Nest entrance closure

Many bee and ant species close their nest entrances at night to protect the nest against nocturnal enemies (Roubik, 1989; Tofilski et al., 2008). However, stingless bee colonies often do not close their nests completely (Roubik, 1989). In our study population, approximately half the colonies closed their nest entrances in the evening after sunset. Furthermore, some colonies had partially closed entrances. Fewer colonies tended to close the nest entrance after a day with a lot of rain. The temperatures were otherwise very similar. Nocturnal predators might be less active after heavy rainfall, thereby reducing the need to close the nest entrance. However, more data are needed to investigate the relationship between weather and nest entrance closure.

Tetragonisca angustula has an intriguing nest defence organisation with two distinct types of nest entrance guards (Kärcher and Ratnieks, 2009). Although our results make a contribution to understanding guarding behaviour and nest defence in *T. angustula*, perhaps their main value is in directing future research in relation to some of the major questions. For example, why does there appear to be division of labour rather than task switching? What is the advantage in having hovering guards? Why do some colonies not close their entrances, and how is entrance closing organised?

Chapter 7 - Killing and replacing queen-laid eggs: measuring the cost of worker policing in the honey bee

Martin H. Kärcher and Francis L. W. Ratnieks

7.1 Summary

Worker honey bees “police” each other’s reproduction by killing worker-laid eggs. Previous experiments demonstrated that worker policing is effective, killing most (*ca.* 98%) worker-laid eggs. However, many queen-laid eggs were also killed (*ca.* 50%) suggesting that effective policing may have high costs. But in these experiments eggs were manipulated by transferring them with forceps into test cells in unrelated discriminator colonies. We measured the survival of unmanipulated queen-laid eggs and the rate at which removal errors were rectified by the queen laying a new egg. Only 9.6% of the queen-laid eggs in drone cells and 4.1% in worker cells were removed in error. Of eggs removed in error, most, 85% in drone cells and 60.5% in worker cells, were replaced rapidly, i.e. within 3 days. Recently removed eggs were replaced sooner: 90% vs. 3.3% of eggs were replaced within 24 hours, once they have been removed from drone cells 0-24 vs. 48-72 hours after laying. Previous studies have shown that worker policing in the honey bee has a high benefit to policing workers because workers are more related to the mother queen’s sons (0.25) than other workers’ sons (0.15). This study shows that worker policing also has a low cost in terms of the killing of queen-laid eggs.

7.2 Introduction

Within insect societies there is extensive potential for conflict over reproduction (Ratnieks and Reeve, 1992; Bourke and Franks, 1995; Ratnieks et al., 2006). One important area of conflict is the production of males (Bourke and Franks, 1995). Since males arise from unfertilised eggs, workers can normally contribute to male production as in most species workers retain ovaries but cannot mate (Bourke, 1988). The proportion of males reared which are sons of the one or several egg-laying queens varies greatly among species (Wenseleers and Ratnieks, 2006a). One factor of importance in causing this variation is variation in the extent of egg policing, the killing of worker-laid eggs by either the mother queen (queen policing) or by workers (worker policing) (Wenseleers and Ratnieks, 2006a, b). Several factors affect whether worker policing is favoured by natural selection (Ratnieks and Wenseleers, 2008). On relatedness grounds it is favoured when the workers are more related to the sons of their mother queen, or queens, than to other workers' sons (Ratnieks, 1988; Pamilo, 1991; Crozier and Pamilo, 1996; Wenseleers and Ratnieks, 2006a). In species with single-queen colonies this occurs when the effective paternity frequency of the mother queen is greater than 2 (Ratnieks, 1988; Foster and Ratnieks, 2001c). Worker policing can also be favoured at effective paternity frequencies below 2 when it helps cause a female-biased sex allocation ratio (Foster and Ratnieks, 2001b) or enhances colony productivity (Ratnieks, 1988), and when the policing workers themselves lay eggs (selfish worker policing) (Bonckaert et al., 2010).

The honey bee, *Apis mellifera*, has a high paternity frequency (Tarpy and Page, 2001; Tarpy and Nielsen, 2002; Tarpy et al., 2004) and has an effective system of worker policing (Wenseleers and Ratnieks, 2006b). Most experimentally transferred worker-laid eggs, 98-99%, are killed within one day (Ratnieks and Visscher, 1989; Ratnieks, 1990b, 1993), and only 0.12% of the adult males reared in colonies with a queen are workers' sons (Visscher, 1989). Effective policing also acts as a deterrent against attempting to reproduce (Wenseleers and Ratnieks, 2006b) and only *ca.* 0.01-0.1% of the workers in a colony with a queen have active ovaries

(Ratnieks, 1993). However, it is estimated that these few egg laying workers could lay up to 25-50 eggs per day, 7% of the male eggs (Visscher, 1996), which would result in a substantial proportion of the colony's male production if not policed (Ratnieks, 1993; Visscher, 1996).

Studies examining the policing of worker-laid eggs in honey bee colonies typically compare the survivorship of eggs that have been experimentally transferred from a queenless colony with egg-laying workers into test cells in an unrelated discriminator colony with similarly manipulated and unrelated queen-laid eggs taken from drone cells (i.e., male eggs). These experiments typically show significantly greater and more rapid killing of worker-laid eggs than queen-laid eggs. But they also show that many queen-laid eggs are killed. In European honey bees, *Apis mellifera*, the proportion of queen-laid eggs still remaining in drone cells ranges from *ca.* 45% after 6 hours (Beekman and Oldroyd, 2005) to 55% after 24 hours (Ratnieks, 1990b) and 61% after 24 hours (Ratnieks and Visscher, 1989). Similar results have been found in other honey bee species, with 30% remaining after 24 hours in *A. florea* (Halling et al., 2001) and 25% after 20 hours in *A. cerana* (Oldroyd et al., 2001) and also in vespine wasps, which, like honey bees, have open hexagonal cells so that eggs can easily be checked. In *Vespula vulgaris* and *Vespa crabro* 67% and 64% of the queen-laid eggs remained after 16 hours, respectively, (Foster and Ratnieks, 2001a; Foster et al., 2002) and in *Vespula germanica* 53% survived for the first 24 hours (Bonckaert et al., 2008).

These results from the above 6 species with worker policing all demonstrate that a large proportion of the queen-laid eggs were killed. This implies that worker policing may have a high cost due to the killing of queen-laid eggs. Although policed eggs are eaten in *A. mellifera*, which will reduce any waste of resources invested in the eggs themselves, the rearing of brood within cells in the brood area, which is thermoregulated at *ca.* 35°C (Seeley, 1985), will be less efficient with more empty cells. This is because for the same number of offspring a larger area of comb would have to be thermoregulated (Ratnieks, 1990a). Alternatively, the high mortality of queen-laid eggs may be due to the experimental procedures used, which are

typically designed to compare the survivorship of queen-laid and worker-laid eggs under controlled conditions, rather than to make absolute estimates of egg survival. In the honey bee studies, eggs were transferred using forceps which damages eggs (Wegener et al., 2010). In addition, eggs may be less well-placed in their new cells than if directly laid by a queen, and are also normally relocated into an empty comb which may result in greater egg removal due to the reluctance of honey bees to rear small patches of brood (FLW Ratnieks, personal observation). In the wasp and hornet studies similar manipulations were made, except that eggs were removed from their original cell by cutting the paper comb so that the egg remained attached to a small piece of the paper nest, which was then glued onto the bottom of the test cell. Finally, in all studies eggs were also transferred into non-nestmate discriminator colonies.

The aim of this study was to investigate the cost of worker policing in the honey bee due to the removal of queen-laid eggs. We did this in two ways. First, we measured the mortality of unmanipulated queen-laid eggs in worker and drone cells. Second, we determined the time taken by the queen to lay a new egg in a worker or drone cell from which an egg had been removed, and how this is affected by the time after laying at which the original queen-laid egg was removed. Our results show that a much smaller proportion of unmanipulated queen-laid eggs are killed, 9.6% in drone cells and 4.1% in worker cells, than in previous experiments using manipulated eggs. Removed eggs were also replaced rapidly. Taken together, these results show that worker policing in the honey bee has a low cost because few recognition errors are made and because these errors are easily rectified.

7.3 Material and Methods

7.3.1 Study organisms, sites and basic methods

Experiments were conducted in June and July 2008 at an apiary of the Laboratory of Apiculture and Social Insects, University of Sussex, England and in April and May 2009 at an apiary near the city of Graz, Austria. During these periods, honey bee colonies in these apiaries were natu-

rally rearing large numbers of drones (males) with the queens readily laying eggs in the large-diameter hexagonal “drone” cells used to rear males². The 2008 experiments were conducted near the end of the main drone rearing season. To ensure that the experimental colonies would adequately care for any male eggs laid by the queens, they were prevented from rearing drones until the time of the experiment by giving them only combs of worker-sized cells and by removing the few drone pupae being reared. The 2009 experiments were conducted at the beginning of the main drone rearing season so that there was no need to prevent colonies from rearing drones prior to data collection. Thus, in both study years the experimental colonies had a high incentive to rear drones.

Six colonies were used in Experiments 1 and 2, the 2008 trial being carried out using 3 *A. m. mellifera* colonies in England and the 2009 trial using 3 *A. m. carnica* colonies in Austria. Experiment 3 used 3 *A. m. carnica* colonies in Austria. The use of two subspecies and locations was to increase the generality of the results, rather than as a controlled comparison. The study colonies were relatively pure representatives of their respective subspecies as shown by wing morphometry (Ruttner, 1996) (*A. m. mellifera*: cubital index = 1.81 ± 0.04 (mean \pm SE), $n = 100$ (colony 1 and 2), $n = 99$ (colony 3), *A. m. carnica*: cubital index = 2.85 ± 0.12 (mean \pm SE), $n = 100$ (colony 4, 5 and 6)). The great majority of workers in the experimental colonies had the body colour typical of their subspecies, (grey, *A. m. carnica*; black, *A. m. mellifera*). All colonies were of similar size, housed in two hive boxes, deep Langstroth in England and Steirisches Einheitsmaß in Austria, each box containing 10 frames of comb. Between the two hive boxes was a queen excluder to confine the queen to the lower box. Brood frames were moved into the

² Mated *Apis mellifera* queens lay fertilised eggs in worker cells and unfertilised eggs in drone cells (Ratnieks and Keller, 1998). Fertilised eggs, that are heterozygous at the sex determination locus, are females, most of which are reared into workers. Homozygotes are diploid males (Beye et al., 2003), which are killed as young larvae (Woyke, 1963). Normal haploid males develop from unfertilised eggs (Beye et al., 2003) and are normally reared in drone cells whether they are the queen’s sons or workers’ sons (Page and Erickson, 1988; Ratnieks, 1993).

upper box during hive inspections so that the brood area also extended above the queen excluder, as needed in Experiment 1.

7.3.2 Experiment 1: Survival of queen-laid eggs

The aim of Experiment 1 was to measure the survival rate of unmanipulated queen-laid eggs. We transferred one empty frame each of worker cells and drone cells from above to below the queen excluder to allow the queen to lay eggs. We checked these combs every 24 hours and when the queen had laid a patch of eggs we removed the frame to the laboratory in order to carefully inspect it under good illumination and to record the presence or absence of an egg in a subset of the cells. We then replaced the frame into the brood area above the queen excluder to prevent additional egg-laying by the queen. This procedure was carried out twice per colony in each study location. Frames were only moved within a colony, never between colonies. The subsets of cells we monitored (one subset per frame: mean = 838.2, range = 592 to 1,333, $n = 22$, data for worker cells of one colony excluded) was a patch of *ca.* 20 rows with *ca.* 40 cells on one side of the frame. Of these subsets, 221 to 708 cells contained an egg (mean = 501.7) and 87 to 906 cells contained no egg (mean = 336.5). To relocate the cells, the leftmost cell in every fifth row received a coloured pin. The presence or absence of an egg was recorded on paper printed with a hexagonal pattern. Counting was performed as quickly as possible, within 15 to 30 minutes, to reduce the possibility of egg dehydration. Because egg-laying by the queen may have taken place any time in the preceding 24 hours, the post-laying ages of the eggs when checked were 0-24 (first inspection), and then 24-48 and 48-72 hours on subsequent inspections. Honey bee eggs hatch after slightly over 3 days (Harbo and Bolten, 1981) and only during the egg stage do workers discriminate between workers' sons and queen's sons (Ratnieks and Visscher, 1989). In total, 59,400 cell inspections were made. In addition to the removal of queen-laid eggs, the inspections also allowed us to obtain data on the presence of newly-laid worker-laid eggs in cells on the second and third inspections.

7.3.3 Experiment 2: Replacement of eggs in empty worker and drone cells by the queen

The aim of Experiment 2 was to determine the probability at which queens laid new eggs in cells from which an egg had been removed. We placed an empty frame of worker cells with two 10x7cm patches of drone cells, *ca.* 140 drone cells per side, below the queen excluder. When the queen had laid eggs, we then removed a small proportion of the eggs in the frame, 10 to 40 eggs each from worker and drone cells, and returned the frame below the queen excluder. We then checked these cells 24 and 48 hours later to determine if they contained a newly-laid egg.

7.3.4 Experiment 3: Replacement of eggs at different time periods after egg removal

The aim of Experiment 3 was to determine whether the timing of egg removal has an effect on the probability of egg replacement. Here we used the same method as in Experiment 2, except that the test comb had only drone cells. We removed 10 eggs per frame and returned the frame below the queen excluder. After a further 24 and 48 hours, we then removed an additional 10 eggs from the same patch. These eggs were now 24-48 and 48-72 hours old, respectively. Each of these groups of 10 empty cells was checked every 24 hours for another two days to quantify the laying of new eggs.

7.3.5 Statistical analysis

Data were analysed and plotted in R 2.10.1 (R Development Core Team, 2009). To avoid pseudo-replication and to control for variation between colonies or cells, we used Generalised Linear Mixed Effects Models (GLMM) for all statistical tests (Bolker et al., 2009; Zuur et al., 2009). For this purpose the lmer function of the lme4 package was applied (Bates and Maechler, 2010). Data were generally binomially distributed apart from the time data in Experiment 2

which were Poisson distributed. Where appropriate, we treated “colony” and/or “cell” as *random effects* and factors, such as “subspecies”, “sex” or “day”, as *fixed effects*. We then compared *random intercept models* with *random intercept and slope models* using Likelihood Ratio Tests and selected the model according to Zuur et al. (2009). Initially, interactions between fixed effects were included but non-significant interactions were removed in the final model. For data visualisation we used the ggplot2 package (Wickham, 2009).

7.4 Results

7.4.1 Experiment 1: Survival of queen-laid eggs

Only a small proportion of queen-laid eggs were removed (Figure 1). In total, 6.0% and 9.6% of the eggs in drone cells ($n = 18,447$) and 2.1% and 4.1% in worker cells ($n = 14,664$) were removed within 24 and 48 hours, respectively, with the removal rate from drone cells being significantly higher ($p = 1.05 \times 10^{-7}$, $n = 33,111$, Figure 1). *A. m. carnica* removed fewer eggs than *A. m. mellifera* ($p = 2.98 \times 10^{-6}$, $n = 33,111$). The removal of queen-laid eggs from drone cells was significantly greater in the first 24 hour period, eggs aged 0-24 hours at the start, than in the next 24 hour period ($p = 8.97 \times 10^{-14}$, $n = 12,298$, Figure 1). There was no such difference for queen-laid eggs in worker cells ($p = 0.0798$, $n = 9,776$, Figure 1).

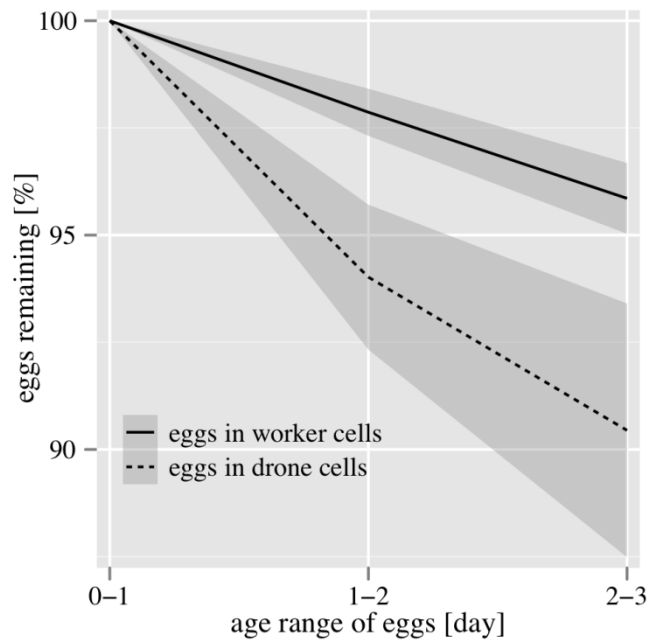


Figure 1: Experiment 1. Natural survival rate of queen-laid eggs in drone and worker cells until hatching. Lines represent means. Grey shaded areas represent standard errors. Note the different decrease of the per cent of eggs in worker or drone cells until hatching.

7.4.2 Experiment 1: Survival of worker-laid eggs

When checking cells we occasionally found eggs in cells that had been empty on the previous day, indicating that they were worker-laid eggs since the queen was confined below the queen excluder. The proportion of worker-laid eggs was higher in *A. m. mellifera* (0.8649% of previously empty cells, $n = 1,503$) than in *A. m. carnica* (0.0649%, $n = 1,540$) ($p = 0.0284$, $n = 3,043$). However, there was no correlation between the proportion of worker-laid eggs appearing in cells and the proportion of queen-laid eggs that were policed ($p = 0.5864$, $n = 12$). Worker-laid eggs that had already survived 0-24 hours in drone cells ($n = 9$) were as likely to be policed (22%) as 0-24 hour old queen-laid eggs in drone cells ($n = 6,149$, 6%) ($p = 0.199$, $n = 6,158$). For this comparison only male eggs in drone cells were used. When inspecting queen-laid eggs at the age of 24-72 hours we found only one newly-laid egg in a worker cell. In total 14 worker-laid eggs were found in drone cells, 13 in cells that had not previously contained a queen-laid egg and one in a cell from which a queen-laid egg had been removed. Four of these 13 eggs

were found when queen-laid eggs were already 48-72 hours old. The other 9 were found when queen-laid eggs were 24-48 hours old so that we could track them for one day, to show that 7 (78%) survived the following 24 hours. These 9 worker-laid eggs were found in 3,029 drone cells containing no egg at first inspections (6,149 drone cells contained a queen-laid egg). Thus, after 24 hours, there were *ca.* 3 worker-laid eggs per 1000 empty drone cells. The actual egg-laying rate would have been higher due to the removal of eggs prior to the daily inspection (Visscher, 1996).

7.4.3 Experiment 1: Unusual death rate of eggs in worker cells of one colony

In one of the colonies, a considerably higher proportion of queen-laid eggs was removed from worker cells. Although a large proportion of these eggs, 93.5%, survived to 24-48 hours only 71.3% survived to 48-72 hours. At the end of the experiment we noted that this colony had a much lower worker population than the other colonies and was not building up in population as expected. Therefore, we excluded the data for queen-laid eggs in worker cells from the analysis. This did not affect our results, such as the conclusion that the proportion of queen-laid eggs that are killed is low.

7.4.4 Experiment 2: Replacement of eggs in empty worker and drone cells by the queen

Eggs were more likely to be replaced by *A. m. carnica* (86.5 ± 5.5 %, mean \pm SE %) than *A. m. mellifera* (63.3 ± 17.0 %) ($p = 0.0252$, $n = 290$). However, there was no significant difference in the chance of an egg being replaced in a drone (85.0 ± 5.1 %) versus a worker (60.5 ± 20.4 %) cell ($p = 0.6534$, $n = 290$). Eggs that were replaced were replaced quicker in *A. m. carnica* than in *A. m. mellifera* ($p = 0.01$, $n = 219$, Figure 2) and in drone cells versus worker cells ($p = 1.80 \cdot 10^{-7}$, $n = 219$, Figure 2). In *A. m. carnica* this took 36.4 ± 0.3 hours for drone and 41.1 ± 2.8

hours for worker cells. In *A. m. mellifera* it took 39.9 ± 2.3 hours for drone and 45.6 ± 4.2 hours for worker cells (Figure 2, each: mean \pm SE).

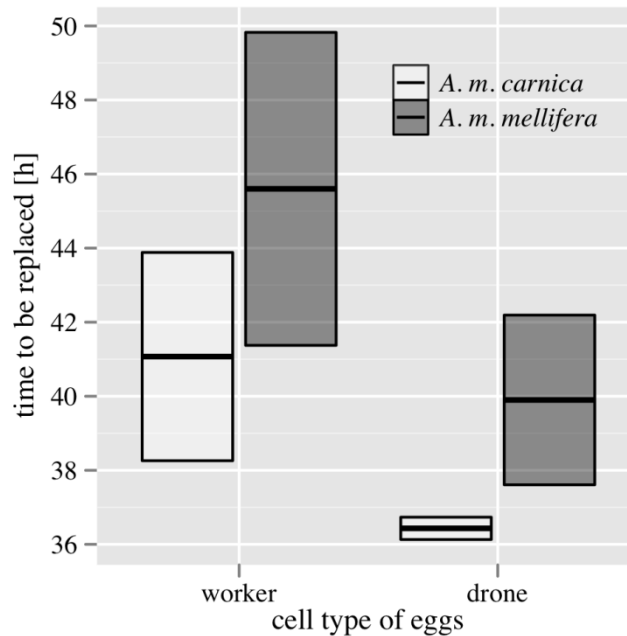


Figure 2: Experiment 2. Replacement, by the queen laying new eggs, of eggs removed from drone and worker cells in *A. m. mellifera* and *A. m. carnica*. Crossbars represent the mean \pm standard error. Note that eggs in drone cells were more quickly replaced than eggs in worker cells, and that *A. m. carnica* was quicker in replacing eggs than *A. m. mellifera*.

7.4.5 Experiment 3: Replacement of eggs at different time periods after egg removal

Queen-laid eggs removed from drone cells 0-24h after laying are more likely to be replaced than eggs removed 1 or 2 days later ($p = 0.000392$, $n = 270$, Figure 3). $40.0 \pm 10\%$ of eggs removed aged 48-72 hours, $53.3 \pm 14.5\%$ removed aged 24-48 hours and $93.3 \pm 3.3\%$ removed aged 0-24 hours were replaced within 72 hours (each: mean \pm SE). Younger eggs were also quicker replaced with $90.0 \pm 0\%$ aged 0-24 hours being replaced within 24 hours versus only $36.7 \pm 16.7\%$ aged 24-48 hours and $3.3 \pm 3.3\%$ aged 48-72 hours (each: mean \pm SE). As a con-

sequence, the increase in egg replacement within the 72 hours after egg removal was higher for eggs removed at a later stage ($p = 0.000632$, $n = 270$, Figure 3).

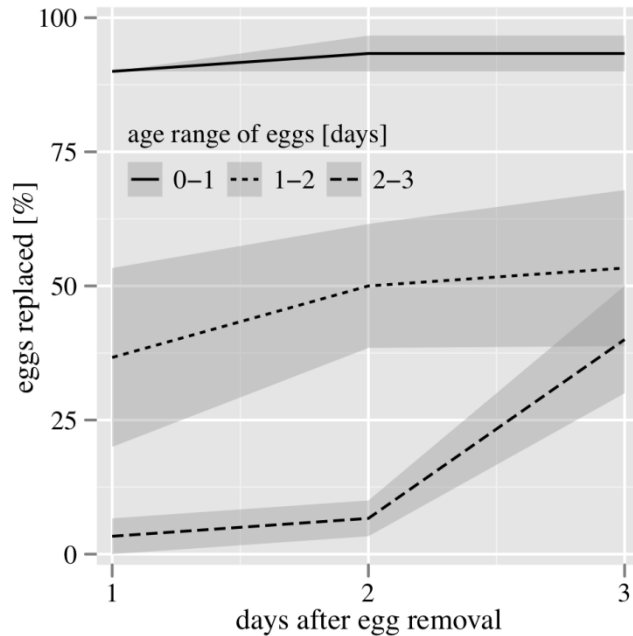


Figure 3: Experiment 3. Replacement, by the queen laying new eggs, of eggs removed at different ages from drone cells in *A. m. carnica*. Lines represent means. Grey shaded areas represent standard errors. Note that eggs removed at the age of 0-24 hours were more likely to be replaced and this happened more quickly.

7.5 Discussion

The results clearly show that worker policing in the honey bee, *Apis mellifera*, has a low cost in terms of the removal of queen-laid eggs in both drone and worker cells. Experiment 1 shows that the vast majority of queen-laid eggs survives. Experiment 2 demonstrates that most eggs that are removed are replaced, and that replacement is rapid. Experiment 2 also demonstrates that eggs are replaced more quickly in drone cells than in worker cells, and Experiment 3 shows that recently removed eggs are replaced sooner.

The results of Experiment 1 indicate that the high percentage of removal, *ca.* 50% after 24 hours, of queen-laid eggs observed in previous studies of worker policing in the honey bee was due to the manipulation and relocation of eggs. This did not occur in the current experiment, which therefore gives a more realistic estimate of the natural proportion of queen-laid eggs that survive. Interestingly, a larger proportion of eggs in drone cells was killed during the first 24 hour monitoring period (eggs were aged 0-24 hours at start) compared to the second 24 hours. However, this was not true for eggs in worker cells. Eggs in drone cells were also twice as likely to be policed as eggs in worker cells. The acceptance threshold model by Reeve (1989) provides a plausible explanation for this: The greater killing of queen-laid eggs in drone cells may be due to a less permissive acceptance threshold in these cells. This would be expected given that workers normally lay eggs in drone cells (this study; Ratnieks, 1993).

Eggs removed from drone cells were replaced more quickly than eggs from worker cells, possibly because eggs in drone cells are more likely to be policed. However, the quicker replacement of queen-laid eggs removed from drone cells than from worker cells probably does not greatly decrease the overall cost of policing errors because many more workers than drones are reared. Our data are in line with the finding that the queen's tendency to replace male eggs is higher compared to female eggs (Wharton et al., 2007). Eggs were more likely to be replaced and at a quicker rate in *A. m. carnica* than in *A. m. mellifera*. Future studies may find it worthwhile to determine whether this is simply due to environmental factors due to the different study locations and conditions or actual biological differences between these two subspecies. However, the overall pattern in egg replacement, as for the other experiments, was similar for both subspecies, which were both studied within their native range.

The sooner after egg-laying an egg was removed from its cell the quicker and the more likely it was to be replaced (Figure 3). A probable reason for this is that the queen is more likely to remain or revisit this area to lay more eggs, given that honey bee queens lay eggs in large patches over several days. Given that eggs are policed between 0 and 3 days after being laid,

the natural rate of egg replacement is some combination of the egg replacement rates at different ages until hatching, 3 days after laying. Our results show that the queen keeps relaying eggs for at least 3 days with decreasing intensity, and probably for longer. Cells may also become vacant due to the removal of larvae of different ages or pupae. For example, diploid male larvae are eaten by workers soon after hatching from the egg (Woyke, 1963).

Our results from Experiments 1, 2 and 3 all demonstrate that the overall cost of worker-policing is very low at the colony level. This is essential in order to maintain a policing system. If costs were too high an “accept all” strategy (Reeve, 1989), that is no policing of eggs, would be a better option. Previous studies show that only 1-2% of worker-laid eggs are mistakenly not destroyed (Ratnieks and Visscher, 1989; Ratnieks, 1990b, 1993). This leads to a combined error rate of *ca.* 6-8% (accept 1-2% of worker-laid eggs + reject *ca.* 4-10% queen-laid eggs). Although not perfect, this is much better than the best combined rate, 100%, due to “guessing” that would occur in the absence of information (Ratnieks, 1991).

Interestingly, Reeve’s model (Reeve, 1989) is also compatible with two other trends in our data. First, the proportion of queen-laid eggs in worker cells that is policed is lower than for drone cells. Reeve’s model would predict that the acceptance threshold should be less permissive in drone cells (i.e., queen-laid eggs get policed at a higher proportion, in error) given that most worker-laid eggs are laid in drone cells. Second, since the cost of accepting a worker-laid egg is greater than the cost of rejecting a queen-laid egg, then the acceptance threshold should be set to make more errors on queen-laid eggs (i.e., police queen-laid eggs) than worker-laid eggs (i.e., accept worker-laid eggs), as seen. Our results also indicate that the mistaken killing of queen-laid eggs has a low cost as eggs are rapidly replaced.

Nine of the 14 worker-laid eggs that we found in drone cells could be tracked for one day. Seven of them (78%) survived for 24 hours which is higher than reported by Ratnieks (1993) where only 15% of similar-aged eggs survived for an additional 24 hours. Eggs transferred with forceps have an even lower survival rate, 2% after 24 hours (Ratnieks and Visscher,

1989). In part, this is because these eggs were manipulated. But, in addition, they had not been exposed to policing prior to introduction into the discriminator colonies since they were taken from queenless egg-source colonies in which worker policing is switched off (Miller and Ratnieks, 2001; Châline et al., 2004). The eggs we monitored, however, had already been exposed to 0-24 hours of worker policing in a queenright colony. The 78% worker-laid eggs that survived from the second to third comb inspections is not significantly different from the survival of 0-24 hour old queen-laid eggs to their next inspection. This indicates that once a worker-laid egg has survived for a certain period of time, 0-24 hours, it is more likely to survive the next 24 hours. Possibly, some worker-laid eggs are harder to recognise and these are the ones that are not policed (Oldroyd and Ratnieks, 2000). Approximately 13.3 times as many worker-laid eggs per drone cell were found in the *A. m. mellifera* than in the *A. m. carnica* study. Further investigation would be needed to determine if this represents a consistent difference among sub species.

In one of our study colonies an exceptionally high proportion (28.71%) of queen-laid eggs in worker cells did not survive to 48-72 hours, compared to the mean of 4.1% ($n = 5$ colonies, Figure 1). Whilst in all other colonies the survival of queen-laid eggs in worker cells was higher than in drone cells, in this colony it was the other way round. We do not know the underlying reason. One potential explanation for this phenomenon might be inbreeding leading to diploid male production (Beye et al., 2003). This is unlikely, however, since the queen of this colony was naturally mated and queens mate with *ca.* 10-20 males (Tarpy and Page, 2001; Tarpy and Nielsen, 2002; Tarpy et al., 2004). More importantly, diploid males are removed as young larvae, and presumably not as eggs (Woyke, 1963). In addition, egg viability plays little role in worker policing. Beekman and Oldroyd (2005) showed that live and dead queen-laid eggs (suffocated with CO₂ for 24 hours) have the same chance of being policed. As the survival probability of queen-laid eggs in drone cells of the same colony was not affected it is possible that the factor causing this unusual egg removal occurred only in fertilised eggs.

Previous studies of worker policing in the honey bee have focused mainly on the benefits to the policing workers in the killing of worker-laid eggs. This benefit is great as workers are more related to their mother queen's sons (0.25) than to other workers' sons (0.15). By showing that one major potential cost of policing, the killing in error of queen-laid eggs, is low, this study indicates that worker policing in the honey bee is favoured due to both high benefit and low cost. This conclusion is probably a broad one and is in agreement with data from vespine wasps. In three wasps species video analyses have revealed that most unmanipulated queen-laid eggs were not policed during the first day after laying: 96% survival for *Dolichovespula sylvestris* (Wenseleers et al., 2005c), 98% for *Vespula rufa* (Wenseleers et al., 2005a) and 92% for *Dolichovespula norwegica* (Bonckaert et al., 2010). In three other vespine species queen-laid eggs were transferred and had a high removal rate: *Vespula vulgaris* (Foster and Ratnieks, 2001a), *Vespa crabro* (Foster et al., 2002) and *Vespula germanica* (Bonckaert et al., 2008). Here we present the first study on a species where we now know both, the proportions of manipulated and unmanipulated queen-laid eggs that are killed.

Chapter 8 - Factors influencing virgin queen survival duration and choice in the stingless bee *Melipona quadrifasciata*

Martin H. Kärcher, Cristiano Menezes, Denise A. Alves, Oliver S. Beveridge, Vera-Lucia Imperatriz-Fonseca and Francis L. W. Ratnieks

8.1 Summary

In *Melipona* stingless bees a large proportion of females, *ca.* 10% in the study species *M. quadrifasciata*, develops into queens. Queen rearing goes on year round and almost all are killed. Occasionally, a new queen will be needed to replace a dead mother queen or to head a new colony during swarming. Here we compared queen execution in queenright and queenless colonies and investigated the effects of queen behaviour, body mass, whether she was nestmate of the workers, and, in queenless colonies, the effect of the time a colony had been queenless, on survival duration and acceptance. None of the 220 virgin queens ever attacked another virgin queen, nor did any of the 88 virgin queens introduced into queenright colonies ever attack the resident queen. In our colonies, which were not swarming, a new queen was only accepted in a queenless colony, never in a queenright. Factors which increased survival duration and acceptance were a) to emerge from the cell about 2 hours after the resident queen has gone, then b) to hide as well as possible whilst c) avoiding any fights in order to live as long as possible. In this way a queen was available when the colony chooses a new queen, 24 to 48 hours after the resident queen was removed. Other behaviours, such as running, walking or resting, antennating or trophallaxis, played little or no role, as did the factors body mass or nestmate. Queen choice lasted for *ca.* 2 hours during which time other virgin queens were kept on being killed. The queen and the bees were extremely agitated and the bees accumulated around the queen. The queen inflated her abdomen possibly to release phero-

mones and some of the workers deposited pheromone on the surface, such as on the glass lid of the observation hive.

8.2 Introduction

Social insect colonies are often only headed by one or a few reproductive females known as queens whilst the majority works (Ratnieks and Helanterä, 2009; Bourke, 2011). This extremely low proportion of females reared into queens is achieved by kinship, coercion and constraint (Foster et al., 2006; Ratnieks et al., 2006; Wenseleers and Ratnieks, 2006b). *Melipona* bees, one genus of stingless bees, cannot coerce female larvae into developing as workers due to their unique brood rearing situation: Larvae are not progressively but mass provisioned receiving exactly the same quantity and quality of food, nor is the fate of female larvae constraint since both worker and queen larvae develop in sealed cells of exactly the same size (Bourke and Ratnieks, 1999; Ratnieks, 2001; Wenseleers et al., 2004). In *Melipona* only single mating occurs (Peters et al., 1999) and kinship seems to be the only factor limiting the proportion of female larvae that develop into queens (Bourke and Ratnieks, 1999; Ratnieks, 2001; Queller, 2006). A great excess of queens is reared given that a colony would only need to rear a few queens per year to allow swarming or to supersede an old queen. The proportions reared are similar to theoretical predictions (Ratnieks, 2001): *Ca.* 12% of the females in *M. quadrifasciata* (Kerr, 1950), *ca.* 23-18% in *M. beecheii* (Darchen and Delage-Darchen, 1975; Wenseleers et al., 2004), *ca.* 7% in *M. subnitida* (Koedam et al., 1999) and *ca.* 10% in *M. favosa* (Koedam, 1999).

Like honey bees, *Melipona* stingless bees only need a new queen to either replace the resident queen once she died or when the colony splits for swarming (Winston, 1987). Both situations are relatively rare which is why the vast majority of virgin queens is slaughtered (da Silva et al., 1972; Koedam et al., 1995b; Wenseleers et al., 2004). Two studies in *M. favosa* have shown, however, that some of the queens also leave their natal colony (Sommeijer et al., 2003b) possibly to attempt to take over a conspecific foreign colony (Sommeijer et al., 2003a). One very recent study in *M. scutellaris* has also shown that colonies are occasionally taken over by a queen from another colony (Wenseleers et al., 2011). One key difference between stingless bees and honey bees is the timing of queen rearing and the number of queens that

are reared. In honey bees only *ca.* 30 queens are reared once a year, none, one or several of which manage to swarm with some fraction of the colony after the mother queen left with the first swarm but one of which always inherits the mother colony once no more swarms are produced (Winston, 1987). In addition, an old resident queen can also be replaced via supersedure. During supersedure the colony typically only rears one or a few young queens. The other key difference is that due to single mating, stingless bee females are much more closely related to each other than are honey bees. This significantly reduces the incentive for direct reproduction (Wenseleers et al., 2003; Ratnieks and Helanterä, 2009). It might also be one factor why no queen fights occur in *Melipona*, as predicted by Peters et al. (1999). However, this explanation does not fit to the exorbitant number of females risking the fate of a queen in the first place. In honey bees, queen fights are common since only one or a few queens are needed during the swarming season. Thus, one would expect at least a certain degree of queen-queen fighting to occur in stingless bees as well.

To date queen execution and the selection of a new queen and the events surrounding this have been the subject of descriptive studies many of which were only case studies with a low sample size (Imperatriz-Fonseca and Zucchi, 1995; Jarau et al., 2010). Also, in no study was the behaviour towards virgin queens compared in queenless versus queenright colonies, although some valuable studies have been done using queenless colonies (Koedam, 1995; van Veen et al., 1999; Wenseleers et al., 2004). An additional confounding factor in this research area, apart from different researchers sharing different opinions, is that *Melipona* consists of 35 species (Silveira et al., 2002), several of which have been studied. A recent study on 2 colonies of *M. beecheii* where queens were kept in cages on brood combs suggests that queen pheromones are not triggering worker aggression (Jarau et al., 2009). By contrast, the virgin queens' behaviour or their inflated abdomen should be responsible for workers attacking her. To test this, queens with and without behaviour were compared. However, the method is problematic since the control group of queens had been freeze killed and these dead queens

were treated by the workers in the same way as dead workers (Jarau et al., 2009). The authors finally propose that the colony selects the strongest virgin queen through workers fighting with queens. Here we did not directly study what causes or triggers the workers to attack particular queens. Instead, we tried to examine the factors that affect a virgin queen's acceptance or survival time, respectively. We only used living queens and used a natural bioassay.

To discover factors influencing queen survival in queenright and queenless *Melipona* colonies and the choice of a new queen in queenless colonies, we investigated a variety of factors concerning the young queens: whether they were nestmates or not; their live body mass; the proportion of time they spent in various behaviours during their first hour after introduction; and whether the colony into which they were introduced was queenright or queenless, and for how long it had been queenless. The factors "nestmate" and "virgin queen live body mass" had no or rather a negative effect, respectively on the chance to survive or at least live longer. However, queens introduced sooner rather than later after resident queen removal had a higher chance of survival or a longer duration of survival. Queens spending more time hiding and being less time under attack lived significantly longer and, generally, were also the queens that were accepted by the workers. Other behaviours, such as "walking or resting", "running" or "antennating combined with trophallaxis" had a slightly positive or no effect on a virgin queen's life time. Queenfights between young queens or against the resident queen were never observed. Besides, this study describes the queen choice of 4 queens in 4 colonies which has not been described in such detail before and seems to be a relatively novel behaviour studied in social insects. We also recorded how virgin queens were killed and presented the caste ratio and live body mass of *ca.* 1,100 individuals. At the end of our discussion we propose a "best strategy" which apparently was used by chosen virgin queens.

8.3 Methods and Material

8.3.1 Study site, colonies and environmental conditions

Data were collected from 11 January to 15 February 2007 at Fazenda Aretuzina, formerly a coffee farm and now a research station owned by Prof. Paulo Nogueira-Neto. It is located in an area of sugar cane fields and dry, secondary forests near the town of São Simão, São Paulo State, Brazil. During the study period weather conditions were hot with maximum temperatures of *ca.* 30°C on most days, with occasional heavy rain. Flowers were abundant, and colonies foraged actively in the daytime.

Four colonies of *Melipona quadrifasciata* were bought from a local beekeeper. Subsequently, each was housed in a wooden observation hive with a glass lid of several slides (approximate inner dimensions: 35 cm (width) x 17 cm (depth) x 9 cm (height)). When observations were not being made the glass was covered with black felt in order to prevent light from confusing the bees and for improved thermal insulation. Additionally, a lamp was placed over each colony in order to maintain warmth. Observation hives were kept indoors, *ca.* 1 m above ground. Each had a plastic tube to the outside through a hole in the wall to allow natural foraging.

Each colony contained *ca.* 300 adult workers, similar to a study of Hikawa and Miyanaga (2009), several brood combs and honey and pollen pots. Natural combs, which are parallel and horizontal, were rearranged into a single horizontal layer to facilitate observation. Sheets of wax-based involucrum covering the combs were also transferred into the observation hives. Newly constructed involucrum was periodically removed in order to observe the bees. A trap for phorid flies, a pest of stingless bees, was placed into each colony since flies could enter during or after colony manipulation. The intersections between the glass slides forming the glass lid were taped to block gaps that would allow the phorids to enter.

Since it has been suggested that a fraction of virgin *M. fava* queens leaves their nest (Sommeijer et al., 2003b) to take over a foreign conspecific colony (Sommeijer et al., 2003a)

and *M. quadrifasciata* is a related species we kept the area around the nest exit free from involucrum to check if virgin queens leave the entrance. However, when continuously and meticulously observing the queens during most part of the day we did never see a virgin queen leaving the nest exit alive. Only dead queens were occasionally dragged outside which we then collected where they were dropped by the workers.

8.3.2 Introduction and observation of virgin queens

In order to obtain data on queen execution we introduced marked virgin queens in study colonies. Initially, during the first 5 days of the study, we introduced on average 12.8 virgin queens per day per colony ($n = 64$) into 4 queenright observation colonies. Virgin queens were collected from the 4 observation hives themselves and from 10 additional queen source colonies. The introduction of virgin queens mimics the natural situation in which queens are being reared continually. In the current study 10.4% of all females emerging from combs were queens. Subsequently, from observation day 6 onwards, we introduced exactly 3 virgin queens per day into each of the 4 colonies. On day 6 colonies were still queenright. On day 7 we introduced virgin queens 2 hours after removing the mother queen from each colony, then 24, 48, and 72 hours after dequeening (days 8, 9, and 10), and then every two days after 120, 168, 216, and 264 hours (days 12, 14, 16, and 18). Another 5 days later, once the new queens had been fully accepted in their colonies, they were removed allowing a second series of observations. Again starting with queenright colonies on the first day queens were introduced 2, 24 and 48 hours after resident queen removal in the subsequent 3 days.

In order to obtain newly-emerged queens we incubated brood combs in Petri dishes. We used brood combs from the colonies we studied (nestmates, $n = 4$) and also from other colonies (non-nestmates, $n = 10$) in order to have sufficient queens. Before a virgin queen was introduced into one of the observation colonies, she was individually marked with one or two different colours, dorsally on her thorax and usually also on her abdomen. In addition, the

following characteristics were recorded: a) her origin, i.e. whether she was a nestmate or not and from which colony she originated, b) her live body mass, c) if the colony that she was introduced to was queenless (plus for how long) or queenright.

After introduction each queen was continuously observed for 1 hour or until she was killed. While observing, one person classified the virgin queen's behaviour as a) walking or resting, b) running, c) being attacked by workers, d) hiding, e) antennating often combined with trophallaxis and f) attacking the mother queen or another virgin queen. At the same time a second person recorded the start and stop times of each behaviour to the nearest second. In this way we obtained a record on the proportion of time spent for various behaviours of accepted or executed virgin queens. Many queens were not killed within the first hour and sometimes hid in empty brood cells, below brood combs, between layers of involucrum, or among the honey and pollen pots. Therefore, we continued to check all 4 observation hives every few hours for marked virgin queens and determined the last time we saw each queen alive. As soon as we could not see a certain virgin queen anymore or found her dead body, we recorded the time and estimated the time of death by adding half of the time of the current interval to the time when we last saw her alive. Applying this method we obtained a record of the virgin queen's life time in a colony, if she was not killed within the 1 hour observation period. Likewise, if a queen hid below a brood comb, for example during the 1 hour observation period, e.g. at 10 minutes, and her dead body was then dragged by workers into an area where we could see her again, e.g. at 30 minutes, the time of death was estimated at 20 minutes.

8.3.3 Descriptive data

The first 1,121 individuals to emerge in the incubator were also used to determine the ratio of workers, drones and queens and also their live body mass. No food was given to them before weighing or introducing them into a colony.

As soon as it was clear that a queen had been killed it was collected to determine possible causes of death. In this way we could also be sure that it was not feigning death (van Veen et al., 1999) with the workers being unable to remove it from the colony or drag it on the garbage area in the nest. Most queens could be collected in this way. Sometimes, however, queens were killed outside our observation periods and removed from the colony. In this case we searched for them outside near the hive entrance and also recorded the distance to the entrance.

8.3.4 Statistical analysis

Data were analysed and plotted in R 2.12.1 (R Development Core Team, 2010). To avoid pseudo-replication and to control for variation between study colonies or virgin queens from different source colonies we used Generalised Linear Mixed Effects Models (GLMM) for all statistical tests (Bolker et al., 2009; Zuur et al., 2009). For three tests the standard Chi-square test had to be used, and this is noted in the results. For the GLMMs the `lme` function of the `nlme` package (Pinheiro et al., 2010) and the `lmer` function of the `lme4` package (Bates and Maechler, 2010) were applied. For analysis the life time data of the introduced queens were transformed into a normal distribution applying the Box-Cox method (Sokal and Rohlf, 1995). The survival data were binomially distributed. “Study colony” and “virgin queen source colony” were treated as *random effects* in all models. Factors such as “hours queenless”, “nestmate” or “per cent of time hiding” were treated as *fixed effects*. *Random intercept models* and, where applicable, *random intercept and slope models* were used. Using Likelihood Ratio Tests they were then compared using the model of Zuur et al. (2009). We analysed each behaviour in a separate GLMM and did not test for interactions between different behaviours. We did not allow for interactions between non-behavioural fixed effects since there was no a priori biological reason to expect an interaction between “hours queenless”, “nestmate” or “live body mass”. For data visualisation we used the `ggplot2` package (Wickham, 2009).

8.4 Results

8.4.1 Queen execution

a) Factors influencing the timing of execution of virgin queens

In queenless colonies the factors nestmate versus non-nestmate ($p = 0.62$) and live body mass ($p = 0.50$) had no effect on the time to execution of introduced queens. The effect of the time that a colony had been queenless ($p = 0.06$, Figure 1) ($n = 123$) was also non-significant, but only slightly above the 95% significance level. Similarly, in queenright colonies nestmate versus non-nestmate ($p = 0.29$, $n = 88$) and live body mass ($p = 0.11$, $n = 12$) were again non-significant. Combining queenright and queenless colony data strengthens these results: Nestmate versus non-nestmate ($p = 0.55$), live body mass ($p = 0.41$), and whether a colony was queenless or queenright ($p = 0.26$) did not affect the time a virgin queen survived until execution ($n = 135$). The latter is also illustrated in Figure 1, where, contrary to the statistical analysis, we split queenless colony data into “recently queenless”, i.e. introduced at 2 hours of queenlessness, and “queenless”, i.e. introduced at 24 hours of queenlessness or later. Note that data for queenright and queenless colonies are overlapping showing a similar decrease in the number of queens surviving. However, virgin queens in recently queenless colonies generally appear to live longer (Figure 1). 50% of the virgin queens in queenright colonies were killed after *ca.* 1,000 seconds (16 min 40 sec). Somewhat later, note the log₁₀ scale where minor grid lines indicate $10^{0.5}$ steps, the same was true for queens in queenless colonies, but almost exactly 9,000 seconds (2 h 30 min) later half of the queens in recently queenless colonies were killed at *ca.* 10,000 seconds (2 h 46 min 40 sec).

In contrast, some of the queen behaviours measured in per cent of time of a queen’s first hour in a colony or until its death, respectively - data of queenless and queenright colonies were combined - did significantly correlate with the time a virgin queen survived until execution: Antennating and trophallaxis did not correlate ($p = 0.43$), being under attack was negatively correlated ($p = 0$), hiding was positively correlated ($p = 0$), running was not significantly

correlated ($p = 0.07$) and walking or resting was slightly positively correlated ($p = 0.04$) ($n = 211$, Figure 2). It is noteworthy that none of the 220 virgin queens that we introduced ever attacked another virgin queen, nor did any of the 88 virgin queens that were introduced into queenright colonies attack the resident queen.

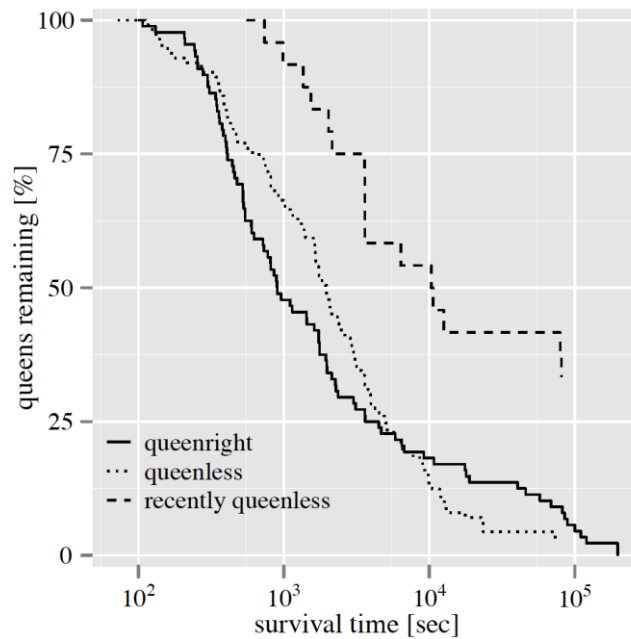


Figure 1: Survival duration of virgin queens in queenright, queenless and recently queenless colonies. 88 virgin queens were introduced into queenright, 108 into queenless and 24 into recently queenless colonies. “Queenless” includes queens introduced at 24 hours of queenlessness or later whilst “recently queenless” refers to queens introduced after 2 hours of queenlessness only.

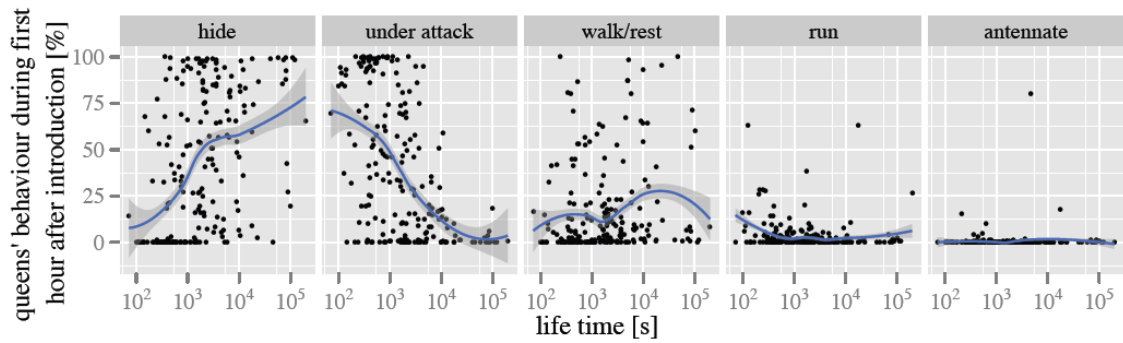


Figure 2: Relationship between virgin queen behaviour and survival duration. Data points represent the life time of single queens and the percentage of time they spent for the specified behaviour. Lines represent means. Grey shaded areas represent standard errors. Life time [s] on the x-axis is log10 transformed.

b) Factors influencing the survival of virgin queens

In queenless colonies, whether a queen was a nestmate or not had no effect ($p = 0.28$) and her live body mass a non-significant negative effect ($p = 0.08$) on the survival of introduced queens ($n = 131$). However, the time (hours) that a colony was queenless did have a strong effect ($p = 0.01$, $n = 131$) (Figure 3). Seven of the 9 accepted queens were accepted when introduced 2 hours after the resident queen was removed. One of these queens disappeared during the study period so that a second queen, introduced at 216 hours of queenlessness, was accepted as a replacement. Only in one case did a colony choose its first new queen from a virgin queen introduced more than 48 hours after removal of the resident queen. Whilst 7 of the virgin queens survived in recently queenless colonies and 2 survived in queenless colonies, none of them survived in queenright colonies (Figure 1) which excludes any meaningful further statistics equivalent to section (a). A Chi-square test demonstrated that queens were more likely to be accepted in queenless than in queenright colonies ($p = 0.03$, 9 : 123 versus 0 : 88 (accepted : rejected), $n = 220$).

Presumably due to the low number of queens that were accepted ($n = 9$) the same behaviours mentioned above (section a) had no significant effect on the survival of a young queen or at least there is a higher level of statistical uncertainty: Antennation or trophallaxis (p

= 0.78), being attacked ($p = 0.07$), hiding ($p = 0.07$), running ($p = 0.30$) and walking or resting ($p = 0.45$) were all below the 95% significance level ($n = 132$). However, comparing the mean time a queen spent for the various behaviours during her first hour or until she was killed in a queenless colony reveals that queens that were accepted differ significantly from queens that were killed (Chi-square test, $p = 6.31 \cdot 10^{-7}$). This is mainly due to the larger amount of time, *ca.* 66%, accepted queens were hiding and the smaller amount of time, *ca.* 3%, they were attacked (Figure 4). Generally, however, the behaviour of queens did not significantly differ in queen-right and queenless colonies (Chi-square test, $p = 0.08$, Figure 4).

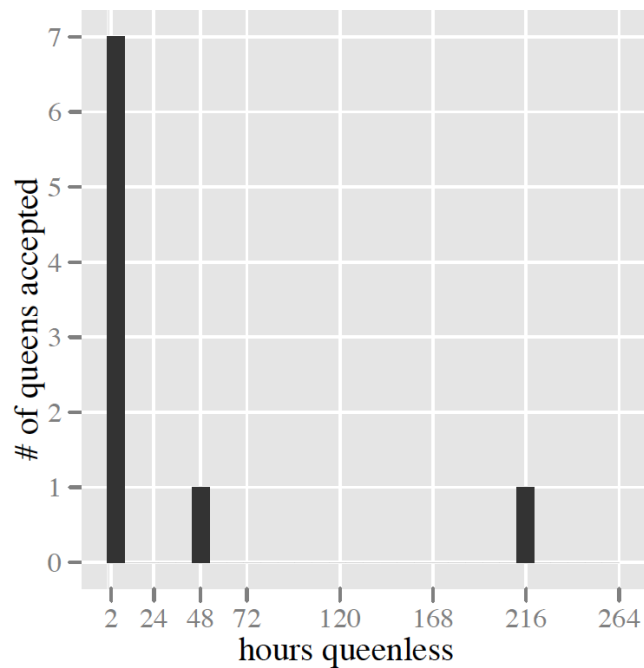


Figure 3: Number of queens accepted that were introduced at various times (hours) after removing the resident queen. 88 queens were introduced into colonies that were 0 hours queenless, i.e. into queenright colonies, 24 queens each into colonies that were 2, 24 and 48 hours queenless, and 12 queens each into colonies that were 72, 120, 168, 216 and 264 hours queenless. Note that the queen accepted after 216 hours was a “replacement queen” after the originally chosen queen disappeared.

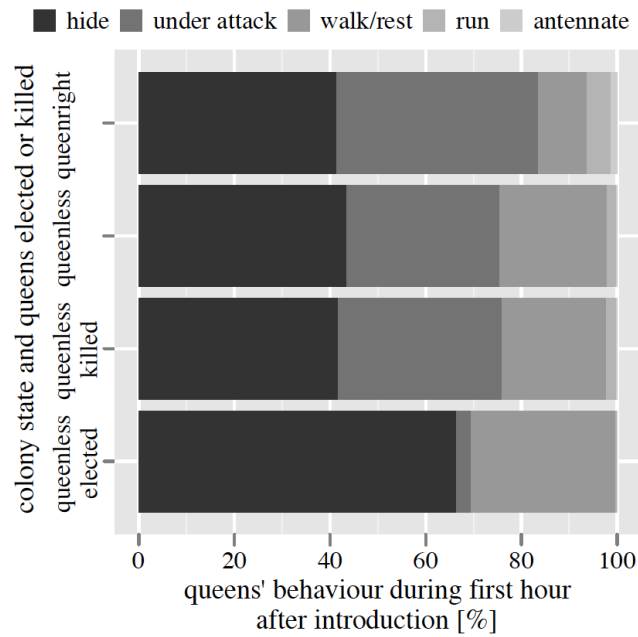


Figure 4: The proportion of time virgin queens performed various behaviours during the first hour after introduction. The top two bars compare the proportions shown in queenright and queenless colonies which were not significantly different (Chi-square test, $p = 0.08$). The bottom two columns compare the behaviour of killed versus chosen queens in queenless colonies which were significantly different (Chi-square test, $p = 6.31 \times 10^{-7}$). Unlike killed queens, elected queens spent much more time hiding, were much less time under attack, spent somewhat more time walking or resting but spent little or no time running or antennating.

8.4.2 Descriptive data

Of 1,121 newly-emerged individuals 867 were workers (77.34 %), 153 were drones (13.65 %) and 101 (9.01 %) were queens. Of the 968 females 10.43 % were queens and 89.57 % were workers. Two workers (0.23 %), 2 males (1.31 %) and 18 queens (17.82 %) died during incubation. These were not used to determine their body mass. Workers weighed on average 80.35 ± 6.46 mg ($n = 865$), drones 73.67 ± 6.98 mg ($n = 151$), and queens 61.23 ± 8.55 mg ($n = 83$) (each mean \pm SD). Drones were on average 8.31 % lighter than workers, queens on average 23.79 % lighter.

Of the 130 dead virgin queens that we collected, 15 had been beheaded, 35 had neck injuries, 3 had their first pair of legs missing, 2 had their abdomen visibly stretched, and in 78

no injuries were visible. 49 had 1 injury and 3 had 2 injuries (2 were beheaded and had their first pair of legs missing, 1 had its neck injured and the first pair of legs missing). Dead virgin queens were found up to 6 m away from the hive entrance.

8.4.3 Queen choice

On the second round of queen introductions into first queenright and then queenless colonies we also recorded the behaviour of the colony and the new colony queen. That is, the queen that had been chosen by the workers. It appeared that the behaviour of the colony and the new queen fell into 4 phases: Phase 1 was an unstable situation where a large number of virgin queens were being killed despite the colony not having a laying queen. It was characterised by occasional antennating and trophallaxis of queens with workers, walking and resting, running, hiding, and being under attack. Phase 2 was characterised by a behaviour, here quantitatively studied for the first time, which appears strongly to represent the choice of a new queen. Previously, only case studies on single queens have been conducted and it was not clear if what was being described was a typical event or an unusual event (Koedam et al., 1995a; Jarau et al., 2010). For about two hours the worker bees were excitable and agitated, accumulating around the queen that they selected. The workers were unevenly distributed within the hive. Their movements were rapid and some of them rubbed the tip of their abdomen on the nest surfaces including the glass cover of the hive where we could most easily observe this additional behaviour. The queen, who at this stage appeared to have an inflated abdomen, performed a dance similar to the honey bee “round dance” (von Frisch, 1965). She repeatedly turned left and right, shaking her abdomen, stroking it over bees standing next to her and occasionally stroking with her third pair of legs over her abdomen. Due to the likely use of pheromones by workers and the queen (Figure 5b) and this highly defined behaviour we termed this phase “queen choice”. In phase 3 the queen had been accepted and her movements and those of the workers were no longer rapid. In contrast to phase 2 there was hardly any physi-

cal contact between the workers and the queen apart from antennation and trophallaxis between one worker and the queen (Figure 5b). This was followed by a period (phase 4) where the queen was often hiding and if present only slowly walking on the combs.



Figure 5: a) Queen execution. Several workers in the process of tugging at a virgin queen's legs for several minutes until dead. Additional workers, such as the one approaching the queen from the front, often try to cut off the queen's head. Only in rare cases can a queen escape being stretched out, and if she does she will usually be killed in the next attack. b) Queen election. One of the few lucky virgin queens that were chosen by a queenless colony as the new resident queen. Note the queen being densely surrounded by workers facing towards her but, unlike the case in the honey bee *Apis mellifera*, generally not contacting her with their anten-

nae, apart from workers engaged in trophallaxis with her or head-to-head antennation. Also note that, unlike the virgin queen in Figure 5a, the chosen queen has inflated her abdomen, possibly to release a volatile pheromone.

Of the 4 chosen queens that we observed in detail, 3 were from queens introduced 2 hours after removal of the resident queen and one from queens introduced after 48 hours (Figure 6). It also shows that the unstable situation, phase 1, varies considerably in duration (4 minutes to 45 hours) and was much shorter for a queen that emerged later, introduced after 48 hours. This reduced the variation of the onset of queen proclamation which took place approximately 24 to 48 hours after the resident queen had been removed. Interestingly, phase 3, ranging from *ca.* 22 to *ca.* 46 hours, led to zero variation in the start of phase 4 at *ca.* 72 hours.

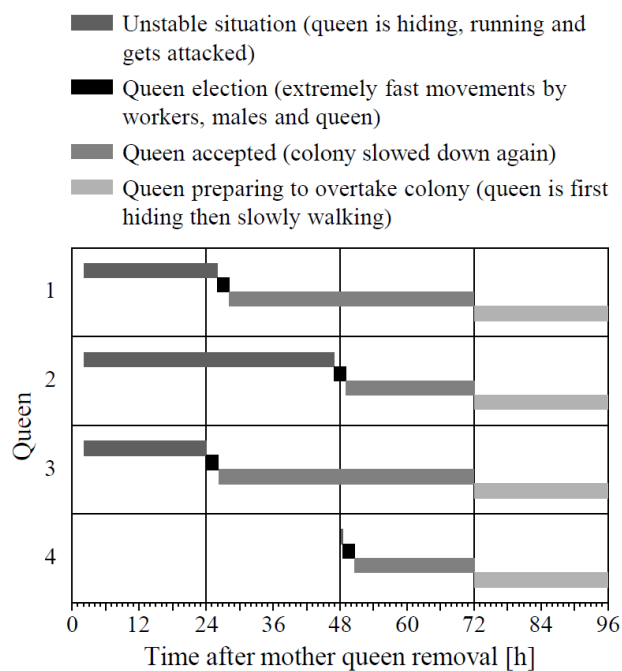


Figure 6: A detailed illustration of the behaviour of virgin queens and the later behaviour of their colonies during the first 4 days after removing the resident queen. Note that colony 4 kept on killing all virgin queens for two days of queenlessness until it finally accepted a queen introduced at 48 hours. Also note how this colony quickly caught up after two days of queenlessness.

8.5 Discussion

Our data clearly demonstrate that accepted queens generally emerged shortly, in our case 2 hours, after the removal of the resident queen, that hiding and avoiding worker attacks was crucial to survive, so that these queens could be chosen as the new resident queens 24 to 48 hours after removing the previous resident queen. This study is also the first to compare queen execution in both queenright and queenless *Melipona* colonies, apart from describing the choice of new resident queens.

Queens were never accepted in queenright but only in queenless colonies, and this difference was significant. However, the time virgin queens survived did not differ in these two states of a colony (Figure 1). Only if they had emerged within a very short period of time after queen removal, in our case after 2 hours, were queens more likely to live longer (Figure 1). Queens of this group were also more likely to get accepted later on (Figure 3).

Behaviours, such as antennating, trophallaxis and running, were not significantly correlated with a virgin queen's life time nor did they significantly affect their chances of survival. Walking or resting did not affect the chance of survival; however, it was slightly positively correlated with the time a virgin queen survived. Hiding had a clear positive ($p = 0$, Figure 2) and a borderline ($p = 0.07$) effect on life time and the chances to survive, respectively. Being attacked had a clear negative ($p = 0$, Figure 2) and a borderline ($p = 0.07$) effect on life time and the chances to survive, respectively. Thus, contrary to Jarau et al. (2009) who showed that alive queens received more attacks than dead queens, these data suggest that evasive behaviour increases the time a queen survives and her chances of being chosen.

Figure 4 gives a similar impression: Queens that were chosen spent far more time hiding during their first hour in the colony than queens that were eventually killed. Queens that were finally selected were, on average, under attack by workers for a shorter period of time, presumably as a consequence of the large amount of time they spent hiding. Contrary to Jarau et al. (2009), who concluded that a colony's workers select the fittest queen by fighting with

them, we propose that *Melipona* colonies select for the most evasive queen: Evasive queens are good in hiding, avoid attacks by workers and stay alive for longer (Figure 2) which means that they have a higher chance of being available when a queen is actually elected, *ca.* 24 to 48 hours after queen removal (Figure 6). Suppose queens are elected once a year within one day (Figure 6), then a queen that lives 24 hours would have 24 times the chance to be in this time window than a queen that lives only 1 hour. Our hypothesis also intuitively makes sense since a) a queen that had to fight with workers before it gets chosen as a new resident queen might be seriously damaged (see descriptive results of this study) and thus have a reduced fitness and b) if workers truly select a queen by fighting with them one would expect queens to be chosen that emerged over a much longer time span. In our case 7 selected queens hatched after 2 hours and only one after 48 hours. The queen that hatched after 216 hours was a replacement queen for an already accepted queen that hatched after 2 hours and later on disappeared (Figure 3).

The workers did not differentiate between nestmate and non-nestmate virgin queens, implying that once an intruding virgin queen managed to pass the guards it has the same chances of inheriting a colony as her native competitors. Neither their chances of being accepted in queenless colonies nor their time surviving in queenless or queenright colonies was different for nestmates or non-nestmates. It is therefore puzzling that, stingless bees, where colonies are occasionally taken over by queens from other conspecific colonies (Wenseleers et al., 2011), have not evolved the ability to recognise non-nestmate virgin queens (this study) and not even mated, older queens (C Menezes, personal communication) within a colony, excluding the blank slate hypothesis (Breed et al., 2004). But honey bees, lacking intraspecific parasitism, would always kill introduced non-nestmate queens even if the colony is queenless and the colony only has a chance to rear a new, but own, queen, i.e. when eggs or young larvae are still available. This is especially surprising since stingless bees are generally good in

nestmate recognition (Kärcher and Ratnieks, 2009) and it would be highly interesting to find out how virgin *Melipona* queens can pass entrance guards of foreign colonies.

Despite receiving the same amount and quality of food as workers, queens have a considerably lighter live body mass, *ca.* 24% (*M. quadrifasciata*, this study) in agreement with previous data from *M. beecheii*, 16% (Wenseleers et al., 2004) and have also a smaller body size (Figure 1 a, where the abdomen is not inflated). In *M. beecheii*, however, queen and worker dry weight were not significantly different. This might be due to a higher loss of water during the development of the queens (Wenseleers et al., 2004) which is also 1-2 days faster than in workers (Velthuis and Sommeijer, 1991). This higher loss of water might indicate that developing a queen specific morphology and physiology is more costly than a worker specific, reflected by their reduced live body mass. It is therefore intriguing but not significant that queens with lighter live body masses were more likely to be chosen ($p = 0.08$, $n = 131$). Given that workers and queens can self-determine their caste (Bourke and Ratnieks, 1999) one obvious possible explanation might be that lighter queens have either more fully developed into queens or made their decision to become a queen earlier leading to better developed queens which are also more attractive for workers.

We never observed fights between the resident queen and a virgin queen or between two virgin queens ($n = 220$ virgin queens). One explanation suggested by Peters et al. (1999) is that through single mating in *Melipona* virgin queens are closer related to each other (0.75) than honey bee queens (*ca.* 0.25) which significantly reduces the potential for conflict and might lead to a lack of queen fights (Peters et al., 1999). However, we are not fully convinced by this explanation since this does not seem to be compatible with the large proportion of females attempting to become a queen but generally resulting in being killed. Whilst it is clear that single mating further reduces conflict we expected fewer but not no queen fights at all. This is because conflict cannot be entirely removed by single mating (Ratnieks et al., 2006). A second explanation, suggested by the same authors, Peters et al. (1999), seems to be more

plausible: Unlike honey bees, stingless bees cannot sting each other to death but would have to fight for a long time presumably also seriously damaging the “winner”. Not only do stingless bee queens lack a sting, which is even more lethal in honey bee queens than in honey bee workers, their mandibles are rather weak too and it is unclear if one virgin queen could kill another individual in this way at all. Consequently, virgin queens are no threat to a resident queen and instead continuously executed by workers. Although virgin queens are executed in queenless colonies in the same way as in queenright colonies, in queenless colonies one single queen is chosen and no spare queens, as reported in several trigonine species (Imperatriz-Fonseca and Zucchi, 1995), are nourished, neither are queens maintained to allow queenfights, and neither were queens maintained in order to actively choose one. Apparently, colonies primarily aim to have a new egg laying queen as soon as possible and no spare queens are needed since more queens are still hatching for a longer period after the removal of the resident queen. By chance we could report such an incident where a replacement queen, emerged after 216 hours, was chosen (Figure 4).

This study also presents a detailed description of the behaviour of 4 colonies choosing 4 virgin queens in *Melipona quadrifasciata*. Contrary to Jarau et al. (2010) who found that the one *Melipona beecheii* queen that was observed showed aggressive behaviour towards workers for three days, we never observed such behaviour. However, our findings are in line with Koedam (1995) who described the acceptance of one *Melipona favosa* queen. This queen, however, was accepted somewhat earlier, i.e. after 15 hours, than the 4 queens we observed that were accepted between 24 and 48 hours. Three of the 4 accepted queens, accepted at 24 to 48 hours of queenlessness, were introduced after 2 hours whilst the 4th queen, introduced after 48 hours, was accepted a few minutes after she was introduced, i.e. at ca. 48 hours (Figure 6). This implies that generally queens need to be available for about one day before being possible candidates that can be selected.

To conclude, the best strategy of a virgin queen in *M. quadrifasciata* seems to be to emerge from the cell about 2 hours after the resident queen has gone, then to hide as well as possible avoiding any fights in order to live as long as possible and be available when the colony chooses a new queen, at the same time possibly establishing a queen pheromone, so that she can finally be chosen 24 to 48 hours after the resident queen was removed. From the bees' perspective, however, this report of a massacre would probably end with: *Ave observator! Morituri te salutant!*

Chapter 9 - Conclusion

In this final section of my thesis I would like to highlight the key questions that led to the projects in this thesis, as well as the key results of these projects, how they have added to or changed our understanding and where this might lead future research.

Chapter 4: To begin with, it was puzzling when we read that *T. angustula* should only possess hovering guards that were unable to detect conspecific intruders (Bowden et al., 1994). An unusual possible explanation in their discussion was that they might be detected inside foreign colonies (Bowden et al., 1994). A second study reported that *T. angustula* used citral as a kairomone when attacked by the robber species *L. limao* (Wittmann et al., 1990). This effect, however, was not compared with a reaction to odours from other allospecific stingless bee species. We thus felt there was enough material that needed further investigation. Our study consequently demonstrated that *T. angustula* possesses two distinct types of guards, “hovering guards” and “standing guards”. Behavioural specialisation in bee guarding has never been found before. Apart from this finding we also proved the finding of citral acting as a kairomone for *T. angustula* (Wittmann et al., 1990). Combining Bowden et al.’s and our study we now know that hovering guards are able to use visual and volatile chemical cues whilst standing guards are able to use non-volatile and volatile chemical cues. Hovering guards are unable to use non-volatile chemical cues and therefore cannot detect conspecifics from other colonies. However, we still do not know if standing guards also use visual cues to detect intruders. Given that this is a common criticism of some journal referees and that *T. angustula* has excellent standing guards, one could offer them black or yellow dummy bees covered with hydrocarbons from the guards’ colony. Given that the body colour of this species is yellow, it would be interesting to determine whether the guards attack or ignore these dummy bees, and whether their colour is a factor. Another question arising from this study, a good example of Karl von

Frisch's magic well, was if guarding in *T. angustula* is only task specialisation or even division of labour (Ratnieks and Anderson, 1999) which we investigated in chapter 6.

Chapter 5: One of the nestmate recognition studies where I took part during my PhD was included in this thesis. Previous research has shown that honey bee guards rejected some of their nestmates, accepted some conspecific non-nestmates, but never accepted worker wasps (Couvillon et al., 2009). This extremely different response from the guards required further investigation. In chapter 5 we demonstrated that harmful intruders such as wasps were not detected by their "harmful odour" but simply because they smelled very different from nestmates. An interesting side result of this study was that woodlice were treated like non-nestmate bees, revealed by measuring acceptance or rejection as a graded response. Since woodlice usually live in moist environments where dehydration is not a threat they have fewer hydrocarbons on their cuticle (Warburg, 1993). This is further evidence that hydrocarbons, which might be sufficient for nestmate recognition, are certainly used in nestmate recognition. This further supports the U-absent mechanism but, importantly, does not exclude a possible co-existing D-present mechanism³. An alternative explanation for the low level of aggression directed towards woodlice might be their shield shaped carapace which bees cannot grasp easily with their mandibles. Thus, using dummies covered with conspecific non-nestmate odour but of different shapes and sizes might reveal whether these factors might have played a role in woodlice acceptance. However, it would also clarify if odours were the only cue used by honey bee guards, perfectly fitting to a possible future study suggested above.

Chapter 6: Despite *T. angustula* possessing a range of fascinating features at their entrances, such as the opening and closing of the wax made entrance tubes in the morning and in the

³ Using the U-absent mechanism, guards reject intruders with undesirable, i.e. different, odours. Using the D-present mechanism, guards accept intruders with desirable, i.e. similar, odours.

evening, we have not found any natural history studies describing the behaviour around their nest entrances. Together with the fundamental question arising from chapter 4 we thought this needed a follow-up study in order to gain a deeper understanding. Results indicate a relatively well separated division of labour where two thirds of the guards always acted as standing guards and one third of the guards performed both, hovering and sometimes standing. Results also indicate that contrary to honey bees where guarding is a task performed by a small group of bees for only *ca.* 1 to 3 days of their life *T. angustula* guards were guarding for *ca.* 20 days, possibly spending most of their life time guarding. In the course of the field work of this study, we observed that standing and hovering guards differed in size. This finding is about to be submitted as a follow-up publication. Additionally, this study has generated a series of other arising questions (C Grüter, personal communication). It therefore seems that chapter 4 has generated a small scale avalanche, if this is an appropriate comparison for social insect science. It also proves one generalisation by my first supervisor: Common species are often the most interesting species.

Chapter 7: Even though worker policing in insect societies has been studied extensively in the past couple of decades research has exclusively focused on the benefit of policing neglecting its cost. The cost, however, is the equally important second half of Hamilton's rule, $r \times b > c$, where r is the relatedness, b the benefit and c the cost, which has to be smaller than the product of r and b to cause the evolution of an altruistic behaviour (Hamilton, 1964a, b; Bourke, 2011). In this thesis we quantified this cost in the honey bee and concluded that this cost is low since a) only a small number of queen laid eggs is killed and b) the majority of these eggs is replaced which is also done quickly. A comparative study looking at the cost of policing in various species of wasps and honey bees might reveal that these three components, r , b and c , depend on each other. In other words, in species where r or b or both of them are high we would also expect c to be high.

Chapter 8: Our final study investigated the factors influencing the acceptance of virgin *Melipona* stingless bee queens or the time they survived before they were slaughtered, respectively. Queen execution or queen choice has been described previously in several species of *Melipona*. However, none of these studies provided a statistically based attempt to explain what makes virgin queens accepted or at least live longer. In summary, our data suggest that in order to get chosen a queen has a) to hatch shortly after the resident queen was removed, unless a virgin queen is needed for a swarm, b) to hide as much as possible and avoid any fights with workers, possibly producing a queen pheromone in the meanwhile making her attractive, so that c) she is still alive and attractive at the time of queen election, 24 to 48 hours after the resident queen was removed. During my PhD I got the impression that, especially in the area of caste fate, queen acceptance and queen execution in stingless bees, there seems to exist a plethora of non-compatible opinions. Thus, using exactly the same methods as ours, including the statistical method, it would be highly interesting if different observers receive similar results in other species of *Melipona*. Furthermore, it would be interesting to investigate more deeply why there are differences between species, looking at differences in the general natural history of various *Melipona* species.

In essence, this thesis adds two findings regarding the mechanism of nestmate recognition: a) Harmful intruders are simply recognised by their different odour, not by their specific odour, and b) Intruders possessing no or fewer hydrocarbons, such as woodlice, are less likely to be detected as non-nestmates, suggesting that the U-absent mechanism is possibly more relevant than the D-present mechanism (Reeve, 1989; Sherman et al., 1997). This thesis also reported the first example of behaviourally specialised entrance guards, i.e. by hovering and standing guards in *T. angustula* and how they work together.

Using honey bees, here we also present the first study measuring the cost of worker policing in eusocial hymenoptera, representing the second half of Hamilton's ground breaking

formula, $r \times b > c$. Finally, we think this study managed to determine how queen execution and queen choice is affected by various factors and what is key for a young virgin queen to be chosen as the new resident queen.

References

- Akino T, Yamamura K, Wakamura S, Yamaoka R.** 2004. Direct behavioral evidence for hydrocarbons as nestmate recognition cues in *Formica japonica* (Hymenoptera: Formicidae). *Applied Entomology and Zoology* 39: 381-387. doi: 10.1303/aez.2004.381.
- Bates D, Maechler M.** 2010. lme4: Linear mixed-effects models using S4 classes. *R package version 0.999375-33*.
- Beekman M, Oldroyd BP.** 2005. Honeybee workers use cues other than egg viability for policing. *Biology Letters* 1: 129-132. doi: 10.1098/rsbl.2005.0294.
- Beye M, Hasselmann M, Fondrk MK, Page RE, Omholt SW.** 2003. The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. *Cell* 114: 419-429.
- Blomquist GJ, Bagnères A-G,** editors. 2010. Insect hydrocarbons. *Cambridge University Press*, New York, NY.
- Blum MS.** 1966. Chemical releasers of social behavior. VIII. Citral in the mandibular gland secretion of *Lestrimelitta limao* (Hymenoptera: Apoidea: Melittidae). *Annals of the Entomological Society of America* 59: 962-964.
- Blum MS, Crewe RM, Kerr WE, Keith LH, Garrison AW, Walker MM.** 1970. Citral in stingless bees: Isolation and functions in trail-laying and robbing. *Journal of Insect Physiology* 16: 1637-1648.
- Bolker BM, Brooks ME, Clark CJ, Geange SW, Poulsen JR, Stevens MHH, White J-SS.** 2009. Generalized linear mixed models: a practical guide for ecology and evolution. *Trends in Ecology & Evolution* 24: 127-135. doi: 10.1016/j.tree.2008.10.008.
- Bonckaert W, Tofilski A, Nascimento F, Billen J, Ratnieks F, Wenseleers T.** 2010. Co-occurrence of three types of egg policing in the Norwegian wasp *Dolichovespula norvegica*. *Behavioral Ecology and Sociobiology*: 1-8. doi: 10.1007/s00265-010-1064-3.
- Bonckaert W, Vuerinckx K, Billen J, Hammond RL, Keller L, Wenseleers T.** 2008. Worker policing in the German wasp *Vespula germanica*. *Behavioral Ecology* 19: 272-278. doi: 10.1093/beheco/arm128.
- Boomsma JJ, Franks NR.** 2006. Social insects: from selfish genes to self organisation and beyond. *Trends in Ecology & Evolution* 21: 303-308. doi: 10.1016/j.tree.2006.04.001.
- Bourke AFG.** 1988. Worker reproduction in the higher eusocial Hymenoptera. *Quarterly Review of Biology* 63: 291-311.
- Bourke AFG.** 2011. Principles of social evolution. *Oxford University Press*, Oxford, NY.
- Bourke AFG, Franks NR.** 1995. Social evolution in ants. *Princeton University Press*, Princeton, NJ.

- Bourke AFG, Ratnieks FLW.** 1999. Kin conflict over caste determination in social Hymenoptera. *Behavioral Ecology and Sociobiology* 46: 287-297.
- Bowden RM, Garry MF, Breed MD.** 1994. Discrimination of con- and heterospecific bees by *Trigona (Tetragonisca) angustula* guards. *Journal of the Kansas Entomological Society* 67: 137-139.
- Breed MD.** 1998. Recognition pheromones of the honey bee: the chemistry of nestmate recognition. *Bioscience* 48: 463-470.
- Breed MD, Garry MF, Pearce AN, Hibbard BE, Bjostad LB, Page RE.** 1995. The role of wax comb in honey bee nestmate recognition. *Animal Behaviour* 50: 489-496.
- Breed MD, Page RE.** 1991. Intra- and interspecific nestmate recognition in *Melipona* workers (Hymenoptera: Apidae). *Journal of Insect Behavior* 4: 463-469.
- Breed MD, Perry S, Bjostad LB.** 2004. Testing the blank slate hypothesis: why honey bee colonies accept young bees. *Insectes Sociaux* 51: 12-16. doi: 10.1007/s00040-003-0698-9.
- Butler CG, Free JB.** 1952. The behaviour of worker honeybees at the hive entrance. *Behaviour* 4: 262-292.
- Châline N, Martin SJ, Ratnieks FLW.** 2004. Worker policing persists in a hopelessly queenless honey bee colony (*Apis mellifera*). *Insectes Sociaux* 51: 113-116. doi: 10.1007/s00040-003-0708-y.
- Conner J, Camazine S, Aneshansley D, Eisner T.** 1985. Mammalian breath: trigger of defensive chemical response in a tenebrionid beetle (*Bolitotherus cornutus*). *Behavioral Ecology and Sociobiology* 16: 115-118.
- Couvillon MJ, Caple JP, Endors SL, Kärcher MH, Russell TE, Storey DE, Ratnieks FLW.** 2007. Nest-mate recognition template of guard honeybees (*Apis mellifera*) is modified by wax comb transfer. *Biology Letters* 3: 228-230. doi: 10.1098/rsbl.2006.0612.
- Couvillon MJ, Ratnieks FLW.** 2008. Odour transfer in stingless bee marmelada (*Frieseomelitta varia*) demonstrates that entrance guards use an "undesirable-absent" recognition system. *Behavioral Ecology and Sociobiology* 62: 1099-1105. doi: 10.1007/s00265-007-0537-5.
- Couvillon MJ, Robinson EJH, Atkinson B, Child L, Dent KR, Ratnieks FLW.** 2008a. En garde: rapid shifts in honeybee, *Apis mellifera*, guarding behaviour are triggered by onslaught of conspecific intruders. *Animal Behaviour* 76: 1653-1658. doi: 10.1016/j.anbehav.2008.08.002.
- Couvillon MJ, Roy GGF, Ratnieks FLW.** 2009. Recognition errors by honey bee (*Apis mellifera*) guards demonstrate overlapping cues in conspecific recognition. *Journal of Apicultural Research* 48: 225-232. doi: 10.3896/ibra.1.48.4.01.
- Couvillon MJ, Wenseleers T, Imperatriz-Fonseca VL, Nogueira-Neto P, Ratnieks FLW.** 2008b. Comparative study in stingless bees (Meliponini) demonstrates that nest entrance size

predicts traffic and defensivity. *Journal of Evolutionary Biology* 21: 194-201. doi: 10.1111/j.1420-9101.2007.01457.x.

Crawley MJ. 2002. Statistical computing: an introduction to data analysis using S-Plus. *John Wiley & Sons Ltda*, Chichester, UK.

Crozier RH, Pamilo P. 1996. Evolution of social insect colonies. Sex allocation and kin-selection. *Oxford University Press*, Oxford, UK.

D'Ettorre P, Wenseleers T, Dawson J, Hutchinson S, Boswell T, Ratnieks FLW. 2006. Wax combs mediate nestmate recognition by guard honeybees. *Animal Behaviour* 71: 773-779. doi: 10.1016/j.anbehav.2005.05.014.

da Silva DLN, Zucchi R, Kerr WE. 1972. Biological and behavioural aspects of reproduction in some species of *Melipona* (Hymenoptera, Apidae, Meliponinae). *Animal Behaviour* 20: 123-&.

Dani FR, Jones GR, Corsi S, Beard R, Pradella D, Turillazzi S. 2005. Nestmate recognition cues in the honey bee: Differential importance of cuticular alkanes and alkenes. *Chemical Senses* 30: 477-489. doi: 10.1093/chemse/bji040.

Darchen R, Delage-Darchen B. 1975. Contribution a l'étude d'une abeille du Mexique *Melipona beecheii* B. (Hymenoptère: Apide). Le déterminisme des castes chez les Mélipones. Beitrag zum Studium einer mexikanischen Biene *Melipona beecheii* B. (Hymenoptera: Apidae). Der Kastendeterminismus bei den Meliponen. *Apidologie* 6: 295-339.

De Jong D. 1990. Insects: Hymenoptera (ants, wasps, and bees). In: Morse RA, Nowogrodzki R, editors. Honey bee pests, predators, and diseases. *Cornell University Press*, Ithaca, NY. pp 135-155.

DeGrandi-Hoffman G, Collins A, Martin JH, Schmidt JO, Spangler HG. 1998. Nest defense behavior in colonies from crosses between africanized and European honey bees (*Apis mellifera* L.) (Hymenoptera: Apidae). *Journal of Insect Behavior* 11: 37-45.

Downs SG, Ratnieks FLW. 1999. Recognition of conspecifics by honeybee guards uses nonheritable cues acquired in the adult stage. *Animal Behaviour* 58: 643-648.

Downs SG, Ratnieks FLW. 2000. Adaptive shifts in honey bee (*Apis mellifera* L.) guarding behavior support predictions of the acceptance threshold model. *Behavioral Ecology* 11: 326-333.

Downs SG, Ratnieks FLW, Badcock NS, Mynott A. 2001. Honeybee guards do not use food-derived odors to recognize non-nest mates: a test of the Odor Convergence hypothesis. *Behavioral Ecology* 12: 47-50.

Downs SG, Ratnieks FLW, Jefferies SL, Rigby HE. 2000. The role of floral oils in the nestmate recognition system of honey bees (*Apis mellifera* L.). *Apidologie* 31: 357-365.

Evans JD, Wheeler DE. 1999. Differential gene expression between developing queens and workers in the honey bee, *Apis mellifera*. *Proceedings of the National Academy of Sciences of the United States of America* 96: 5575-5580.

- Foster KR, Gulliver J, Ratnieks FLW.** 2002. Worker policing in the European hornet *Vespa crabro*. *Insectes Sociaux* 49: 41-44.
- Foster KR, Ratnieks FLW.** 2001a. Convergent evolution of worker policing by egg eating in the honeybee and common wasp. *Proceedings of the Royal Society of London Series B-Biological Sciences* 268: 169-174.
- Foster KR, Ratnieks FLW.** 2001b. The effect of sex-allocation biasing on the evolution of worker policing in hymenopteran societies. *American Naturalist* 158: 615-623.
- Foster KR, Ratnieks FLW.** 2001c. Paternity, reproduction and conflict in vespine wasps: a model system for testing kin selection predictions. *Behavioral Ecology and Sociobiology* 50: 1-8.
- Foster KR, Wenseleers T, Ratnieks FLW.** 2006. Kin selection is the key to altruism. *Trends in Ecology & Evolution* 21: 57-60. doi: 10.1016/j.tree.2005.11.020.
- Free JB.** 1977. The social organization of the honey bees. *Edward Arnold*, London, UK.
- Fröhlich B, Riederer M, Tautz J.** 2001. Honeybees discriminate cuticular waxes based on esters and polar components. *Apidologie* 32: 265-274.
- Getz WM.** 1981. Genetically based kin recognition systems. *Journal of Theoretical Biology* 92: 209-226.
- Getz WM.** 1991. The honey bee as a model kin recognition system. In: Hepper PG, editor. Kin Recognition. *Cambridge University Press*, Cambridge, UK. pp 358-412.
- Grosso AF, Bego LR.** 2002. Labor division, average life span, survival curve, and nest architecture of *Tetragonisca angustula angustula* (Hymenoptera, Apinae, Meliponini). *Sociobiology* 40: 615-637.
- Halling LA, Oldroyd BP, Wattanachaiyingcharoen W, Barron AB, Nanork P, Wongsiri S.** 2001. Worker policing in the bee *Apis florea*. *Behavioral Ecology and Sociobiology* 49: 509-513.
- Hamilton WD.** 1964a. The genetical evolution of social behaviour. I. *Journal of Theoretical Biology* 7: 1-16.
- Hamilton WD.** 1964b. The genetical evolution of social behaviour. II. *Journal of Theoretical Biology* 7: 17-52.
- Harbo JR, Bolten AB.** 1981. Development times of male and female eggs of the honey bee. *Annals of the Entomological Society of America* 74: 504-506.
- Hikawa M, Miyanaga R.** 2009. Effects of pollination by *Melipona quadrifasciata* (Hymenoptera: Apidae) on tomatoes in protected culture. *Applied Entomology and Zoology* 44: 301-307. doi: 10.1303/aez.2009.301.
- Hölldobler B, Wilson EO.** 2009. The superorganism: The beauty, elegance, and strangeness of insect societies. *W. W. Norton & Company*, New York, NY.

- Hunt GJ, Guzman-Novoa E, Uribe-Rubio JL, Prieto-Merlos D.** 2003. Genotype-environment interactions in honeybee guarding behaviour. *Animal Behaviour* 66: 459-467. doi: 10.1006/anbe.2003.2253.
- Imperatriz-Fonseca VL, Zucchi R.** 1995. Virgin queens in stingless bee (Apidae, Meliponinae) colonies: a review. *Apidologie* 26: 231-244.
- Jarau S, Van Veen JW, Aguilar I, Ayasse M.** 2009. Virgin queen execution in the stingless bee *Melipona beecheii*: The sign stimulus for worker attacks. *Apidologie* 40: 496-507. doi: 10.1051/apido/2009022.
- Jarau S, van Veen JW, Aguilar I, Ayasse M.** 2010. A scientific note on virgin queen acceptance in stingless bees: evidence for the importance of queen aggression. *Apidologie* 41: 38-39. doi: 10.1051/apido/2009045.
- Jeanne RL.** 1986. The organization of work in *Polybia occidentalis*: costs and benefits of specialization in a social wasp. *Behavioral Ecology and Sociobiology* 19: 333-341.
- Kajobe R, Roubik DW.** 2006. Honey-making bee colony abundance and predation by apes and humans in a Uganda forest reserve. *Biotropica* 38: 210-218. doi: 10.1111/j.1744-7429.2006.00126.x.
- Kärcher MH, Ratnieks FLW.** 2009. Standing and hovering guards of the stingless bee *Tetragonisca angustula* complement each other in entrance guarding and intruder recognition. *Journal of Apicultural Research* 48: 209-214. doi: 10.3896/ibra.1.48.3.10.
- Kelber A, Zeil J.** 1997. *Tetragonisca* guard bees interpret expanding and contracting patterns as unintended displacement in space. *Journal of Comparative Physiology A - Neuroethology, Sensory, Neural, and Behavioral Physiology* 181: 257-265.
- Kerr WE.** 1950. Genetic determination of castes in the genus *Melipona*. *Genetics* 35: 143-152.
- Koedam D.** 1995. Behavioural and physiological implications of queen dominance in stingless bees [Ph.D. thesis]: *Universiteit Utrecht*, pp.
- Koedam D.** 1999. Production of queens, workers and males in the stingless bee *Melipona favosa* (Apidae: Meliponinae): Patterns in time and space. *Netherlands Journal of Zoology* 49: 289-302.
- Koedam D, Aarts T, Sommeijer MJ.** 1995a. Queen acceptance in *Melipona favosa* (Apidae: Meliponinae); morphological changes and behavioural development of virgin queens. Behavioural and physiological implications of queen dominance in stingless bees, PhD thesis, Koedam, D. *Universiteit Utrecht*, pp 83-100.
- Koedam D, Contrera FAL, Imperatriz-Fonseca VL.** 1999. Clustered male production by workers in the stingless bee *Melipona subnitida* Ducke (Apidae, Meliponinae). *Insectes Sociaux* 46: 387-391.
- Koedam D, Monge IA, Sommeijer MJ.** 1995b. Social interactions of gynes and their longevity in queenright colonies of *Melipona favosa* (Apidae: Meliponinae). *Netherlands Journal of Zoology* 45: 480-494.

- Lockey KH.** 1988. Lipids of the insect cuticle: origin, composition and function. *Comparative Biochemistry and Physiology Part B: Biochemistry & Molecular Biology* 89: 595-645.
- Lubbock J.** 1882. Ants, bees, and wasps: a record of observations on the habits of the social Hymenoptera. *Kegan Paul, Trench, Trubner & Co.*, London, UK.
- Michener CD.** 1974. The social behavior of the bees. *Harvard University Press*, Cambridge, MA.
- Michener CD.** 2007. The bees of the world. *The John Hopkins University Press*, Baltimore, MD.
- Miller DG, Ratnieks FLW.** 2001. The timing of worker reproduction and breakdown of policing behaviour in queenless honey bee (*Apis mellifera* L.) societies. *Insectes Sociaux* 48: 178-184.
- Moore AJ, Breed MD, Moor MJ.** 1987. The guard honey bee: ontogeny and behavioural variability of workers performing a specialized task. *Animal Behaviour* 35: 1159-1167.
- Moritz RFA, Kirchner WH, Crewe RM.** 1991. Chemical camouflage of the death's head hawkmoth (*Acherontia atropos* L.) in honeybee colonies. *Naturwissenschaften* 78: 179-182.
- Morse RA, Nowogrodzki R.** 1990. Honey bee pests, predators, and diseases. *Cornell University Press*, Ithaca, NY.
- Nowbahari E, Lenoir A, Clement JL, Lange C, Bagnères A-G, Joulie C.** 1990. Individual, geographical and experimental variation of cuticular hydrocarbons of the ant *Cataglyphis cursor* (Hymenoptera: Formicidae): Their use in nest and subspecies recognition. *Biochemical Systematics and Ecology* 18: 63-73.
- Oldroyd BP, Halling LA, Good G, Wattanachaiyingcharoen W, Barron AB, Nanork P, Wongsiri S, Ratnieks FLW.** 2001. Worker policing and worker reproduction in *Apis cerana*. *Behavioral Ecology and Sociobiology* 50: 371-377.
- Oldroyd BP, Ratnieks FLW.** 2000. Evolution of worker sterility in honey-bees (*Apis mellifera*): how anarchistic workers evade policing by laying eggs that have low removal rates. *Behavioral Ecology and Sociobiology* 47: 268-273.
- Oldroyd BP, Wongsiri S.** 2006. Asian honey bees. *Harvard University Press*, Cambridge, MA.
- Ono M, Igarashi T, Ohno E, Sasaki M.** 1995. Unusual thermal defence by a honeybee against mass attack by hornets. *Nature* 377: 334-336.
- Ozaki M, Wada-Katsumata A, Fujikawa K, Iwasaki M, Yokohari F, Satoji Y, Nisimura T, Yamaoka R.** 2005. Ant nestmate and non-nestmate discrimination by a chemosensory sensillum. *Science* 309: 311-314. doi: 10.1126/science.1105244.
- Page RE, Erickson EH.** 1988. Reproduction by worker honey bees (*Apis mellifera* L.). *Behavioral Ecology and Sociobiology* 23: 117-126.
- Pamilo P.** 1991. Evolution of colony characteristics in social insects. II. Number of reproductive individuals. *American Naturalist* 138: 412-433.

- Peters JM, Queller DC, Imperatriz-Fonseca VL, Roubik DW, Strassmann JE.** 1999. Mate number, kin selection and social conflicts in stingless bees and honeybees. *Proceedings of the Royal Society of London Series B-Biological Sciences* 266: 379-384.
- Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RDC.** 2010. nlme: Linear and nonlinear mixed effects models. *R package version 3.1-97*.
- Queller DC.** 2006. To work or not to work. *Nature* 444: 42-43. doi: 10.1038/444042a.
- R Development Core Team.** 2008. R: A language and environment for statistical computing. *R Foundation for Statistical Computing* Vienna, Austria.
- R Development Core Team.** 2009. R: A language and environment for statistical computing. *R Foundation for Statistical Computing* Vienna, Austria.
- R Development Core Team.** 2010. R: A language and environment for statistical computing. *R Foundation for Statistical Computing* Vienna, Austria.
- Ratnieks FLW.** 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. *American Naturalist* 132: 217-236.
- Ratnieks FLW.** 1990a. The evolution of polyandry by queens in social Hymenoptera: the significance of the timing of removal of diploid males. *Behavioral Ecology and Sociobiology* 26: 343-348.
- Ratnieks FLW.** 1990b. Worker policing in social insects. 11th International Congress - IUSSI1990, India pp 365-366.
- Ratnieks FLW.** 1991. Facultative sex allocation biasing by workers in social Hymenoptera. *Evolution* 45: 281-292.
- Ratnieks FLW.** 1993. Egg-laying, egg-removal, and ovary development by workers in queenright honey bee colonies. *Behavioral Ecology and Sociobiology* 32: 191-198.
- Ratnieks FLW.** 2001. Heirs and spares: caste conflict and excess queen production in *Melipona* bees. *Behavioral Ecology and Sociobiology* 50: 467-473.
- Ratnieks FLW, Anderson C.** 1999. Task partitioning in insect societies. *Insectes Sociaux* 46: 95-108.
- Ratnieks FLW, Foster KR, Wenseleers T.** 2006. Conflict resolution in insect societies. *Annual Review of Entomology* 51: 581-608. doi: 10.1146/annurev.ento.51.110104.151003.
- Ratnieks FLW, Helanterä H.** 2009. The evolution of extreme altruism and inequality in insect societies. *Philosophical Transactions of the Royal Society B-Biological Sciences* 364: 3169-3179. doi: 10.1098/rstb.2009.0129.
- Ratnieks FLW, Keller L.** 1998. Queen control of egg fertilization in the honey bee. *Behavioral Ecology and Sociobiology* 44: 57-61.

- Ratnieks FLW, Reeve HK.** 1992. Conflict in single-queen Hymenopteran societies: the structure of conflict and processes that reduce conflict in advanced eusocial species. *Journal of Theoretical Biology* 158: 33-65.
- Ratnieks FLW, Visscher PK.** 1989. Worker policing in the honeybee. *Nature* 342: 796-797.
- Ratnieks FLW, Wenseleers T.** 2005. Policing insect societies. *Science* 307: 54-56. doi: 10.1126/science.1106934.
- Ratnieks FLW, Wenseleers T.** 2008. Altruism in insect societies and beyond: voluntary or enforced? *Trends in Ecology & Evolution* 23: 45-52. doi: 10.1016/j.tree.2007.09.013.
- Reeve HK.** 1989. The evolution of conspecific acceptance thresholds. *American Naturalist* 133: 407-435.
- Ribbands CR.** 1953. The behaviour and social life of honey bees. *Bee Research Association*, London, UK.
- Roubik DW.** 1989. Ecology and natural history of tropical bees. *Cambridge University Press*, New York, NY.
- Ruther J, Meiners T, Steidle JLM.** 2002. Rich in phenomena-lacking in terms. A classification of kairomones. *Chemoecology* 12: 161-167.
- Ruttner F.** 1996. Zuchttechnik und Zuchtauslese bei der Biene: Anleitungen zur Aufzucht von Königinnen und zur Kör- und Belegstellenpraxis. *Ehrenwirth*, München.
- Sakagami SF, Roubik DW, Zucchi R.** 1993. Ethology of the robber stingless bee, *Lestrimelitta limao* (Hymenoptera: Apidae). *Sociobiology* 21: 237-277.
- Seeley TD.** 1985. Honeybee ecology: A study of adaption in social life. *Princeton University Press*, Princeton, NJ.
- Seeley TD.** 1997. Honigbienen. Im Mikrokosmos des Bienenstocks. *Birkhäuser Verlag*, Basel Boston Berlin.
- Sherman PW, Reeve HK, Pfennig DW.** 1997. Recognition systems. In: Krebs JR, Davies NB, editors. Behavioural Ecology. fourth ed *Blackwell Science*, Oxford, UK. pp 69-96.
- Silveira FA, Melo GAR, Almeida EAB.** 2002. Abelhas Brasileiras: Sistemática e Identificação, first ed. *MMA/Fundação Araucária*, Belo Horizonte.
- Singer TL.** 1998. Roles of hydrocarbons in the recognition systems of insects. *American Zoologist* 38: 394-405.
- Sokal RR, Rohlf FJ.** 1995. Biometry: the principles and practice of statistics in biological research, third ed. *W. H. Freeman and company*, New York, NY.
- Sommeijer MJ, de Bruijn LLM, Meeuwsen FJAJ.** 2003a. Reproductive behaviour of stingless bees: solitary gynes of *Melipona favosa* (Hymenoptera: Apidae, Meliponini) can penetrate existing nests. *Entomologische Berichten* 63: 31-35.

- Sommeijer MJ, de Bruijn LLM, Meeuwsen FJA, Slaa EJ.** 2003b. Reproductive behaviour of stingless bees: nest departures of non-accepted gynes and nuptial flights in *Melipona favosa* (Hymenoptera: Apidae, Meliponini). *Entomologische Berichten* 63: 7-13.
- Suka T, Inoue T.** 1993. Nestmate recognition of the stingless bee *Trigona (Tetragonula) minangkabau* (Apidae: Meliponinae). *Journal of Ethology* 11: 141-147.
- Tarpy DR, Nielsen DI.** 2002. Sampling error, effective paternity, and estimating the genetic structure of honey bee colonies (Hymenoptera: Apidae). *Annals of the Entomological Society of America* 95: 513-528.
- Tarpy DR, Nielsen R, Nielsen DI.** 2004. A scientific note on the revised estimates of effective paternity frequency in *Apis*. *Insectes Sociaux* 51: 203-204. doi: 10.1007/s00040-004-0734-4.
- Tarpy DR, Page RE.** 2001. The curious promiscuity of queen honey bees (*Apis mellifera*): evolutionary and behavioral mechanisms. *Annales Zoologici Fennici* 38: 255-265.
- Tofilski A, Couvillon MJ, Evison SEF, Helantera H, Robinson EJJ, Ratnieks FLW.** 2008. Preemptive defensive self-sacrifice by ant workers. *American Naturalist* 172: 239-243. doi: 10.1086/591688.
- van Veen JW, Sommeijer MJ.** 2000. Colony reproduction in *Tetragonisca angustula* (Apidae, Meliponini). *Insectes Sociaux* 47: 70-75.
- van Veen JW, Sommeijer MJ, Monge IA.** 1999. Behavioural development and abdomen inflation of gynes and newly mated queens of *Melipona beecheii* (Apidae, Meliponinae). *Insectes Sociaux* 46: 361-365.
- van Zweden JS, d'Ettorre P.** 2010. Nestmate recognition in social insects and the role of hydrocarbons. In: Blomquist GJ, Bagnères A-G, editors. *Insect hydrocarbons*. Cambridge University Press, New York, NY. pp 222-243.
- Velthuis HHW, Sommeijer MJ.** 1991. Roles of morphogenetic hormones in caste polymorphism in stingless bees. In: Gupta AP, editor. *Morphogenetic hormones of arthropods*. Rutgers University Press, New Brunswick, NJ. pp 346-383.
- Visscher PK.** 1989. A quantitative study of worker reproduction in honey bee colonies. *Behavioral Ecology and Sociobiology* 25: 247-254.
- Visscher PK.** 1996. Reproductive conflict in honey bees: A stalemate of worker egg-laying and policing. *Behavioral Ecology and Sociobiology* 39: 237-244.
- von Frisch K.** 1965. *Tanzsprache und Orientierung der Bienen*. Springer Verlag, Heidelberg.
- Warburg MR.** 1993. *Evolutionary biology of land isopods*. Springer Verlag, Berlin, Germany.
- Wegener J, Lorenz MW, Bienefeld K.** 2010. Differences between queen- and worker-laid male eggs of the honey bee (*Apis mellifera*). *Apidologie* 41: 116-126. doi: 10.1051/apido/2009061.

- Wenseleers T, Alves DA, Franco TM, Billen J, Imperatriz-Fonseca VL.** 2011. Intraspecific queen parasitism in a highly eusocial bee. *Biology Letters* 7: 173-176. doi: 10.1098/rsbl.2010.0819.
- Wenseleers T, Badcock NS, Erven K, Tofilski A, Nascimento FS, Hart AG, Burke TA, Archer ME, Ratnieks FLW.** 2005a. A test of worker policing theory in an advanced eusocial wasp, *Vespula rufa*. *Evolution* 59: 1306-1314.
- Wenseleers T, Hart AG, Ratnieks FLW, Quezada-Euan JGG.** 2004. Queen execution and caste conflict in the stingless bee *Melipona beecheii*. *Ethology* 110: 725-736.
- Wenseleers T, Ratnieks FLW.** 2006a. Comparative analysis of worker reproduction and policing in eusocial hymenoptera supports relatedness theory. *American Naturalist* 168: 163-179.
- Wenseleers T, Ratnieks FLW.** 2006b. Enforced altruism in insect societies. *Nature* 444: 50-50. doi: 10.1038/444050a.
- Wenseleers T, Ratnieks FLW, Billen J.** 2003. Caste fate conflict in swarm-founding social Hymenoptera: an inclusive fitness analysis. *Journal of Evolutionary Biology* 16: 647-658. doi: 10.1046/j.1420-9101.2003.00574.x.
- Wenseleers T, Ratnieks FLW, Ribeiro MD, Alves DD, Imperatriz-Fonseca VL.** 2005b. Working-class royalty: bees beat the caste system. *Biology Letters* 1: 125-128. doi: 10.1098/rsbl.2004.0281.
- Wenseleers T, Tofilski A, Ratnieks FLW.** 2005c. Queen and worker policing in the tree wasp *Dolichovespula sylvestris*. *Behavioral Ecology and Sociobiology* 58: 80-86. doi: 10.1007/s00265-004-0892-4.
- Wharton KE, Dyer FC, Huang ZY, Getty T.** 2007. The honeybee queen influences the regulation of colony drone production. *Behavioral Ecology* 18: 1092-1099. doi: 10.1093/beheco/arm086.
- Wickham H.** 2009. ggplot2: Elegant graphics for data analysis. *Springer*, Heidelberg, London, New York.
- Wilson EO.** 1971. The insect societies. *The Belknap Press of Harvard University Press*, Cambridge, MA.
- Winston ML.** 1987. The biology of the honey bee. *Harvard University Press*, Cambridge, MA.
- Wittmann D.** 1985. Aerial defense of the nest by workers of the stingless bee *Trigona (Tetragonisca) angustula* (Latreille) (Hymenoptera: Apidae). *Behavioral Ecology and Sociobiology* 16: 111-114.
- Wittmann D, Radtke R, Zeil J, Lubke G, Francke W.** 1990. Robber bees (*Letstrimelitta limao*) and their host. Chemical and visual cues in nest defence by *Trigona (Tetragonisca) angustula* (Apidae: Meliponinae). *Journal of Chemical Ecology* 16: 631-641.
- Wood MJ, Ratnieks FLW.** 2004. Olfactory cues and *Vespula* wasp recognition by honey bee guards. *Apidologie* 35: 461-468. doi: 10.1051/apido:2004040.

Woyke J. 1963. What happens to diploid drone larvae in a honey bee colony? *Journal of Apicultural Research* 2: 73-75.

Zuur AF, Ieno EN, Walker NJ, Saveliev AA, Smith GM. 2009. Mixed effects models and extensions in ecology with R. *Springer science+business media*, New York, NY.