Differentiation in reproductive potential and chemical communication of reproductive status in workers and queens of the ant *Myrmecia gulosa*

Dissertation zur Erlangung des

naturwissenschaftlichen Doktorgrades

der Bayerischen Julius-Maximilians-Universität Würzburg

vorgelegt von

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aus

Mulhouse

Würzburg 2002

Eingereicht am: .....

Mitglieder der Promotionskommission:

Vorsitzender: .....

Gutachter: .....

Gutachter: .....

Tag des Promotionskolloquiums: .....

Doktorurkunde ausgehändigt am: .....

Acknowledgements

I am grateful to Pr. Bert Hölldobler for providing excellent work facilities and for his unconditional support throughout my work. I thank Dr. Christian Peeters to whom I owe my introduction to the world of ants in general and “primitive” ants in particular. The fruitful discussions with him considerably improved my knowledge of ant societies.

This thesis is not exclusively the product of my work. I tried to associate several disciplines of science to understand certain aspects of the social organisation of ant societies and I therefore needed help in areas outside my competences, either to compensate for gaps in my knowledge or to help me develop new skills. To this many people contributed:

I thank Dr. Graeme Jones and Richard Beard for their determining contribution to the chemical part of my work. All the chemical analyses and compound synthesis on which this study is based are the work of Richard Beard. I am indebted to him for the completion of this Herculean task. Thanks also goes to Pr. Jacobus Boomsma, for his work in coordinating the European Community “Training and Mobility of Researchers” and “Improving Human Potential” networks, which made this cooperation possible. Pr. Abraham Hefetz has shared with me his experience of ant-applied chemistry and I thank him very much for this.

It is in histology that Pr. Johan Billen, Dr. Bruno Gobin and Karin Möller shared their knowledge with me, and I am grateful to them. For the same reason, as well as for valuable technical assistance and continuous support, I owe much to Malu Obermaier and I thank her.

In an area to which the exercise of scientific activity is unavoidably bound, namely administration, Dr. Volker Neese has been an indispensable guide and master to me. I am indebted to him for his help and availability and thanks go to him.

Discussions with Dr. Jürgen Gadau, Dr. Matthias Sanetra, and Virginie Cuvillier-Hot helped me in developing my ideas. Dr. Bruno Gobin, Dr. Thibaud Monnin, and Pr. Diana Wheeler commented on chapters of this thesis and helped improve it.

In most of the above-mentioned categories and even more, the name of Dr. J. Liebig should have appeared. To avoid repetition, I express here my deep gratitude to him, for contributing greatly to this thesis.

I am grateful to Pr. M.P. Schwarz, Dr. K. Hogendoorn, Dr. R. Leijs, Dr. S. Shattuck and A. McArthur for logistic and technical support as well as hospitality during my fieldwork in Australia. I would also like to thank those many people outside Academia in this country, who offered their help willingly on many occasions and for their kindness. Among these, I particularly acknowledge Russell, warden from the Coutts Camp, Waterfall, New South Wales.

Richard Gabriel, Jan Geisendorfer, Arnold Kloos, Annette Laudahn, Dr. Christina Sauer, and Valenska Wenzel fed the ants, allowing me to avoid painful stinging and to occasionally leave the institute. Some of them managed to avoid the stings, some did not, but sincere thanks go to all of them for their help. Gerhard Eisenmann and Helga Heilman provided valuable technical help, I thank them, as well as all the people from the Institute who contributed in various ways to my work.

I am indebted to Lalitha Thomas-Oppenländer for revising my English and to Anett Endler for the German translation.

I am grateful to Anett, Chrimie, Christof, Christoph, Corinna, Jacqueline, Karl, Klaus, Olav and Walter and many others who contributed to a nice work and extra-work environment during my stay in Würzburg.

Je remercie aussi chaleureusement mes parents pout leur soutient indéfectible tout au long de ces années.

Last but not least, I thank Virginie for her heroic behaviour and her most precious help.

Differentiation in reproductive potential and chemical communication of reproductive status in workers and queens of the ant *Myrmecia gulosa*

I. Introduction

The most complex form of social organisation, outside humans, has been called eusociality (i.e. true society). Eusocial species are characterised by overlapping generations, cooperative brood care and division of reproductive labour among colony members (Wilson 1971). Ants and termites are all eusocial, as some bee, wasp, aphid, thrips, ambrosia beetle, shrimp and mole-rat species (Choe and Crespi 1997, Jarvis 1981, Jarvis and Bennett 1994, Jarvis et al. 1994, Burda et al. 2000, Duffy 1996). Typically, one or a few individuals reproduce, while the majority of their nestmates altruistically give up personal reproduction and help with the rearing of the reproductives' offspring. The occurrence of a sterile helper caste represented a problem to Darwin's theory of natural selection. As he considered individuals as units of self-interest trying to maximise their number of offspring, altruism and sterility could not be selected for. However, he realised that this could indeed be the case, provided that helpers allow for the production of extra sexual offspring, which would transmit the trait of worker-production (Bourke and Franks 1995, p. 11). A theoretical basis to this explanation was given by Hamilton (1964a, b). His inclusive fitness theory demonstrated that altruism and sterility could evolve if helpers contribute to the rearing of related offspring, thus gaining indirect (inclusive) fitness instead of direct fitness.

Although eusocial insects are often considered examples of harmonious societies where altruism prevails, genetic differences among nestmates of equal reproductive potential create conflicts of interest (reviewed in Bourke and Franks 1995). In the great majority of species, workers lost the ability to mate and hence the potential to produce diploid, female offspring. There is therefore no conflict between queens and workers about who should produce females (Ratnieks 1988). In contrast, workers of most species have functional ovaries and can lay viable haploid eggs giving rise to males (Bourke 1988, Choe 1988). They are therefore able to compete with the queen for their production and a conflict over the parentage of the males produced by the colony is expected to take place (Hamilton 1964b, 1972, Trivers and Hare 1976). This conflict opposes not only queens to workers, but also workers among them, as they have equal reproductive potentials and are more related to their own sons than to their nephews (i.e. sons of their sister nestmates)(Trivers and Hare 1976, Ratnieks 1988). However, in spite of diverging interests among colony members, there is rarely open conflict and workers concur in rearing their brothers. Thus, the conflict is not expressed (i.e. is not

actual), because of processes that reduce the divergence in interest (Ratnieks and Reeve 1992).

Several factors can account for the sterility of workers in queenright colonies. Increasing caste dimorphism during evolution improved queen specialisation in egg laying, whereas it limited reproductive options in workers to the point they became sterile, thereby eliminating the conflict. Determination of the reproductive potential of workers is thus accomplished at the larval stage in only a few genera of ants (Bourke and Franks 1995, p. 228). As already mentioned, in most ants, despite morphological specialisation, workers can produce males and regulation of their reproduction has to take place at the adult stage. Agonistic interactions rarely occur among ant workers and their queens, and the latter were rarely reported to destroy worker-laid reproductive eggs (e.g. Oliveira and Hölldobler 1990, but see Dietemann and Peeters 2000). Chemical communication is therefore thought to play a central role in the regulation of worker reproduction. It was indeed shown that queens produce chemicals in whose presence workers do not reproduce (Carr 1962, Passera 1980, Hölldobler and Wilson 1983). In polyandrous or polygynous species, workers can benefit more from raising brothers (the queen's sons) than nephews, as they are more closely related to the former. They are therefore expected to prevent each other from reproducing, i.e. to police each other, which can lead to self-restraint (Ratnieks 1988). Independent of relatedness effects, self- or worker policing should also evolve when worker reproduction is costly and decreases colony productivity (Cole 1986, Ratnieks 1988). Despite the importance of the division of labour in the evolution and maintenance of sociality in the Hymenoptera, mechanisms of regulation of worker reproduction are poorly understood.

Suitable model species to investigate the proximate regulation mechanisms of worker reproduction should have workers with functional ovaries, conferring on them the ability to challenge the queen's monopoly on male production. They should however, not reproduce under queenright conditions, thus indicating that a regulation takes places and that the conflict over the parentage of males is settled. "Primitive" ants often show these features: workers are not much smaller than queens and they can produce males with fecundity close to the queen's, yet they do not reproduce in her presence. Furthermore, "primitive" ants have weakly specialised queens (Peeters 1997). Correspondingly, the regulation mechanisms of division of reproductive labour might be simple and closer to those that occurred in early stages of the evolution of sociality in ants. Their small colony size also allows for relatively easy culturing and for following the behaviour of each individual.

The subfamily Myrmeciinae represents, together with the Ponerinae, the most "primitive" ants. These two subfamilies belong to two clades of the Formicidae that diverged early on, providing two independently derived "lower" groups for the study of the elaboration of social structure (Peeters 1997). The reproductive biology of the Ponerinae has recently been extensively investigated (reviewed in Peeters 1993, Monnin and Ratnieks 2001), whereas the Myrmeciinae remain poorly studied. Researchers focussed mainly on the singular ponerine species in which mated workers can produce diploid offspring and replace the queen. These ants drew considerable attention because of the maximal potential reproductive conflicts occurring among morphologically identical individuals that compose the societies (Peeters 1993, Monnin and Ratnieks 2001), and because they represent the closest situation to the hypothetical monomorphic ancestor of ants (Haskins and Haskins 1950, Peeters 1997). On the other hand, little is known on how morphologically specialised queens maintain their reproductive monopoly in "primitive" and in "advanced" species.

This study focuses on the modalities of the conflict over male production and how it is resolved in the Myrmeciinae *Myrmecia gulosa*. In this species, colonies reach the size of a few thousand individuals. Worker size varies and queens are distinctly larger than the largest workers. Furthermore, workers lay reproductive eggs when orphaned (Haskins and Haskins 1950). *M. gulosa* represents a good model species to investigate the conflict over male production in a system with intermediate social complexity between the "higher ants" and most the Ponerinae studied to date. Moreover, its study will shed light on the reproductive biology of the second taxon of "primitive" ants, which has largely been neglected until now.

The aim of this work is first to characterise the morphological specialisation of queens and workers, and to determine the differences in reproductive potential associated with this specialisation. This will help to understand the evolution of queen-worker dimorphism and worker polymorphism in ants and provide a basis for the investigation of the regulation of worker reproduction, which constitutes the main topic of this thesis.

The behavioural regulation mechanisms of worker reproduction are examined, as well as the chemical cues mediating recognition of reproductive individuals (both queens and workers), on which the regulation is based. In particular, the role of the cuticular hydrocarbons is examined. In several Hymenopteran species cuticular hydrocarbon profiles change with reproductive status of individuals. Such fertility markers could mediate the recognition of egg-layers by nestmates and constitute a basis for the regulation of reproduction (Peeters et al. 1999, Cuvillier-Hot et al. 2001, Sledge et al. 2001). Although these examples concern species without morphologically specialised queens, the same

mechanism could occur where morphological dimorphism between queen and worker castes occurs (Liebig et al. 2000). Identifying the pheromonal mechanisms by which queens regulate reproduction in their colonies would contribute to fill important gaps in our understanding of how division of labour is regulated in the Hymenoptera (e.g. Winston and Slessor 1998, Vargo and Hulsey 2000).

II. Generalities

II.A. The genus *Myrmecia*

The scientific history of the genus *Myrmecia* started with the discovery of Australia by the expedition led by Captain James Cook in 1770. The Royal Society of Britain financed an expedition whose mission was to perform astronomical observations in this part of the world. A transit of the planet Venus across the sun was to be observed from different parts of the globe in order to calculate the distance between Sun and Earth. As a side project, Captain Cook was charged with discovering and exploring a wanting continent: the "*Terra Australis Incognita"*. Augustus Darlymphe proposed its existence because of an imbalance of the known oceanic mass with the known land mass in the south Pacific. As with all great exploration and discoveries, the journey was rich in troubles as well as strokes of luck. It was a storm that, by accident, sent the ship too far North on the return journey and brought it close to the shores of "New Holland". Thus, Captain Cook and his crew were able explored the East coast of Australia. The botanists Joseph Banks and naturalist Daniel Solander collected the first *Myrmecia* specimens during the first days of exploration in Botany Bay (May 1770). Their ship later almost wrecked on the Great Barrier Reef and could not be properly repaired before November of that year. It nevertheless reached home in July 1771 with loads of unknown plant and animal material for biologists to examine.

Although Banks did not mention these large and ubiquitous ants in his journal, he described in detail aspects of the biology of the green ants *Oecophylla* and other, smaller species. He referred to the ants he observed as

> "*an industrious race who in all countries have for that reason been admird by man, tho probably in no countrey more admirable than in this*".

Famous taxonomists such as Brown, Clark, Emery, Forel, Mayr, Smith and Wheeler later worked on the description and classification of *Myrmecia* specimens. The most recent revision of the genus has been the work of Ogata and Taylor (Ogata 1991, Ogata and Taylor 1991). They recognised 89 species which they divided into 9 groups. They also provided a phylogeny of the genus, based on morphological characteristics.

Ants of the genus *Myrmecia* are the sole living representatives of the sub-family Myrmeciinae. Fossil members of the subfamily belong to the genera *Prionomyrmex* and *Ameghinoa* from the Baltic and Argentina Oligocene amber respectively (see Ogata 1991) and to the genus *Cariridris* from the Brazilian early Cretaceous amber (Brandão et al. 1989).

Their present distribution however, is limited to Australia and New Caledonia. In addition to their natural distribution, a mainland species colonised New Zealand after its introduction by humans. On the mainland, these ants are abundant and diverse in the southern regions, but rare in the tropic (Shattuck 1999). Two groups, commonly named bulldog ants and Jack-jumpers are well-known to Australians. The former are large and aggressive ants that build imposing mounds, sometimes with a diameter of more than a meter (plates 1 and 2). These characteristics constitute efficient warning for humans who can thus avoid their painful stinging. Jack-jumpers, on the other hand, are smaller and more cryptic ants that are able to hop. They move quickly in an unpredictable manner and are more difficult to spot. This together with the fact that they have a large distribution range makes them responsible for most of the medical complications following stinging. *Myrmecia* stinging can indeed induce sever allergic and occasionally lethal reactions in humans (Clarke 1986, Taylor 1988, Douglas et al. 1998).

Apart from the medical aspect, *Myrmecia* has triggered scientific interest mainly because they are considered as representing some of the most “primitive” ants living today. All ants are eusocial, the transition from solitary to social stages can therefore not be studied in the Formicidae. However, “primitive” species that are closer in both morphology and behaviour to the presumed ancestor of ants can help understanding the elaboration of complex social organisation (Haskins and Haskins 1950, Peeters 1997). Examples of morphological ancestral characteristics of *Myrmecia* workers are the occurrence of ocelli, of a functional sting, a segmented thorax (Ogata 1991) and a weak queen/worker dimorphism (Peeters 1997). Their solitary hunting habits as predators, their relatively small colony sizes compared to “higher” ants and the partially claustral mode of colony founding represent other primitive characteristics (Wilson 1971). The biology of several species has been studied and typical features for the genus can be summarised. Most species live underground in more or less complex nests (Wheeler 1932, Gray 1971a, 1974, Barnett 1974). Although they are predators that hunt by sight, these ants also collect nectar from flowers; as most species do not perform trophallaxis, it is thought that they use nectar for their own consumption (Wheeler 1932, Haskins and Haskins 1950, Haskins and Whelden 1954, Gray 1971b, Crosland 1988). Transfer of food is realised by sharing of trophic eggs (Freeland 1958, Barnett 1974, Sisson 1974). A majority of *Myrmecia* species forage by day and show reduced activity when larvae are not present in the nest, usually during the winter (Freeland 1958, Douglas and Brown 1959, Gray 1971b, Barnett 1974, Taylor et al. 1993). Workers of the same nest show size variations in some species (Wheeler 1932, Haskins and Haskins 1950, Gray 1971c, 1973, Ito

et al. 1994). Their notoriously aggressive behaviour alone has been the subject of several studies (Robertson 1971, Haskins et al. 1973, Via 1977, Sture-Ericksson 1985, Crosland 1989).

II.B. *Myrmecia gulosa*

M. gulosa was the among the first ant of the subfamily to be described by Fabricius in 1775, together with *M. forficata* (1787) and *M. esuriens* (1804) (Ogata 1991). Shuckard designated *M. gulosa* as type species of the genus (Swainson and Shuckard 1840). It was chosen by Ogata and Taylor as representative of the largest group of species in the genus. The 42 species that form this group have regionally varying common names: bull dog ants, bull ants, bull Joes, inch ants, inch men sergeant ants, soldier ants etc (Ogata 1991, Ogata and Taylor 1991). *M. gulosa* is found in sandstone area and its distribution range corresponds to the following regions: North East and South East coastal regions, Murray-Darling Basin, Queensland, New South Wales and Australian Capital Territory (Ogata and Taylor 1991). Their colony size can reach 2000 individuals and the species in monogynous (Haskins and Haskins 1950). It is the best-studied species of the genus. Various aspects of its behaviour and biology (Haskins and Haskins 1950, Freeland 1958, Robertson 1971, Haskins et al. 1973, Sisson 1974, Via 1977, Haskins and Haskins 1980, Crosland et al. 1988), chemistry (Cavill et al. 1964, Cavill and Williams 1967, Cavill et al. 1970, Ewen and Ilse 1970, Brophy and Nelson 1985, Billen 1988, Jackson et al. 1989, Street et al. 1994, Mackintosh et al. 1998) and morphology (Billen 1990) have been investigated.

II.C. General methodology

The methods used throughout this study are described here. Methods that are specific to the experiments or observations described in following sections of this study will be detailed where it is appropriate.

II.C.a. Ant Collection and laboratory rearing

Collection *M. gulosa* nests consist of mounds up to 1m in diameter, made of excavated soil and plant litter. The entrances are situated on top or at the sides of the mounds (plate 1). Colonies of *M. gulosa* were excavated in sandstone areas (plate 3) close to Waterfall (n=13) and Glenorie (n=1), New South Wales, Australia, between September 1998 and October 2000. Adults and brood were collected and counted. Given the complex structure of the nests, some workers may have been missed in unexplored tunnels. In order to minimise this problem, the walls of the excavation were checked on the following days for tunnels reopened by buried workers. Stray foragers were also collected. Most of the brood was collected from the numerous chambers of the mound and directly under the mound (plate 4). From there, tunnels ramified deeper underground, connecting chambers that were further away from each other, compared to those in the mound. These tunnels could be about 2 meters long and took any direction (plate 5). The architecture and depth (about 1meter) of the nests were limited by sandstone blocks, which had to be removed to collect the entire colony. The queen could generally be found at intermediate depth. Since colonies possessing the biggest mounds were not excavated, the average colony size reported in our study is probably an underestimate. Given the large size of these ants, the number of individuals brought back for laboratory rearing was reduced in colonies larger than 1000 workers. Together with the queen and all the eggs found, 500 to 1000 workers, and up to 250 larvae and 100 cocoons were randomly selected. The remaining workers were frozen for later measurements and dissection.

Laboratory rearing Ants were kept in plaster-of-Paris nests into which chambers had been moulded and covered with glass plates to allow observation. These nests were connected to foraging arenas where food (pieces of cockroaches or entire crickets and honeywater) was deposited every 1-2 days. The photoperiod was set at 10:14h (light:darkness) cycles and a high humidity was maintained inside the nests by regularly moistening the plaster. The temperature was maintained at 24±1°C for most of the year. Because the ants originated in a temperate area, artificial winter was created by lowering the temperature to 17±1°C for 4 to 8 weeks once a year.

These conditions proved to be favourable for the ants, which produced several hundred new individuals over the 36 month study period.

Ant marking To recognise workers individually and to follow their behaviour, they were marked with enamel paint (Revell, Bünde, Germany). Four dots of various colours applied on the thorax of each ant in the colony constituted individual codes. The paint dried quickly and did not affect the workers' behaviour and survival in an obvious manner.

II.C.b. Chemical analysis

Cuticle extraction Individuals to be extracted in hexane were killed by freezing at –70°C for 5 minutes. After thawing, they were dipped in 1 ml hexane and shaken gently for two minutes. They were then removed from the vial with clean forceps.

The alternative Solid Phase MicroExtraction (SPME, Arthur and Pawliszyn 1990) technique was used to extract CHCs from live individuals. This method was recently adapted to the study of pheromone production in insects by Malosse et al. (1995), Mozuraitis et al. (1996), Frérot et al. (1997). Monnin et al. (1998) used it for the first time in social insects in order to extract cuticular hydrocarbons in the ant *Dinoponera quadriceps*. During extraction, the ants were immobilised by blocking their post-petiole in a split cardboard piece. This reduced the mobility of the ant's gaster and allowed the rubbing of a solid-phase microextraction fibre (SUPELCO, coated with a 7-µm polydimethylsiloxane film) on the tergites. The extraction was performed for 30 seconds, in a standardised manner. As no solvent was required, individuals survived the extraction. In the present study, this non-destructive technique allowed for the extraction of individuals, especially queens, while making possible further behavioural studies of the limited number of colonies available. This permitted optimal management of the ant keeping and collection of samples in large enough number to perform statistical analysis. It also allowed for the collection of CHCs without contamination from internal products and for repeated extraction of the same individuals.

Gas chromatography A volume of 0.5µl of the hexane extracts was injected in the port (260°C) of a Carlo Erba 8130 gas Chromatograph equipped with a DB-1 nonpolar capillary column (J&W Scientific, Folsom, CA, 30m x 0.32mm x 0.25µm). Helium was used as a carrier gas with a column head pressure of 95 kPa. Samples were run in the splitless mode. The column temperature

was maintained at 60°C for 4 minutes and then raised to 250°C at a rate of 20°C min^{-1} and from 250°C to 300°C at 2.5°C min^{-1}. Flame ionisation detector temperature was set at 310°C. When SPME was used, the fibres were directly injected into the injection port of the chromatograph and the same temperature program was used.

Gas chromatography/mass spectrometry (GC/MS) GC/MS analyses were performed in Keele, UK. Electron Ionisation GC/MS was undertaken on a Hewlett-Packard 5890 Gas Chromatograph coupled to a Hewlett-Packard 5970 Series Mass Selective Detector controlled by a Hewlett-Packard ChemStation. The column used with all GC data was an Rtx-5 (Crossbond 5% diphenyl-95% polysiloxane column), 15m, 0.25 mm ID, 1.0 μm df. The temperature programs varied with the samples analysed and will be given in the methods of the concerned sections.

Statistical analyses of GC results Cuticle and glands are composed of numerous chemical compounds. When comparing profiles of different groups of individuals by statistical multivariate analysis, the number of variables to be used was constrained by the requirements of the tests used. A subjective rule was thus used to select the compound peaks used as variables. Only peaks that were present in the majority of individuals of each group compared were used. Furthermore, they had to represent more than a predetermined percentage of the total peak area. Details are given in the method sections for each analysis. The relative areas of the peaks selected were then restandardised to 100% and transformed following Reyment's formula (Reyment 1989), to avoid the use of compositional data: $Z_{i,j} = \ln[X_{i,j}/g(X_j)]$, where $X_{i,j}$ is the area of peak i for the ant j, $g(X_j)$ the geometric mean of all peaks for the ant j, and $Z_{i,j}$ the transformed area of peak i for the ant j (Liebig et al. 2000). Visual checking of the chromatograms showed that peaks with a zero value were not totally absent, but were present in minute amounts and not integrated as peaks. As the quantity detected was below the integration threshold, the software calculated no area for these peaks. Zeros were therefore replaced by a value slightly inferior to the integration threshold.

III. Caste specialisation and differentiation in reproductive potential

III.A. Introduction

A large proportion of hymenopteran societies are structured by important morphological differences among female members. This specialisation increases the efficiency of the division of labour (Wilson 1971), and one aspect of its evolution is the capacity of larvae to initiate developmental changes in response to nutritional factors, combined with the social regulation of larval nutrition (Wheeler 1986). The existence of divergent queen and worker castes is the clearest manifestation of physical specialisation, but some ants exhibit also marked phenotypic diversity among the adult workers. This is restricted to 15% of ant genera (45/297) and includes both continuous size variation and polymorphism (Hölldobler and Wilson 1990). Polymorphism, defined by Wilson (1953) as allometry occurring over a sufficient range of size variation within a normal mature colony to produce individuals of distinctly different proportions, occurs only in eight independent lineages (Hölldobler and Wilson 1990). Comparative data are needed to understand the evolution of physical castes in ants, and phylogenetically "primitive" species are crucial for this, because they represent the early stages of increasing complexity of social organisation (Peeters 1997).

Ants in the subfamilies Myrmeciinae and Ponerinae are characterised by a number of ancestral morphological traits and are commonly believed to be less complex socially than "higher" ants (Brown 1953, Wilson 1971, Taylor 1988, Ogata 1991, Peeters 1997). The Myrmeciinae includes a single genus *Myrmecia*. Its colonies vary from a few dozens ants to a few thousands (Haskins and Haskins 1950, Gray 1974, Higashi and Peeters 1990, Ito et al. 1994). Variation in worker size has been reported in various species, and sometimes a bimodal distribution of their size frequency (Haskins and Haskins 1950, Gray 1973). Wilson (1953) considered these features to be the first steps leading toward the evolution of worker polymorphism. The queens of *Myrmecia* are generally not much larger than the largest workers (Wheeler 1932, Haskins and Haskins 1955, Gray 1971c, Ito et al. 1994). Workers have functional ovaries and produce trophic eggs in the presence of the queen (Freeland 1958, Barnett 1974). They lay male-destined eggs once orphaned, as in many ants from other subfamilies (Bourke 1988, Choe 1988, Dietemann and Peeters 2000).

Morphological specialisation was studied in *M. gulosa*. Differences in body size and body proportions of queens, large and small workers, as well as in their ovaries and egg laying rates are described. The results are compared with the available data on morphologically "primitive" ants

and the evolution of queen-worker dimorphism and worker polymorphism in the Myrmeciinae is discussed.

Associated with limited queen-worker dimorphism is the retention of the spermatheca in the majority of ponerine species (Peeters 1991). In about a hundred of them, workers kept the ability to mate and to produce diploid offspring (Peeters 1993, 1997). In a proportion of these species, these gamergates co-occur with queens, whereas in others, queens are no longer produced. Gamergates have never been reported outside the Ponerinae, but were expected to occur in *Myrmecia*, since workers seem morphologically competent (Crosland et al. 1988, Peeters 1991a, Peeters 1997). Occurrence of gamergates is reported here for the first time in the Myrmeciinae.

III.B. Methods

Queen/worker and worker/worker size variation Workers and queens are distinct in *M. gulosa*, but dealate virgin queens are indistinguishable from mated queens without dissection. We therefore call queen any individual having the queen morphology, either mated or not (Peeters and Crozier 1988). When the distinction has to be made, the terms virgin queen or mated queen are used. Queen-worker dimorphism and worker polymorphism were assessed by morphometric data and ovarian dissection.

Outerorbital distance is measured in full-face view through the points of highest convexity of the eyes. Head length is measured in full-face view from the midpoint of a transverse line connecting the anteriormost points of the clypeal anterior projections to the midpoint of the occipital margin of the head, excluding the occipital carina (Ogata and Taylor 1991). The outerorbital distance and head length of 157 workers, 10 mated queens and 17 virgin queens was measured. In the Myrmeciinae, the tergites and sternites of the first gastral segment (abdominal segment IV) are fused to form a tubular structure (Taylor 1978). As stated by Ito et al. (1994), gaster width can therefore be used as a morphometric feature as it is less or not subject to size variation due to trophic state or ovarian activity of the individuals. The maximal width of this segment, viewed dorsally, was measured in 49 of the above-mentioned workers, in 16 virgin queens and 6 mated queens. All mated queens were collected from the field, unlike virgin queens, which pupated in the laboratory. The workers were randomly chosen among individuals frozen just after field collection. They all belonged to the same colony. In contrast, virgin queens and mated queens originated from 2 and 10 colonies respectively. Data were plotted on log-log scales and regression lines calculated for workers and queens separately, as well as for small and large

workers separately. Plotting both queens and workers on the same graph give information about the caste differentiation process in a species with limited queen-worker dimorphism.

Ovarian morphology Dissections were done in Ringer solution. The number of ovarioles per ovary and number of clearly visible oocytes (with and without yolk) per ovarioles was counted for 4 mated queens, 438 workers reared in the laboratory (from 10 colonies), and 153 workers (from 3 colonies) which were frozen immediately after field collection. The correlation between gaster width and number of ovarioles was calculated for 49 workers and 2 mated queens all born in the field, as well as for 10 virgin queens partially reared under lab conditions. Maturity of oocytes is defined by their basal position in the ovariole and their size. Oocyte and egg length were measured under a binocular microscope with an accuracy of 0.1mm. Mature trophic as well as reproductive oocytes (n=385 and n=222 respectively) of large and small workers (from 5 queenright and 3 orphan colonies) were measured in the ovarioles. Presence of yellow bodies was checked in 476 and 234 workers from the queenright and orphan colonies respectively. Age classes of workers were known in 2 of the queenright colonies (n=234 workers), allowing examining the correlation of yellow body load with age. Reproductive eggs of workers and queens (n=262 from 6 colonies and n=420 from 7 colonies respectively) were collected in the nests and measured. Normal distributions of oocytes and egg length were tested with the Shapiro-Wilks test and compared with an ANOVA, Tukey post hoc test for unequal sample sizes. The same test was used to compare the lengths of queen-laid eggs for interindividual differences among queens. Since trophic eggs lose shape once laid and have a thin membrane that is easily punctured during handling, it was impossible to measure them. Spermathecae of all workers were checked for presence of sperm. Additionally, in order to check if workers active outside of the nest produced trophic eggs, 16 individuals observed returning to their nest (n=3) after a foraging trip in the field and 110 foragers or guards picked up on the colony mounds (n=2) were dissected. Their ovarian development was classified in three groups: ovaries containing mature, sub-mature or no oocytes.

Egg-laying activity Individuals laying reproductive eggs (queens or workers) did not systematically bend their gaster forward between their legs, as typically shown by ovipositing ants in ponerine species. In addition, queens could lay several eggs at a time. Reproductive egg-laying rates as well as the duration of the queens' oviposition cycles were therefore deduced from the daily counts of eggs present in the nests (8 colonies over 207 days) and not from direct observation of ovipositions. Egg production was monitored during the first days of oviposition activity, when egg number was small enough to ensure precise counting. As workers laying

trophic eggs characteristically bend their gaster forward and seize the egg with their mandibles, trophic egg-laying rate was measured by direct observation. Trophic egg laying as well as the fate of the eggs in colonies with brood (n=5) were monitored over a total of 83 hours. Furthermore, the egg laying rates of large and small workers were compared in 4 groups of 200 workers (100 small with 100 large individuals) without brood, over a total of 44 hours. Trophic eggs laid after solicitation from the queen were not counted since there may be preferential solicitation of the small workers surrounding the queen (cf. section IV.B.c.).

In a total of 23 groups of 20 workers and in 5 larger groups containing at least 100 workers, workers' egg-laying activity was followed once they are separated from their queen. Their behaviour was regularly monitored. The reproductive egg-laying rate of workers was deduced from the eggs counted in 14 orphaned groups of 20 workers over periods ranging from 3 to 80 days of oviposition activity. At the end of the study, all workers in the groups were dissected to count the number of individuals possessing reproductive oocytes in their ovaries, hence the number of egg-layers. Six of these groups contained exclusively small workers, and the 8 other groups contained exclusively large workers. The fertility of each class of individuals could thus be assessed.

III.C. Results

III.C.a. Colony size

Average size of the societies collected was 992±551 workers (mean±st.dev., n=14, range 134-1859), and usually one dealate queen (table III.1.). A group of small workers constantly surrounded the queen. In 2 colonies out of 14, two dealate queens were found peacefully cohabiting (table III.1.), and small workers gathered around both queens. One of these digynous colonies was split into two with one queen in each part. Both queens laid eggs and produced new workers, revealing that both were mated. In the second colony, only one queen was dissected and her spermatheca was full.

Table III.1.: demography of 14 colonies of *M. gulosa*.

collection date	number of dealate queens	workers	cocoons	larvae
14/09/98	2	761	69	120
19/09/98	1	209	3	88
15/10/99	1	1435	51	562
16/10/99	1	313	14	106
17/10/99	1	1318	36	40
18/10/99	1	411	41	169
19/10/99	1	1523	145	450
20/10/99	1	134	3	25
21/10/99	1	913	30	261
22/10/99	1	1361	33	256
03/11/99	2	1429	>112	>353
04/11/99	1	1264	279	550
10/10/00	1	955	27	>137
12/10/00	1	1859	46	>260
average		991.8		
st.dev.		551.0		

> in front of a figure indicates that brood has not been totally collected

III.C.b. Queen/worker and worker/worker polymorphism

External morphology Worker size (from tip of mandibles to gaster) in *M. gulosa* varied from 14 to 23 mm. The measurements of outerorbital distance of 157 workers showed a bimodal size distribution around 2.4-2.5 and 3.8-3.9 mm (figure III.1.). Two classes of workers were therefore differentiated, hereafter referred to as small and large workers. Individuals with an outerorbital distance of at least 3.4mm were considered as large workers. Although it was not systematically quantified, this pattern was constant in all the colonies collected. Workers were sorted out according to their size and counted in 2 freshly collected colonies. These were composed half of large workers and half of small workers (51% and 49% respectively).

Mated (n=10) and virgin queens (n=17) differed neither in their outerorbital distances nor in their head lengths (U=79, p=0.76 and U=74, p=0.58 respectively) and were therefore pooled together. Queens' outerorbital distance overlapped with the largest workers measured (figure III.1.). The plot of outerorbital distance versus head length shows that workers (n=157) and queens (n=27) fit on a single regression line (r^2=0.99). The regression coefficient (0.93) was significantly different from 1 (t=9.54, p<0.01), the relative growth is thus slightly allometric (figure III.2.), with outerorbital distance increasing faster than head length. When regressions were calculated for workers and queens separately, the lines obtained have similar slopes (t=0.16,

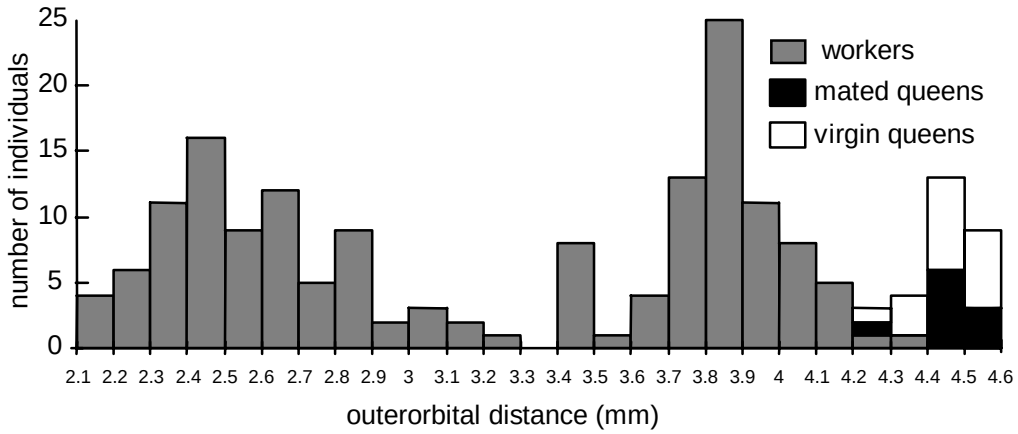


Figure III.1.: size distribution of *M. gulosa* workers, virgin queens and mated queens using outerorbital distance. Workers (n=157) and queens (n=10) were collected in the field, whereas virgin queens (n=17) pupated in the laboratory.

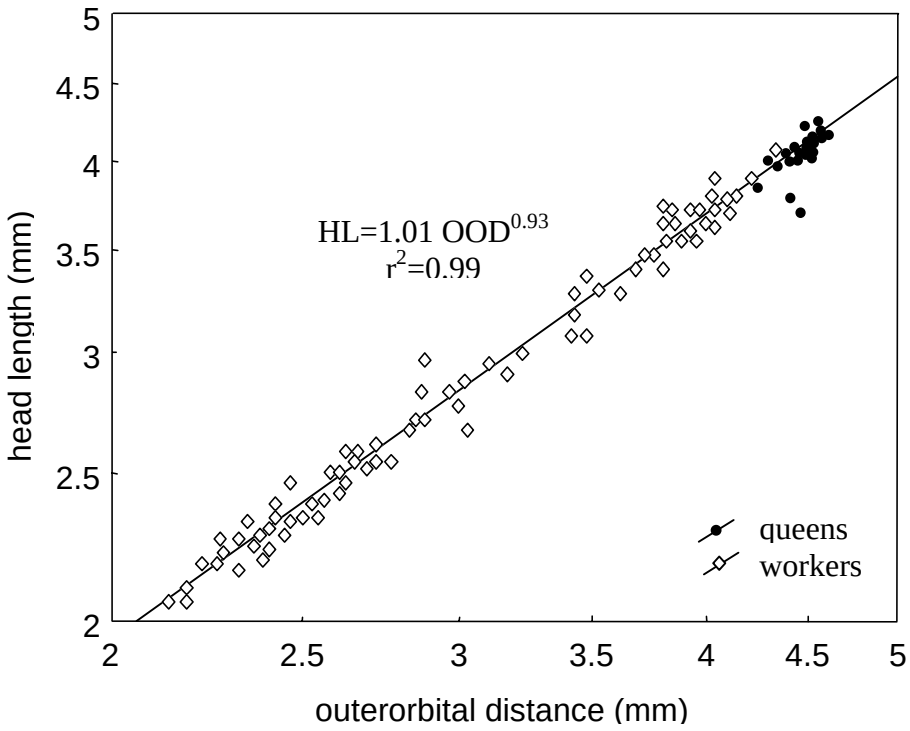


Figure III.2: log-log plot of outerorbital distance (OOD) vs. head length (HL) in workers (n=157), and queens (n=27, 10 mated + 17 virgin queens) of *M. gulosa*.

p=0.87) and intercepts (t=1.98, p=0.05). If only workers were considered and a regression equation was calculated for small (outerorbital distance < 3.4mm) and large individuals (outerorbital distance > 3.4mm) separately, neither their slope nor their intercepts differed significantly (t=0.45, p=0.65 and t=0.22, p=0.83 respectively).

Gaster widths of mated (n=6) and virgin queens (n=16) were similar (U=31, p=0.21) and pooled together. The comparison of the plots of gaster width vs. outerorbital distance between queens and workers (n=22 and n=49 respectively, figure III.3.) reveals a larger difference in their morphology. The regression coefficients are not significantly different from each other (t=0.38, p=0.70), but the intercepts are distinct (t=21.40, p<0.01). The regression coefficient for workers is significantly different from 1 (t=13.75, p<0.01), indicating allometric growth. For queens, the difference is not significant (t=1.10, p=0.28); this was most likely due to the low number of individuals available and to their small size range, and is probably not a real phenomenon. Outerorbital distance increases faster than gaster width, and with the same rate in both queens and workers. However, queens have wider gasters than hypothetical worker of same head size. For example, an exceptionally large worker having the average outerorbital distance of queens (4.45mm) would have a 3.81mm wide gaster, instead of 4.54mm for an average sized queen. When small workers were compared with their larger nestmates and a regression was calculated for each of the size classes, again no differences between them could be found (t=1.16, p=0.25, t=0.19, p=0.85 for slopes and intercepts respectively).

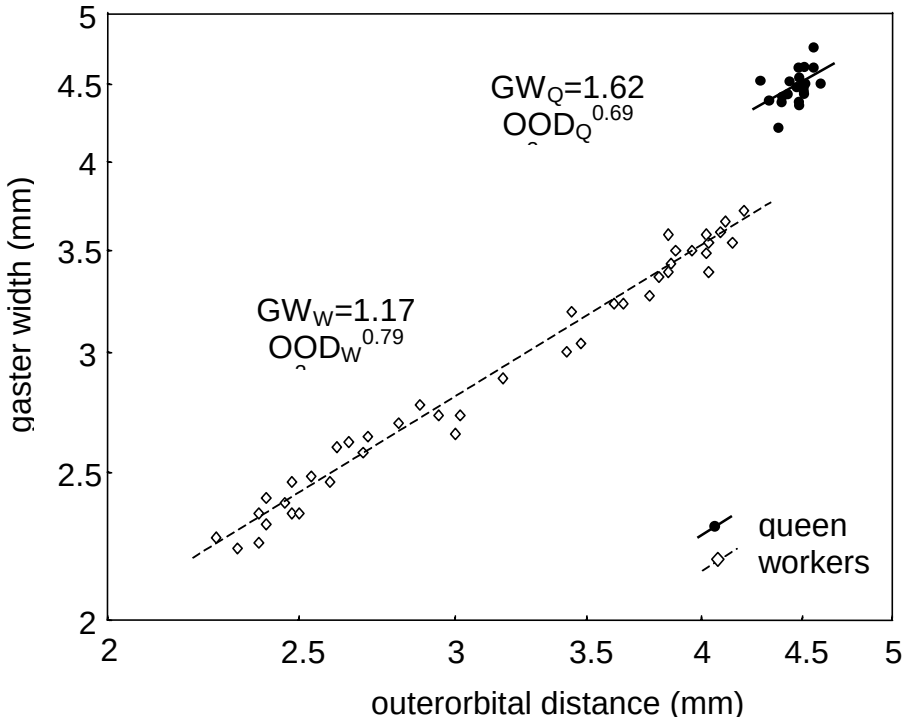


Figure III.3.: log-log plot of outerorbital distance (OOD) versus gaster width (GW) in workers (n=49) or queens (n=22, 6 mated + 16 virgin queens) of *M. gulosa*.

Ovarian morphology Mated and virgin queens had the same number of ovarioles per ovary (n=4 and n=10 respectively, U=60.0, p=0.3). The average number (±st.dev.) of ovarioles per ovary was 22.4±2.8 for queens (n=14). Large workers have more ovarioles per ovary (7.1±1.7, n=210) than small workers (4.3±0.9, n=228) (plate 6). The regression lines of gaster width against number of ovarioles were plotted for workers and queens separately (n= 49 and n=13 respectively, figure III.4.). Although the correlation between gaster width and number of ovarioles in queens was not significant (p=0.08), the number of ovarioles in queens is about twice that of workers with comparable gaster width. Although no colony was completely dissected, all the workers (n=579 from 14 colonies) possessed an empty spermatheca, whereas egg-laying queens' spermathecae (n=4) were full with sperm.

III.C.c. Egg-laying activity

Queens The number of mature oocytes was significantly higher in mated queens than in workers (F=234, p<0.01, figure III.5.), each ovariole containing one mature oocyte. On average, large

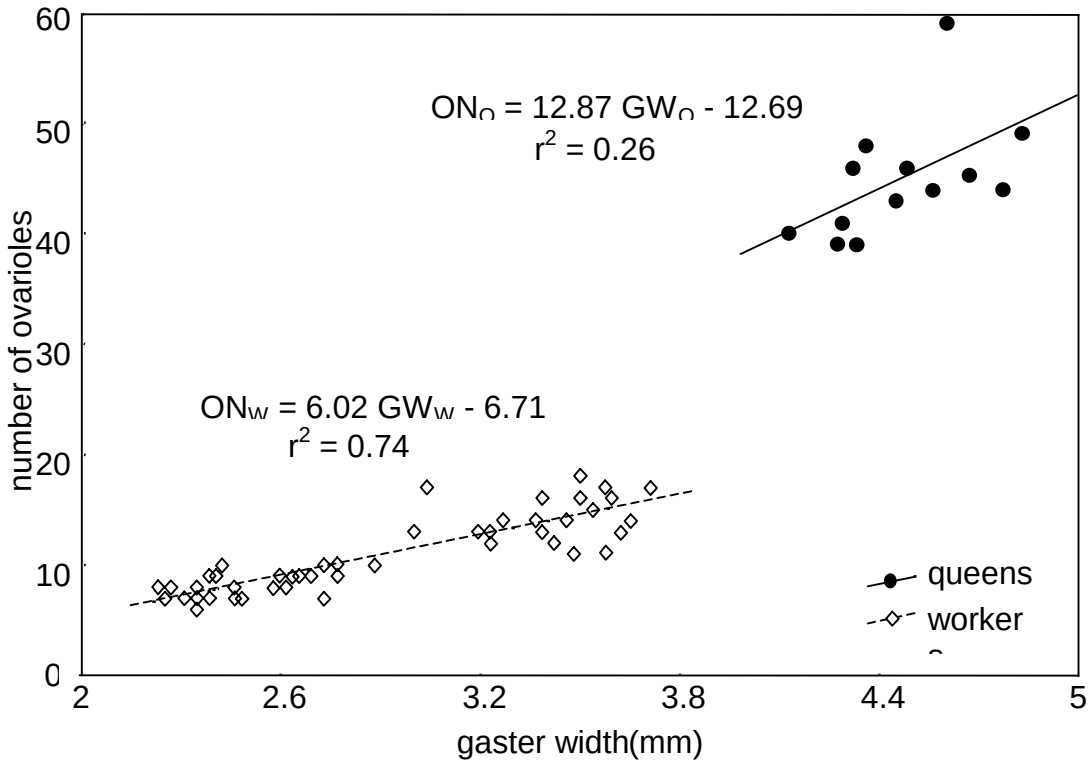


Figure III.4.: correlation between gaster width (GW) and ovarioles number (ON) in workers (n=49) and queens (n=13, 3 mated + 10 virgin queens) of *M. gulosa*.

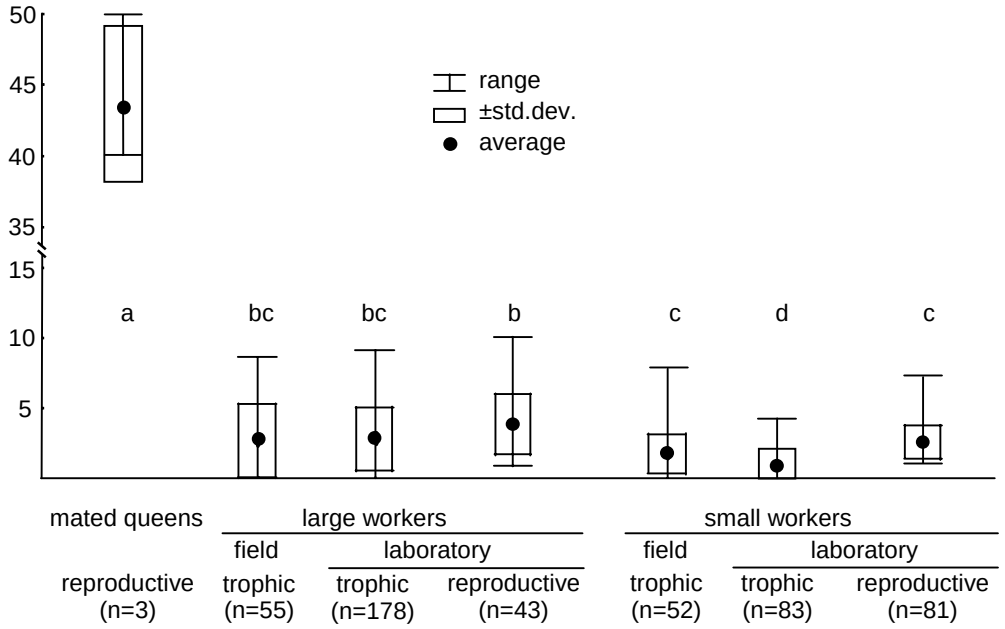


Figure III.5.: number of oocytes found in mated queens, large and small workers of *M. gulosa.* Workers producing trophic or reproductive eggs were considered separately, as well as workers freshly collected from the field or laboratory reared. Sample size is given below group designation. Different letters indicate significant differences at the level 0.05 (Tukey HSD post hoc test for unequal sample sizes).

workers had more mature oocytes than small individuals, although not significantly so in all the cases. Individual ovarioles of mated queens contain more developing oocytes compared to workers (20-25 compared to 3-15 for workers, $F=55$, $p<0.01$).

Queens of *M. gulosa* do not systematically bend their abdomen forward during oviposition. Indeed, eggs were sometimes ejected while the queen was in a resting position. She did not collect the eggs between her mandibles and they dropped onto the floor. They were then picked up by workers and deposited on the pile. Furthermore, queens were observed to lay up to 4 eggs at a time, these being glued together in a row and ejected in few seconds. The total number of eggs counted from one day to the next in the egg piles gave a median rate of 10 eggs laid per day (1st quartile=7, 3rd quartile=13, range 1-21, n=72 days). Eggs were present in the nests for periods of 31.7$\pm$11.4 days (mean$\pm$st.dev., range 22-63 days, n=22 periods assessed) and after they all hatched, it took another 20.2$\pm$9.5 days (range 8-39 days, n=22 periods assessed) before the new egg-laying period started and new eggs accumulated. The first larva appeared 19$\pm$2.4 days after the first egg was laid. This indicates that the active and inactive egg-laying periods of queens last approximately 12.7 and 39.2 days respectively. In a typical laboratory reared monogynous nest of *M. gulosa*, around 140 eggs could be counted on the pile before the first larva appeared. This gives

a second estimate for the egg-laying rate of 11 eggs a day. This figure is close to the first rate calculated by daily egg counts. When the length of the eggs (n=233) laid by different queens were compared, it appeared that each queen laid eggs of constant size, and that this size varied among individuals (range: 1.08-1.15mm, $F=45.36$, $p<0.01$).

Workers

Trophic egg-laying In queenright colonies, both small and large workers laid exclusively trophic eggs (200 hours of observation on 13 colonies, over 3 years of study). These eggs were ejected in seconds as the egg-layer bent the gaster forward between her legs. When it was still partly in the gaster, the ant seized it in her mandibles; the translucent egg then lost shape, appearing as a fragile yolk sac without a rigid chorion. In contrast, reproductive eggs were opaque, possessed a thick chorion and kept their shape after oviposition. Trophic eggs were fed to larvae (which received 66.9% of the eggs produced), to other workers (28.1%, plate 7), to virgin queens (2.8%), or to the queen (0.9%). In addition, 1.4% were consumed by the egg-layers themselves. Large workers laid 2.4 times more eggs than small workers (n=44 hours of observation on 3 colonies). Trophallaxis does not occur in *M. gulosa* and trophic eggs constitute an important channel of food exchange in the colonies.

The characteristics of trophic eggs could already be detected in the ovaries, at the oocyte stage. They were easily distinguished from reproductive oocytes, as they were surrounded by a much thinner layer of follicular cells and were longer than 1.25mm (which corresponded to the maximum size of 95% of the reproductive oocytes measured, n=222). They were also distinguished by their partly translucent yolk. Yolk of reproductive oocytes appeared dense and homogeneous. The dissections (n=681 large workers, 200 small workers) confirmed that queenright workers produce only trophic oocytes. Only some of their ovarioles contained mature or developing oocytes. At most, two yolky oocytes could be found in an ovariole, a basal mature one and a sub-mature one, followed by several non-yolky oocytes. The proportion of laboratory reared workers able to lay trophic eggs (i.e. which have mature oocytes) in a colony was 67%. A less conservative estimate was 92% of the individuals if workers with sub-mature oocytes, but without mature oocytes are included (table III.2.). These data permit to calculate an average trophic egg-laying rate of 1.0 egg/worker/day. A smaller proportion of field collected workers possessed yolky and mature oocytes. In laboratory reared colonies, more large workers had mature or yolky oocytes in their ovaries than small workers. For workers dissected soon after field collection, the trend was opposite (table III.2.). The differences observed may be explained by the greater availability of food in the laboratory.

Trophic oocytes were significantly longer than reproductive oocytes and eggs produced by either the queens or the workers. Trophic oocytes produced by large workers were longer than those produced by small workers ($F=590$, $p<0.01$, figure III.6.). Yellow bodies were found in 89.5% of 476 laboratory reared workers, showing that trophic egg-laying activity produces yellow bodies. Similarly, 95.4% of 153 individuals frozen just after collection possessed yellow bodies. Yellow bodies load and age were correlated in laboratory reared individuals. They were found in 57, 80 and 99% of the workers of less than one month (n=7), 1-4 months (n=91) and more than 6 months old (n=136) respectively.

Table III.2.: ovarian development of laboratory reared and field collected workers of *M. gulosa*, expressed in proportion of workers having only sub-mature / sub-mature and mature trophic oocytes in their ovarioles.

	laboratory	field
large workers	92.7 / 82.1 (n=108)	62.1 / 36.2 (n=58)
small workers	89.7 / 40.0 (n=60)	82.0 / 44.0 (n=50)
all workers	91.7 / 66.8 (n=168)	71.3 / 39.8 (n=108)

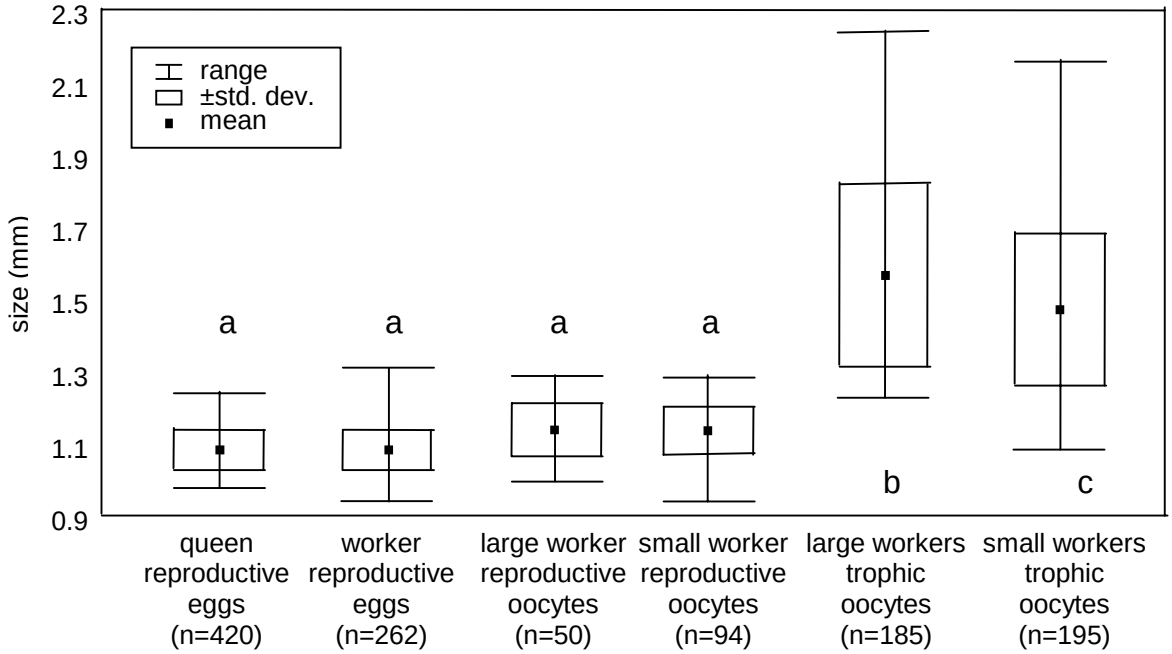


Figure III.6.: egg and mature oocyte length in *M. gulosa*. Different letters indicate significant differences at the level 0.01 (Tukey HSD post hoc test for unequal sample sizes). Sample size is given below group designation.

Seventy five percent of the field workers returning from a foraging trip or active on the nest mounds, possessed yolky oocytes (10/16 and 84/110 respectively). Thirteen and 63% of them respectively were able to oviposit (i.e. had mature oocytes, ready to be laid).

Reproductive egg-laying In 91.3% (21 out of 23) of the orphaned workers groups (20 individuals per group), workers displayed infrequent agonistic behaviour (antennal boxing and biting) 18.1±6.8 (mean±st.dev.) days after they were separated from their queen. Worker-laid male eggs accumulated in all nests but two. The first eggs were laid after 25.0±7.4 days. These reaction delays were shorter if larger colonies became orphaned. Indeed, in the latter situation, agonistic interactions already appeared after 3-6 days and eggs were laid after 11-14 days (n=3 colonies containing 200-300 individuals).

Dissections of all the workers in the 23 groups showed that 0-8 individuals shifted to the production of reproductive haploid eggs while the others continued to produce trophic eggs. Assuming that all workers having reproductive oocytes in their ovaries oviposited, the median egg-laying rate was estimated to be 1.0 eggs/worker/day for large workers (1^{st} quartile=0.4, 3^{rd} quartile=1.0, range 0-6, n=78 days) and 0.7 eggs/worker/day for small workers (1^{st} quartile=0.3, 3^{rd} quartile=0.7, range 0-4, n=73 days). Workers that shifted to the production of reproductive oocytes had the same number of ovarioles as trophic egg-layers ($t=0.22$, $p=0.83$ and $t=0.92$, $p=0.36$ for large and small workers respectively). Four large groups of orphaned workers were reared long enough to follow brood maturation. All the groups reared a high number of worker-produced males. Complete dissection of these groups (total of 290 large and 190 small individuals) showed that the proportion of small workers (39.0%) that shifted to the production of reproductive eggs was higher than that of large workers (16.5%).

Not all the ovarioles of workers were fully active, some being without yolky oocytes. The ratio of mature oocytes to ovarioles falls from 1 in queens to 0.58 and 0.53 for small and large workers in presence of their queen, respectively. In small and large reproductive egg-laying orphaned workers, these ratios are 0.21 and 0.42 respectively. For small and large individuals dissected soon after collection (trophic egg-layers), the values 0.52 and 0.40 respectively are obtained. Mature reproductive oocytes measured in small and large orphaned workers' ovarioles have the same size (Tukey HSD test, $p=1.00$), unlike the trophic oocytes (Tukey HSD test, $p<0.01$). Reproductive eggs laid by workers have the same size as queen eggs (Tukey HSD test, $p=1.00$, figure III.6.). A large proportion (90.4%) of the workers having reproductive oocytes in their ovaries possessed yellow bodies.

When diploid eggs and larvae produced by the queen before her death or removal where left in orphaned groups, an undetermined proportion of this brood was reared into virgin queens.

III.C.d. Alternative reproductive strategy: gamergates

A colony of *M. pyriformis* (belonging to the gulosa group) was excavated near Calga, New South Wales, in an area where no other nest of this species had been found. The colony's queen was not collected and it was not known whether this colony was naturally orphaned, or if the queen had not been found during excavation. Production of workers continued for 6 months of culture in the laboratory, suggesting the presence of an individual producing diploid offspring in absence of queen. To ensure that no queen-laid eggs remained in the colony, all eggs were removed. Newly-laid eggs appeared again soon and 30 of them accumulated in a pile. They developed into females (workers and gynes). Observations permitted the identification of a worker displaying behaviour typical of a reproductive. It was the first individual to react by fleeing upon disturbance of the nest, and more importantly, it induced crouching posture in nestmates upon physical contacts (cf. section IV.B.). The colony was subsequently divided in two equal parts. In the part lacking the suspected reproductive individual, eggs appeared soon again, suggesting the presence of several fertile workers in the original colony. This was confirmed by the rearing of workers from the newly produced eggs. Again, a worker could be identified as presumed egg-layer in this group. Removal of these individuals for dissection (see below) gave the opportunity for one more worker to start reproducing in the first group. Production of new workers and dissection confirmed again the mated status of this third individual. No eggs accumulated in the second group.

Whether these workers reproduced by thelitokous parthenogenesis (production of diploid offspring from unfertilised eggs) or were gamergates (mated workers) was verified. First, isolation of callow workers (which had no opportunity to mate) showed that unmated young individuals neither produced diploid offspring nor males, suggesting that thelitoky is not involved and that virgin workers do not reproduce. Second, the individuals suspected of laying diploid eggs were dissected and the presence of reproductive oocytes and of sperm in the spermatheca was checked for. All these individuals had reproductive oocytes and yellow bodies of large size and number, indicative of egg-laying activity. Although it could not be determined with certainty whether the spermatheca of the first individual was empty or contained only small amount of sperm, the results were clear for the second and third individual. Their spermathecae were full with sperm, showing that they had mated.

Gynes and intercastes (n=8) were reared from the worker-produced diploid brood. This allowed for the comparison of true gyne and reproductive individuals' external as well as internal morphology. Gynes were winged and had typical large thoraces, whereas the gamergates (reproductive workers) had a worker-like slender thorax (plate 8). They were, furthermore, smaller than some of their nestmate workers. The number of ovarioles in the gamergates (n=16.0±3.6, n=3) was lower than in gynes (33.3±2.1, n=3) and non-reproductive workers of comparable size (22.1+6.1, n=109). All the workers dissected, either reproductive or non-reproductive, had a spermatheca.

III.C.e. Morphological aberration: ergatandromorphs

A colony of *M. gulosa* produced, in the laboratory, two individuals of aberrant morphology. They were male on one side and worker on the other side (plate 9), therefore corresponding to the definition of ergatandromorphs. The separation line of the 2 regions of different sexes exactly corresponded to the longitudinal axe of the body. This was strikingly shown by a darker coloration of the worker side. The male side (left side in one individual, right side in the other) possessed the normal male attributes: small mandible, large ocelli (in one, but not the other individual), long antenna with short scapes and long funiculi, wing buds (wings were probably torn off by workers), shorter legs and typical colour pattern of the V^{th} gaster segment. The thoraces and gasters were more worker-like in size and segmentation. The male side of the thoraces were not as bulky as in normal males, and the gasters lacked a supplementary segment. The different shape and relative proportion of the heads induced them to be oriented in an odd manner. These individuals were sometimes attacked by nestmates, but were tolerated. Their manipulation for examination however provoked increased intensity of aggressions by their nestmates and they were finally killed.

Dissection showed that the internal morphology corresponded only partly to the sex indicated by the corresponding side's phenotype. Both individuals possessed a sting, two ovaries and developed poison as well as Dufour glands (plate 10). Only some of the sclerites of the sting apparatus were male. The presence of yellow bodies in the older individual revealed that it had been able to oviposit.

III.D. Discussion

Queen-worker dimorphism Queen-worker dimorphism in most morphologically "primitive" species is weak (Peeters 1997), and the developmental basis of this divergence is poorly known (Dietemann and Fresneau submitted). According to Wheeler (1991), worker caste systems in social insects can be generated through regulation of three aspects of larval growth: critical size (size at which metamorphosis is initiated), growth parameters, and reprogramming of these factors. She suggests that the shape of the size frequency distribution of individuals in a colony, together with log-log plots, are reflections of developmental events in caste determination. To represent queen and worker castes on the same plot of body measurements may similarly allow the identification of some of the developmental parameters differentiating both castes. Queens of *M. gulosa* are not much larger than the largest workers. The size frequency distribution places the queens as a third mode, but with an overlap with the largest workers. The log-log plot of outerorbital distance vs. head length shows similar regression lines for queens and workers. Likewise, regression lines of outerorbital distance vs. gaster width for queens and workers were parallel, but they were discontinuous and displaced. The number of ovarioles increases twice as fast with gaster size in queens compared to workers. This suggests that queen and worker developmental pathways diverge in some but not all features, producing individuals of comparable sizes but nevertheless morphologically specialised. According to Wheeler's (1991) model of the evolution of worker castes in ants, the first step consists in the reprogramming of critical sizes (i.e. the size at which a larva initiates metamorphosis). Our data suggest that the queen and worker castes might have derived from the monomorphic ancestor by the same mechanism. Indeed queen-destined larvae might increase their food consumption after they attained their critical size and until prepupation, or might have their critical size reset so that they grow larger before initiating pupation. In addition, a resetting of the initial size occurred during larval development (regression lines are displaced), but the growth rate remained unchanged (regression lines are parallel).

Ovarian queen-worker dimorphism appears more pronounced that external morphological dimorphism. In queens, each of the 43 ovarioles on average contains a mature oocyte, whereas in workers, only about half of their 8-14 ovarioles are active and contain oocytes. Queen fecundity was estimated to be approximately 10 times higher than that of workers, with 10-11 eggs laid per day. Queens monopolise reproduction in their colonies and workers only produce trophic eggs in their presence. Oviposition by *M. gulosa* queens is cyclical: active periods are 13 days long, during which approximately 140 eggs are laid. Active periods are separated by 39 days long

intervals. Cyclical egg-laying activity seems to be common in *Myrmecia* as it occurs in several other species from different species-groups (Barnett 1974, pers. obs.).

Differences in external and ovarian morphology of queens and workers have been described by Ito et al. (1994) for *M. froggatti*, belonging to the *aberrans* group of species. This group is the most basal within the genus (Ogata 1991). Worker length varies from approximately 10 to 15mm (pers. obs.) and the colonies contain 38 individuals on average (Ito et al. 1994). The results obtained here were reanalysed in order to compare them with those found by Ito et al. (1994). Using their regression equation, a *M. froggatti* queen of 3mm gaster width has 13 ovarioles, whereas a worker of 2.5mm gaster width (16.7% difference) has 9 ovarioles (31.2% difference). According to our results, for the same relative difference in size (16.7%), a *M. gulosa* queen has 31.8% more ovarioles than the corresponding worker. Therefore, the correlation between body size and number of ovarioles is similar in these species. The divergence in ovariole number between workers and queens (workers / queens: 4-15 / 15-18 [Ito et al. 1994]; 6-24 / 39-59, this study) occurs in at least two other species (*M. pilosula* and *M. simillima*, pers. obs.) and suggests that it is an ancestral trait of the genus. This contrasts with the subfamily Ponerinae in which the two castes often have the same number of ovarioles (Peeters 1993, Ito and Ohkawara 1994). In *Harpegnathos saltator*, females have only eight ovarioles, but queens' are longer than workers' (queens lay twice as many eggs, Peeters et al. 2000). A proportion of ponerine species nevertheless exhibit marked queen specialisation in ovariole number (Peeters 1993, Ito and Ohkawara 1994).

In 2 colonies of *M. gulosa* out of 14, two dealated and most probably mated queens cohabited. Although facultative polygyny was never reported in the *gulosa* group, our data suggest that two queens can reproduce in the same colony. Colonies were regularly spaced and their density was high, suggesting habitat saturation. Under such conditions, newly mated queens may found colonies pleometrotically or may be adopted in existing nests. The only other known example of polygyny in the genus is found in the *M. pilosula* complex of species (Craig and Crozier 1979, pers. obs.).

Worker size variation and egg-laying activity Worker size frequency distribution of *M. gulosa* is bimodal (Haskins and Haskins 1950, this study). Although adults and not pupae were measured, as recommended by Wheeler (1991), the two modes in worker size distribution are not consequences of different nutritional conditions for successive cohorts of brood: the differences in

worker size were consistent among colonies (pers. obs.) and not seasonal (Haskins and Haskins 1950). Allometry of workers is weak and monophasic.

Seventy-one percent of the 992±551 workers composing a colony have active ovaries and at least 40% of them can lay trophic eggs at a rate of approximately 1 egg/individual/day. Most of these trophic eggs are given to larvae, which also feed on the insect prey retrieved to the nest. Alternatively, trophic eggs are offered to workers or to the queen, or solicited by them. Although trophallaxis occurs in the genus (Haskins and Whelden 1954), it is absent in *M. gulosa* and trophic eggs constitute the main channel of food exchange among colony members. In *M. froggatti*, large workers possess disproportionately wider gasters and more ovarioles than small workers (Ito et al. 1994). Similarly, *M. gulosa* large workers possess more ovarioles than small workers and laid 2.4 times more eggs than their smaller nestmates. Large workers forage almost exclusively (Haskins and Haskins 1950) and hence have direct access to nutrients. They collect nectar from flowers and hunt for insect prey. Our dissection of field collected individuals showed that although a larger proportion of foragers had oocytes in their ovaries compared to workers found in the nests, the oocytes of the latter were more often mature. Whether this indicates a higher egg-laying rate or a lower production rate is not known, hence whether foragers are the primary producers of trophic eggs remains to be verified. The importance of large workers in the nutrients intake and sharing is also supported by several other facts: the trophic eggs produced by large individuals are slightly larger than the ones produced by small workers, and they produce more of them per capita. Large workers are in charge of caring for larvae, which they feed with trophic eggs and prey, whereas small workers mostly care for eggs and cocoons, which are non-feeding stages. Some small workers form a retinue-like group around the queen, but she rarely solicits or obtains trophic eggs from them (pers. obs.). The central role that large individuals play in the trophic balance of a colony has been highlighted in several species belonging to different subfamilies. In *Crematogaster smithi*, large workers or intercastes (intermediates between workers and queens) produced more unfertilised eggs than their smaller nestmates (Heinze et al. 1999). These eggs were then fed to the larvae or to the queen. In *Acanthomyrmex ferox*, a few major workers produce most of the trophic eggs that are fed to other workers or to larvae (Gobin and Ito 2000).

As in many other ants, workers are able to lay male-destined haploid eggs when the queen dies or is removed. The shift between trophic and reproductive eggs takes at least 11 days. In the case of reproductive egg-laying, large workers have a higher output. They laid on average 1.6 eggs/individual/day compared to 1.0 egg/individual/day for small workers. However, when a large group of workers becomes orphaned, a larger proportion of small workers shifts to the production of reproductive eggs, while most of the large workers continue to produce trophic eggs. Thus,

although small workers are less fecund than their larger nestmates, they reproduce more readily. The divergence of worker sizes in *M. gulosa* has therefore not been accompanied by a reduction in reproductive potential of workers. Ovarian polymorphism was also reported in species with marked worker castes (*Atta*, *Acanthomyrmex*, *Camponotus*, *Crematogaster*, *Oecophylla*). In contrast, in other genera (*Eciton*, *Pheidole* and *Solenopsis)* exhibiting worker polymorphism (Hölldobler and Wilson, 1990, table 8-2), workers lack ovaries altogether (Bourke and Franks, 1995, p 228).

Alternative reproductive strategy: gamergates The occurrence of mated workers or gamergates, producing diploid offspring is reported for the first time in the subfamily Myrmeciinae. Several authors predicted their occurrence in these "primitive" ants (e.g. Crosland et al. 1988, Peeters 1997), but no evidence of their existence was found until this study. However, in an early study of *M. tarsata* (gulosa group), McAreavey (1948) unambiguously described the mating of a worker with a male:

"*… it was a surprise to find that the males, excited by the heat, were attempting to mate with the workers. During the time they were observed only one male succeeded in mating, though for a considerable time, as many as nine workers, each with a male firmly attached to the thorax, was repeatedly solicited. There could be no doubt about the actual mating of one of the pairs. At first the worker remained still for some seconds during which the male gradually relaxed his hold until he rested on his curved extended gaster with its body upright, almost at right angles to the worker, and with his wings and legs limp in the air*".

He moreover excluded any doubt about the identity of the females by stating that, although the queens were subapterous in this species and had reduced thoraces (McAreavey 1948, Peeters pers. comm. and pers. obs.), they were easily recognisable. In his words:

"*Of the twelve queens removed, two had small undeveloped wings, the others were wingless, but not difficult to distinguish from the smaller workers*".

Worker produced males of *M. gulosa* were sometimes observed to attempt mating with their orphan mothers or aunts in a similar manner (plate 11). The workers were reluctant and aggressive towards the insistent males. No successful mating could be observed. Males were often found dead a few days after their attempts. Motivated males were exposed to foreign orphan workers and virgin queens, but these consistently rejected them, indicating that inbreeding avoidance was not the reason for the females unwillingness.

The reproductive individuals found in a queenless nest of *M. pyriformis* during this study were neither ergatoïd queens nor intercastes (Peeters 1991b). Their small size, as well as thoracic and ovarian morphology were typical of those of workers. These reproductive workers can therefore be called gamergates. The number of eggs (approx. 30) counted at any time in the colony or groups of workers created from it, as well as the behaviour of individuals suggested that only one gamergate reproduced in the colony, whereas the other mated workers were reproductively inactive (according to Peeters and Crozier 1988, mated workers are called gamergates only if they reproduce). This situation is unusual compared to the know examples of regulation of mating and of reproduction in the species with gamergates of the subfamily Ponerinae. Indeed, in queenless monogynous species, the gamergate is the only mated individual in the colony. The only other reported case of mated workers co-occurring with a gamergate is *Pachycondyla (= Bothroponera) sp.* from Java (Ito 1993). On the other hand, non-reproductive, but mated individuals are common in polygynous colonies (Peeters 1993). Serial polygyny occurs in *Diacamma cyaneiventre* (André et al. 2001). In this species, a single gamergate reproduces in a colony. After its death or after colony fission, a dominant worker mates and becomes the new gamergate. In the colony of *M. pyriformis* examined here, several non-reproducing mated workers were present in the colony and replaced the gamergate when it was removed. Succession of reproductives could therefore be rapid, without delay necessary for mating. This should be confirmed by studying whether a dominance hierarchy is established among the mated workers and whether it regulates egg-laying. This example adds to the diversity of the regulation mechanisms of reproduction and to the occurrence of alternative reproductive "gamergate" strategy in ants.

From the literature and from the collection realised for this study, it appears that the number of complete *Myrmecia* colonies collected is only about 140 for 21 species. Among these, only a portion has been subjected to behavioural observations and even fewer have been dissected. The chances of finding gamergates with such sample might be faint and explain why they have not been discovered earlier. Clearly, more behavioural observations as well as dissections of complete colonies of *Myrmecia* are necessary to determine whether the occurrence of gamergates is exceptional or is part of the colony life cycle in *Myrmecia*.

Morphological aberration: ergatandromorphs The occurrence of an ergatandromorph in *M. gulosa* has already been reported by Crosland et al. (1988). Differences in repartition of the male and workers attributes described differ from the present study. The individual examined by Crosland et al. (1988) had male type antennae, the gaster possessed a supplementary segment and

the mandible on the worker side had an unusual shape. In contrast, the individuals described here had antenna and mandibles corresponding to the side's sex, but their gasters possessed the number of segments characteristic of workers. Furthermore, the ocelli of one of the ergatandromorph were worker-like. Both ergatandromorphs were dissected in this study. Bilateral sex differences in internal morphology were only partial. They possessed a pair of ovaries and worker glands, and only some sclerites were male in both individuals.

Nothing is known about the mechanism or dysfunction that produced these aberrant individuals. The occurrence and origin of sex-mosaics is better documented in the honeybee. The primary mechanism leading to these "gynandromorphs" is polyspermy, with cleavage and subsequent development of an accessory sperm nucleus. This nucleus leads to the production of the male regions, while the zygotic nucleus produces the female tissue. Other gynandromorphs had biparental female parts and maternal male parts, apparently arising from one of the haploid polar bodies. Yet another type of "gynandromorphs" produced by virgin queens was reported. They were formed by the union of egg pronuclei to from biallelic female tissue and independent development of a haploid nucleus generated male tissue (Laidlaw and Page 1997). Given that *M. gulosa* is rarely reared in the laboratory, the frequency of occurrence of these aberrant forms appears relatively high.

Evolution of worker polymorphism Morphometric studies of several *Myrmecia* species reveal variability in the degree of worker size variation. In the basal aberrans group of species, Gray (1973) described *M. froggatti* as a monomorphic species (isometric relative growth), which was confirmed by Ito et al. (1994). In the *pilosula* group, *M. dispar* (Gray 1971c) and *M. varians* (Gray 1973) are monomorphic with little size variation of workers. However, size frequency distribution of *M. dispar* workers (n=37 colonies measured) varied between colonies from uni- to bimodality, indicating an unstable pattern among colonies, which might be due to environmental variation external to the colony. Species in the derived *gulosa* group show a bimodal size frequency distribution of workers over large size ranges (Haskins and Haskins 1950, Gray 1973, Higashi and Peeters 1990, this study). The relative growth measured deviated only slightly from isometry and is similar to that described for *M. brevinoda* (Higashi and Peeters 1990). Thus *M. gulosa* and *M. brevinoda* cannot be considered as true cases of polymorphism. The various *Myrmecia* species studied seem to represent different steps toward the evolution of morphological diversity within the worker form, but none show true polymorphism (sensu Wilson 1953).

The weak allometry measured in *M. gulosa* was equal for both small and large individuals. Reprogramming of growth parameters (Wheeler 1991) is therefore not necessary to explain the occurrence of two worker size classes. The question arising is which small or large workers derived from the other. An alternative hypothesis is that both classes derived from individuals of intermediate size. Wheeler (1991) suggests that diminution in critical size is not likely and would not produce individuals of markedly smaller size than the original workers. Instead, additional castes that evolve must be derived from the pool of larvae that continues to develop. Large *M. gulosa* workers would therefore derive from small ones, indicating that queen-worker dimorphism was originally more important. A survey of queen-worker dimorphism in the genus and a better knowledge on larval development is needed to clarify the direction of size divergence in *Myrmecia*.

Comparable cases of worker size variation occur in some ponerine ants. In *Paraponera clavata*, workers show a wide range of size variation (though size frequency distribution is unimodal). The large workers are guards and foragers, whereas the small workers remain inside the nest (Breed and Harrison 1988). In *Megaponera foetens*, worker size frequency distribution is bimodal (Crewe et al. 1984). The existence of small and large workers is here more closely associated with task partitioning during foraging raids on termite colonies. The small workers enter termite galleries and large workers bring bundles of immobilised termites back to the nest (Longhurst and Howse 1979).

Worker polymorphism is thought to increase productivity of colonies by improving task performance and division of labour efficiency (Wheeler 1910, Wilson 1953). One can then wonder why worker polymorphism is not more widespread in ants. Behavioural or physiological constraints were proposed to explain this paradox. Behavioural flexibility was shown to be a more important mechanism of adaptation to short-term environmental variations than changes in physical caste distribution. Modifications in caste structure could be considered as a slowly reacting, hard-wired pattern characteristic for the species, which could reduce the scope of conditions favourable to the evolution of caste polyethism (Schmid-Hempel 1992). Wheeler (1991) suggested that morphological commitment is made at the expense of individual flexibility. The advantage of increased ergonomic efficiency provided by additional physical specialisation may not offset the disadvantage of decreased flexibility in response to environmental and demographic variability. Wheeler (1986) also proposed that developmental factors constrain the evolution of worker caste complexity. Some system of gyne determination may not be compatible with further evolution of morphological complexity. As the form of each subsequent caste is

dependent on the one preceding it, gyne determination may occur late in larval development and prevent extensive changes in worker development. Whether gyne differentiation occurs early in *M. gulosa* and leaves scope for diverging worker development requires a better knowledge of larval development and in particular the timing of gyne determination.

Furthermore, the developmental basis, as well as the driving evolutionary forces, of morphological specialisation within a worker caste may be different in ants in which workers are capable of producing males (Wheeler 1986). If physical specialisation for work is developmentally linked with a reduction in reproductive ability, the direct fitness acquired by workers through reproduction (in orphaned colonies) may outweigh the gain in ergonomic efficiency (i.e. inclusive fitness), and may therefore represent a further constraint on the evolution of morphologically complex worker castes. However, workers of some "higher" ants showing complex polymorphism are still able to reproduce (e.g. *Oecophylla smaragdina*, Wilson 1953, Hölldobler and Wilson 1983), which indicates the two phenomena are not mutually exclusive.

Beshers and Traniello (1994, 1996) proposed that size variations might have been pre-adaptations for division of labor. Among the correlates of worker body size that may affect colony fitness, Beshers and Traniello (1994) listed for large workers a general better resistance to stressful conditions, lower per unit energetic costs of activity, better performances in certain tasks, or greater food or water storage. These arguments can be applied to *M. gulosa* where large workers are the exclusive foragers (Haskins and Haskins 1950, pers. obs.) and are therefore exposed to environmental stress. Furthermore, our results show that large workers have indeed important food transfer function, as they have more ovarioles and produce more trophic eggs than small individuals. They could also act as buffer against periods of food shortage by delivering trophic eggs that can be considered as food stores. In addition, colonies could gain in efficiency by producing "cheaper" small workers, which can take care of brood and perform maintenance tasks inside the nest, where less resistance is needed because environmental stress is buffered against harsh external abiotic conditions.

Although they belong to different clades of the Formicidae, the morphologically "primitive" subfamilies Myrmeciinae and Ponerinae are comparable in that they share a mixture of ancestral morphological traits (Ogata 1991) and derived features (this study, Peeters 1997). Among the latter are early forms of worker polymorphism (Wilson 1953).

IV. Queen pheromones

IV.A. Introduction

In most insect societies workers progressively change behaviour after the loss of their queen*. Colony cohesion, activity or productivity may be reduced (Page and Erikson 1988 for bees; Berton et al. 1992 for ants; Landolt et al. 1977, Akre and Reed 1983 for wasps), workers often start to fight among each other (e.g. Dietemann and Peeters 2000 for ants; Premnath et al. 1996, Sumana and Gadagkar 2001 for wasps), to reproduce (e.g. Verheijen-Voogd 1959 for bees; Bourke 1988a, Choe 1988 for ants; Landolt et al. 1977, Greene et al. 1978, Akre and Reed 1983 for wasps; Van Doorn 1989 for bumble bees) and to rear new alate queens with the brood left by the queen (e.g. Butler 1954 for bees; Gösswald and Bier 1954, Brian and Carr 1960, Colombel 1978, Passera and Suzzoni 1979, Passera 1980, Bartels 1988, Vargo 1988, Kikuchi et al. 2000, Dietemann and Fresneau submitted, section III.C.c.). Detection by workers of the presence of their queen is therefore fundamental for colony organisation. However, the way workers perceive the presence of their queens is poorly understood. Because queens rarely, if ever, interact agonistically with workers or perform behavioural displays, it is widely acknowledged that they produce pheromones that influence their nestmates behaviour or physiology. But despite the importance of the pheromonal regulation in the insect societies' organisation, few queen pheromones have been identified and their effect characterised (Vander Meer and Alonso 1998, Vargo 1998, Vargo and Hulsey 2000).

The term pheromone was originally proposed by Karlson and Luscher (1959) and was defined as a substance secreted by an organism outside its body that causes a specific reaction in a receiving organism of the same species. Wilson and Bossert (1963) distinguished two major classes of pheromones: **releaser** pheromones acting on the nervous system of the receiver and eliciting an immediate response, and **primer** pheromones affecting the physiology of individuals and therefore having effects on the longer term. This distinction will be used to organise the results in this chapter. Queen pheromones are likely regulating many functions in the colony. I will focus here only on the pheromones responsible for worker aggregation around their queen or for her recognition (releaser effects), on pheromones preventing worker and virgin queen reproduction (primer effects). Much of what is known about pheromones concerns releaser compounds (Vargo and Laurel 1994, Vander Meer et Alonso 1998), for which bioassays can easily be set up and results obtained within minutes of the experiment.

* the term queen designates hereafter the functional mated queen heading a colony

Fifty years of research made the honeybees the best-understood system regarding queen pheromone based regulation mechanisms. Workers form a retinue around their queen, the queen mandibular pheromone together with an unknown pheromone produced in the head are responsible for the aggregation (Winston and Slessor 1998, Slessor et al. 1998). When licking or antennating their queen, workers become contaminated with queen mandibular pheromone (e.g. Butler 1954, Naumann et al. 1991). When moving inside the hive, these messenger workers (Seeley 1979) transmit the pheromone to their nestmates during physical interactions (Naumann et al. 1991). Moreover, workers can perceive pheromones that the queens deposit onto the wax (Juška 1978, Lensky and Slabezky 1981, Naumann et al. 1991, 1992). The queen mandibular pheromone is composed of a blend of at least five active compounds (Slessor et al. 1988); it is involved in the regulation of the behaviour and of the physiology of workers (Winston and Slessor 1992, Winston and Slessor 1998). In combination with semiochemicals originating in the head of queens, pheromone produced by their Dufour and tergal glands elicits retinue formation and affects worker reproduction (Wossler and Crewe 1999a, 1999b, Katzav-Gosansky et al. 2001). Brood pheromones have also been reported affecting ovarian development in workers (Jay 1968, 1970, Arnold et al. 1994). In ants, most of our knowledge of queen pheromones comes from the fire ants, *Solenopsis invicta*. The queen's poison gland produces a blend of chemicals released during oviposition, which elicits retinue formation (Vander Meer et al. 1980, Rocca et al. 1983a, 1983b, Obin et al. 1988). Investigation of primer pheromonal regulation mechanisms is facilitated in this species by the availability of queens in large numbers and by early markers of physiological response to orphanage (e.g. wing shedding prior to reproduction by virgin queens, Fletcher and Blum 1981a; larval growth resulting from sexualisation, Vargo 1988). The primer pheromones regulating these phenomena have the same source and releasing mechanism as the recognition pheromone (Fletcher and Blum 1981a, Obin et al. 1988, Vargo and Fletcher 1986, Vargo 1997).

Honeybees and fire ants represent highly derived systems in which coordinating the behaviour of thousands of individuals must involve complex regulation mechanisms. As the understanding of these mechanisms progressed, researchers discovered that a queen pheromone may have several functions depending on current circumstances, and that the signals she produces can be redundant (Winston and Slessor 1998, Slessor et al. 1998, Vargo and Hulsey 2000). Despite the remarkable amount of work done on the honeybee, the regulatory effects of live queens on worker reproduction are not yet fully understood and

could not be reproduced to the same extent with synthetic pheromones (Winston and Slessor 1998).

The model species chosen for this study, *M. gulosa*, belongs to a phylogenetically "primitive" subfamily (Brown 1953, Wilson 1971, Taylor 1988, Ogata 1991). "Primitive" ants exhibit the early stages of the evolutionary divergence between reproductive and helper castes (Peeters 1997) and their study can thus contribute to the understanding of the evolution of the mechanisms underpinning division of labour in ants. In *M. gulosa*, workers aggregate around queens in a retinue like behaviour and reproduce when orphan, providing bases for the study of queen releaser and primer pheromones affecting worker reproduction. Release, perception and action modes of these pheromones are examined.

Reports in the literature showed that the pheromones involved in worker aggregation or regulation of worker and gyne reproduction as well as gyne rearing in social insects are chemicals of low volatility that are spread over the cuticle of the queens. Good candidates for the role of pheromones are the long-chained hydrocarbons found on the epicuticle of insects (Lockey 1988, de Renobales et al. 1991). Their volatility is low and physical contact is certainly necessary for their detection. Furthermore, the cuticular hydrocarbon (CHC) profiles have been shown to correlate with ovarian status in various solitary as well as social insects (Dillwith et al. 1983, Trabalon et al. 1990, Schal et al. 1994, Ayasse et al. 1995, Peeters et al. 1999, Liebig et al. 2000, Cuvillier-Hot et al. 2001, Sledge et al. 2001). They could therefore contribute to the recognition of reproductive individuals in a colony and function as signals on the basis of which workers adjust their behaviour (Keller and Nonacs 1993, Liebig et al. 2000). However, no proof has yet been established that they are indeed used by the ants for such a purpose. Promisingly, it has been demonstrated that CHCs encode a colony specific signature and represent the basis of the nestmate recognition mechanism in three species of ants (Lahav et al. 1999, Thomas et al. 1999, Wagner et al. 1999). Importance of CHCs in queen recognition and in regulation of worker reproduction in *M. gulosa* is examined.

IV.B. Queen releaser pheromones: queen recognition and worker aggregation

IV.B.a. Introduction

Hymenopteran queens are often surrounded by workers, which provide protection and tending (Hölldobler and Bartz 1985). As queens specialised in egg-laying, they lost the ability to perform other common tasks, such as feeding or defending herself and caring for the eggs she produces. They thus greatly benefit from the continuous presence around them of workers that take over these tasks. These workers can furthermore contribute to the distribution of queen pheromone in the colony, as in the honeybee (Seeley 1979, Naumann et al. 1991, 1992). It was experimentally demonstrated that queens emit pheromones that are responsible for the aggregation of workers. These experiments consisted of extracting substances from the queen's body and presenting them to workers. They then gathered around or licked the dummy on which the chemicals were applied, as they would do with their queen (Schneirla 1953, Stumper 1956, Watkins and Cole 1966, Jouvenaz et al. 1974, Fowler and Roberts 1982, Glancey et al 1984 for ants; Butler 1954, Pain 1954, Groot and Voogt 1954 for bees; Emerson 1939 for termites; Ishay et al. 1965 for a wasp). Workers also aggregated around queen corpses, excluding any behavioural factor in the triggering of the recognition or aggregation (Watkins and Cole 1966 for army ants; Butler 1954, Velthuis 1972 for bees).

The pheromone responsible for the aggregation of workers around their queens and the pheromone mediating queen recognition are intimately linked. Whether they can be distinguished at all is uncertain. Both effects may well be induced by a single substance or blend of substances. Researchers frequently interchanged the two terms (Lofgren et al. 1983, Edwards and Cambers 1984, Cariou-Etienne et al. 1992, see also Vander Meer and Alonso 1998). Given that retinue formation is bound to queen recognition, and that recognition is generally defined by the observer on the basis of worker aggregation, I will not strictly distinguish both concepts. I will nevertheless use each term when it is more adapted to the phenomenon studied.

Researchers designed experiments to identify with more or less precision the source of the pheromone responsible for this aggregation, but rarely isolated the active chemicals (Vander Meer and Alonso 1998 for a review). The origin and identity of the aggregation pheromone emitted by *M. gulosa* queens was investigated. As a first step, the part of the queens' body from which it is available and by which part they are emitted was determined using non-destructive techniques. On the other hand, identification of their source necessitated

destructive bioassaying of gland products and is limited by the number of colonies available. The role of the releaser queen pheromone eliciting worker aggregation and whether it is involved in the regulation of worker reproduction is discussed.

IV.B.b. Perception distance of queens by workers

Introduction The gathering of workers around their queens is commonly attributed to queen "attractiveness". The term attraction implicitly refers to a remote pheromonal activity of queens on workers. However, none of the experiment designed to study this type of pheromones allowed to clearly differentiate between attractant and arrestant effects. Indeed, there is no need for a volatile attractant pheromone, since workers could contact the queen by chance and be stimulated to aggregate around her (Vander Meer and Alonso 1998).

Workers were reported to detect the presence of their queen at a distance in the honeybee (Velthuis 1972), in *Vespula consobrina* (Akre et al. 1983), *Myrmica rubra* (Coglitore and Cammaerts 1981), *Pachycondyla* cf. *"inversa"* (Tenschert et al. 2001), *P. apicalis* (pers. obs.), and *Harpegnathos saltator* (Liebig pers. comm.). In these species, workers show a specific behaviour as they approach the queen at close range, testifying that recognition takes place without actual contact. However, this distance has never been measured and whether it can be interpreted as an attraction is not clear. The same phenomenon of remote recognition occurs in *M. gulosa*. I measured the distance from the queen at which workers display a typical posture of body and antennae stretching, which indicate that they perceived the queen's presence. Whether this distance perception can be considered as an attraction and plays a role in regulation of worker reproduction is discussed.

Methods As workers walk in the nest, they scan the space in front of them with continuous sweeping movements of their antennae. The angle between scape and body axe varies between 45 and 90 degrees approximately, while the angle between scape and funiculus varies between 45 and 135 degrees. This pattern is modified when workers approach their queen: as they come in close range to her, they stop walking forward, and stretch their legs to bring their head closer to the queen, while their antennae are stretched in her direction. The angle between scape and funiculus increases until approximately 170 degrees, when the two funiculi are almost parallel, and the angle between both antennae decreases (plate 12). However, no physical contact is made, the antennae do not touch the queen's body.

Twenty workers (10 large and 10 small individuals) of 6 colonies were taken from brood chambers of their nest. They were placed together with their queen in a glass Petri dish (240Øx80mm) with slightly moist filter paper on the bottom. The behaviour of workers in the proximity of the queen was videotaped for 3 hours. The distance at which both large and small workers (n=40 measurements) detected the queen was measured on the monitor's screen when playing back the tapes. Using the frame-by-frame play mode, the image was paused when the workers shifted from the scanning antennal movement, to a queen directed antennation (i.e. as the angle between the scapes and funiculi reached 170 degrees and the antennae both pointed in the queen's direction). The shortest distance from the tip of a worker's antenna to the queen's body outline was then measured. Only distance to head, thorax or gaster was considered; antennae, legs and mandibles were excluded. This distance was only measured when workers encountered the queen without being disturbed by the proximity of a nestmate or of the Petri dish wall. Measurements on the screen were scaled to the real distances. As workers were not marked, their perception distance could be measured several times. Perception distances were averaged for large and small workers of each colony and compared with a Wilcoxon test for paired samples. In order to determine the importance of vision in this behaviour, occasional control observations were realised under red light, in the rearing nest. Whether body stretching was specifically elicited by the queen's proximity was also examined during these observations.

Results Workers displayed the characteristic body stretching as they approached their queen, but not when approaching nestmate workers or virgin queens. This behaviour took place under red light, as under daylight. Large and small workers had similar perception distances of their queen (Wilcoxon test, T=2.0, p=0.08, figure IV.B.1.). All measurements can therefore be averaged (large and small individuals from all the colonies) to give an estimate of this distance: workers reacted to the presence of the queen 12.2$\pm$5.4mm (mean$\pm$st.dev., range 0-29.6) away from her. Although it was not assessed precisely, it was obvious that workers initiated the stretching behaviour when approaching the queen from any side.

After perception of the presence of the queen, workers either backed away rapidly without making physical contact with the queen, or walked forward and touched her with their antennae. Upon contact, workers adopted a crouched position and retract their antennae along the side of their head.

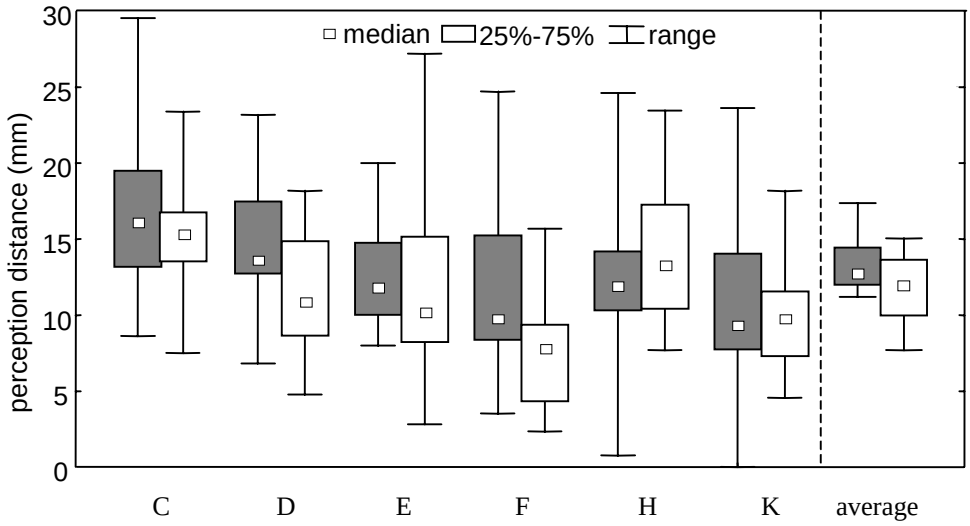


Figure IV.B.1.: perception distances of their queen by large and small workers in 6 colonies of *M. gulosa*. The last bars on the right represent the average of the measures. Average perception distances of large and small workers were not significantly different (Wilcoxon test, p=0.08).

Discussion Workers did not show any particular change in motion when they encountered another worker or a virgin queen as they did with a functional queen. However, occasional observations indicated that the behaviour of body and antennae stretching measured here is not exclusively triggered in the vicinity of the queen. It is a characteristic reaction observed when the ants perceive a stimulus from a distance (i.e. by other sensory channels than direct antennal contact: vision, mechanoreception or olfaction); they then seem to proceed cautiously forward toward the source of the stimulus to make physical contact and use contact chemoreception to presumably gain more information. Observations under red light and immobility of the queens demonstrated that neither vision nor behaviour are involved in triggering the sudden change in behaviour shown by workers when they approach their queen. This suggests that queens emit slightly volatile chemicals, which might mediate their identity or simply trigger the cautious approach of workers. The behaviour following this extension of the body indicates that the queens are recognised: workers either cautiously move forward and made antennal contact or swiftly run away without making physical contact (signification of this behaviour will is further discussed in section IV.D.). Large and small workers have the same perception distance of their queen, indicating similar behavioural threshold concentration (Wilson 1970).

In a majority of cases, workers approached the queen during random explorative movements in the Petri dish. Although the distinction remains subjective, they antennated the

ground rather than the air as one would expect when a worker follows a gradient of volatile pheromone up to its source. Given the short range of action of this pheromone, its designation as an attractant seems inappropriate. I will therefore use the terms “aggregation” instead of the commonly used term “attraction” to describe the phenomenon of workers gathering around the queen and “arrestant” instead of “attractant” to describe the type of pheromone involved. Queens do not seem to be able to attract workers from a long distance. Whether the short-range recognition pheromone highlighted here plays a role in regulating worker reproduction cannot be determined until it is chemically identified and bioassayed.

Most workers do not stay at the queen’s side after perception of her presence, and this independently of whether they touched her or not. There is however, a group of workers that characteristically stay close to the queen and spend most of their time at a distance that is inferior to the perception distance of 12mm measured here. They must therefore be continuously aware of the queen’s presence. Their behaviour is described in the following section.

IV.B.c. The retinue

Introduction As in many ant species, the queen of *M. gulosa* is continuously surrounded by a group of workers (plate 13). The gathering of worker honeybees around their queen has been termed “retinue” (e.g. Velthuis et al. 1965) and given the similarity of the phenomena, the same term will be used here. The number of workers forming the retinue around *M. gulosa* queens was assessed, as well as the frequency of their presence at her side. Workers were also exposed to the corpse of their queen and their interest was monitored. In addition to the identification of the characteristics of the retinue formed around a live queen, this experiment can help to determine the mode of release of the arrestant pheromone and the role of the queen’s behaviour in worker aggregation. The function of the retinue is discussed.

Methods

Number of workers in the retinue Behaviour of retinue workers was observed throughout this study (over 200 hours of observation). Retinue workers typically stay close to the queen, with their heads pointing in her direction. They form a group of individuals clearly organised around her, distinct from the individuals staying further away: the workers behind the more or less regular circle formed by the retinue do not orient themselves toward the queen and thus

do not seem to detect her presence. Only workers oriented toward the queen and with their head at a maximum distance of 30mm from her were considered as belonging to the retinue. The number of individuals forming the retinue was counted at 10 minute intervals for one hour (n=42 hours in total, in 3 colonies: A, B and C). In order to compare the number of workers gathering around the queen or a random worker, counting was repeated by focusing on a control individual instead of the queen. Although the behaviour of workers toward a nestmate is qualitatively different (there is no orientation toward it), the comparison of how many individuals stay within the same perimeter (30mm) around a nestmate worker helps to characterise the retinue formed around the queen. The control individual was chosen according to its large size (comparable to the queen's), its position near the brood (similar to the queen's in order to control for the effect of brood), but as far as possible from her to avoid any interference. Queens are often stationary, whereas workers continuously move in the nest. The control worker was therefore also chosen according to its immobility. If it started moving during the observation, a neighbour worker was chosen according to the same rules and the observations resumed. Observations focusing on control workers consisted of 2 hours bouts on one day and a 1 hour bout on the next day. Colony A was observed twice and colony B once. The results were compared with those obtained for the queen during observation periods matched for duration and frequency. Nest size was modified in colony A in order to test the role of density on the number of workers in the retinue or around a nestmate (original nest area 770cm^2 down to a 380cm^2 nest). Ten days were allowed for adaptation in the new nest, after which the counting was repeated. In order to determine the role played by the shape of the queen, the behaviour of workers toward virgin queens was examined. As virgin queens are rarely produced in the laboratory, these observations were occasional.

Frequency of presence in the retinue All the workers in colonies A and B were individually marked with colour dots on the thorax. The identity of workers in the queen retinue and around a control worker was noted during the above-mentioned observations. The presence of workers beside the queen could therefore be quantified in term of frequency. Again, the effect of density was measured after the density in colony A was changed.

Arrestant properties of queen corpses Dead queens (n=4) were tested for their ability to trigger a retinue. The queens from two colonies (C and D) were killed by freezing and reintroduced in the nest after thawing. The number of workers in close proximity to the queen and with their head and antenna clearly oriented toward her, sometimes antennating her, was

counted at one-minute intervals for ten minutes both before and after they were killed. In the other colonies (A and G), the queen died naturally and her corpse stayed 1-2 days unnoticed in the nest.

Results Virgin queens never elicited formation of a retinue. The retinue surrounding functional queens were mostly composed of small workers, whereas large workers only occasionally took part to the retinue. From 4 to 18 (mean$\pm$st.dev.=10.3$\pm$3.8) individuals continuously sat in a crouched posture next to a queen. When she was at rest, one of the retinue workers typically sat under her body, between her legs, whereas the others stayed a few millimetres away from her. The latter rarely made physical contact with her or groomed her. Contact occurred when the queen was moving and hit the workers with her legs or when she antennated individuals next to her. When the queen oviposited, her eggs dropped to the ground. The retinue workers then seized and carried them to the pile. However, a retinue was continually present, independently of the oviposition activity of the queen. The number of workers in the retinue was different among the three colonies studied (Kruskal-Wallis test, n=262, H=172.9, $p<0.01$). When density of nest A was doubled, the proportion of workers in the retinue remained constant (Wilcoxon test on the arcsine transformed proportions, n=45, H=475.0, p=0.63).

The number of nestmates next to the queen or next to a control worker was similar in both 3 hour observation periods in colony A (Wilcoxon test, $n_{queen}=18$, $T_{queen}=49.0$, $p_{queen}=0.33$; $n_{worker}=18$, $T_{worker}=44.5$, $p_{worker}=0.94$). The data were therefore pooled together. Colony B was observed for a single 3 hours period. Significantly fewer workers were observed next to a large immobile worker than beside the queen in the two colonies studied (Wilcoxon test, $n_A=32$, $T_A=3.5$, $p_A<0.01$, $n_B=16$, $T_B=0.0$, $p_B<0.01$, figure IV.B.2.). The cumulated number of individuals counted after three hours of observation was higher at a worker's side in both colonies (control worker vs. queen: A_1: 47 vs. 40, A_2: 42 vs. 28, B: 36 vs. 34). This indicates a higher turnover of individuals around another worker than around the queen.

The phenomenon was confirmed by the observation that workers were present more frequently beside the queen (a typical frequency distribution is presented in figure IV.B.3.). That is, they stayed for longer next to the queen. An individual's frequency of presence at the queen's side varied from a single visit to almost continuous over the observation period (three hours over two days). A higher density in nest A resulted in a general decline of the frequency that individuals were observed near the queen, whereas the global frequency of presence next

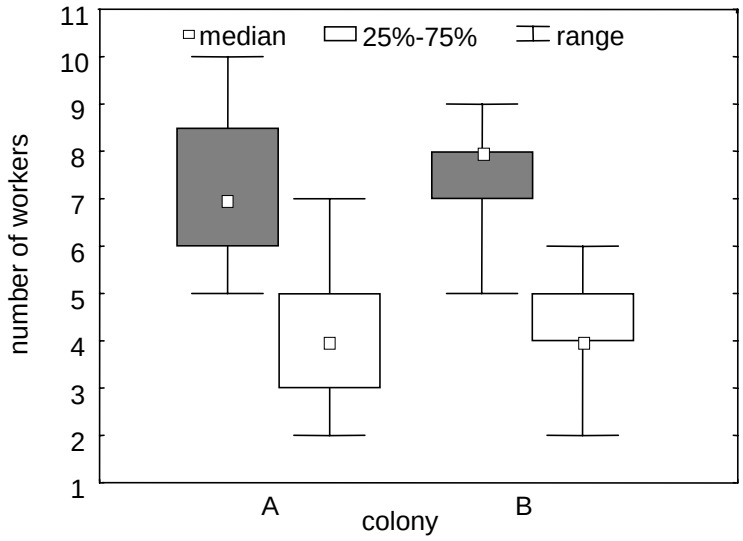


Figure IV.B.2.: number of individuals present next to the queen (plain boxes) or next to a control worker (open boxes) in two colonies. The difference in numbers of workers surrounding the queen or the worker is significantly different in both colonies (Wilcoxon test, $p<0.01$).

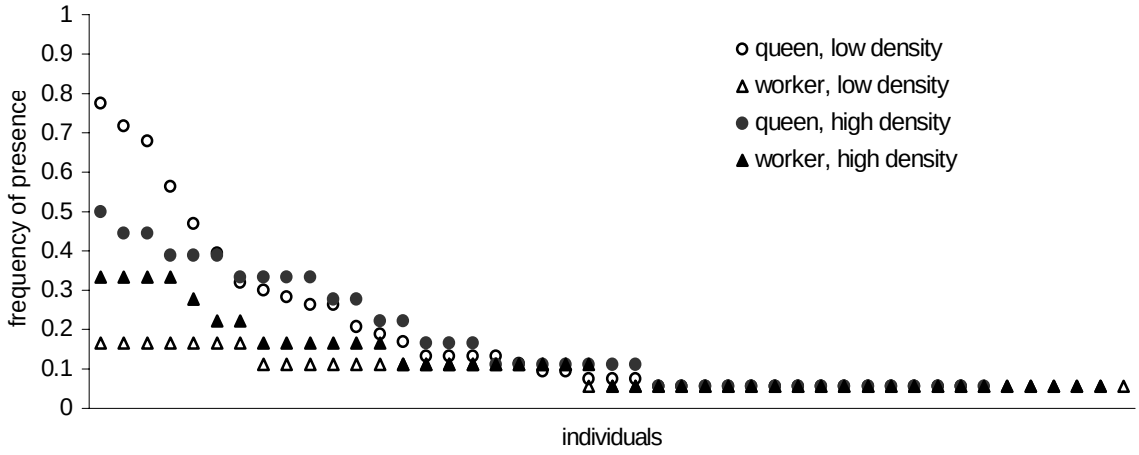


Figure IV.B.3.: typical distribution of frequency of presence of workers at the side of either their queen or a large, immobile worker over a total of 45 observations in one colony. Each symbol represents a worker, but two symbols with the same x value do not correspond to the same individual. For clarity, the individuals were classified in the descending order of frequency values. The observations were repeated ten days after the density was doubled in the nest.

to a worker increased (figure IV.B.3.). In the three cases studied, the frequency of presence of an individual beside the queen was correlated with the number of physical contacts that occurred with the queen (coefficients of determination ranging from 0.88 to 0.96).

On four occasions, gathering of workers around queen corpses could be observed. In the two cases where queens could be tested both alive and dead, the number of workers surrounding a queen corpse was higher than that surrounding the live queens (Wilcoxon test, n_C=10, T_C=0.0, p_C<0.01 and n_D=10, T_D=0.0, p_D<0.01). Corpses were antennated much more intensely than the live queens. In both these colonies, the number of workers surrounding queen corpses was significantly higher than that counted around a worker corpses (Mann-Whitney U-test, n_C=10, U_C=0.0, p_C<0.01, n_D=10, U_D=0.5, p_D<0.01) (figure IV.B.4.). When 2 queens died naturally and their corpse remained in the nest, workers aggregated around them for at least 1-2 days after death.

Discussion Retinue workers point their heads and antennae in the direction of the queen and thus form a more or less regular circle around the queen. Mostly small individuals compose the retinue and large workers only occasionally take part to it. Some workers stay "faithful" to their queen in that they almost constantly remain at her side. In contrast, a higher number of individuals can be observed next to a worker over the same period. There is a constant turn-over of individuals that move inside the nest, so that they do not stay for long next to a

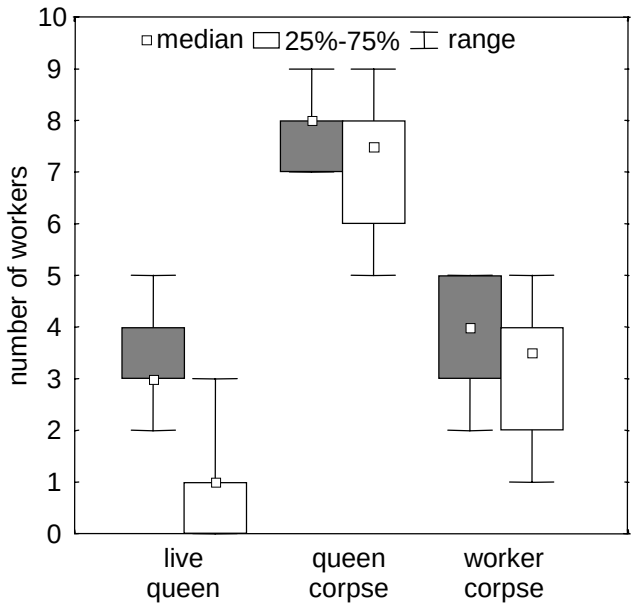


Figure IV.B.4.: cumulated number of workers gathering around a live queen, her corpse, or around the corpse of a large worker in 2 colonies (C: plain boxes, D: open boxes). The number of workers in the aggregation was counted at one-minute intervals for 10 minutes.

nestmate. Their behaviour is qualitatively different from that of retinue workers, as they do not orient toward their nestmates and generally ignore their presence. There are therefore fewer individuals surrounding a queen than expected by chance, which is at least in part explained by the repulsive effect she possesses on most individuals (section IV.B.b.).

Density did not affect the proportion of workers in the colony that took part to the retinue, but did affect the duration they spent next to a nestmate. As density was increased, workers were observed less often next to the queen, but stayed longer beside a worker. This last effect is well explained by congestion in a smaller nest, but the link between congestion and the reduction in frequency of presence in the retinue of the queen is not clear. Repeating the manipulation is necessary to draw conclusions on the effects of colony size and density.

In the laboratory virgin queens were only occasionally produced. As they cannot disperse, they stay in the nest and behave as workers. Workers do not show interest in virgin queens as they do for mated queens. As in all the ant species studied, the shape of a gyne alone was not enough to elicit the formation of a retinue (e.g. Coglitore and Cammaerts 1981, Edwards 1987). Although no virgin queens were available to test the effect of their corpses on the aggregation of workers in these two colonies, they never triggered the interest of workers to a remarkable degree in other colonies. In contrast, when queen corpses are present or reintroduced in their colonies, they elicit a gathering of workers. Moreover, more individuals assemble around them than around large nestmate worker corpses. This gives evidence that dead queens are still recognised and that a pheromone, but not behaviour is involved in this recognition. Recognition and retinue formation still took place 1-2 days after death. The substance involved therefore remains on the cuticle after death. Two mechanisms can explain this persistence: the chemical could passively ooze out of a reservoir for some time after the queen's death or could remain on her cuticle after its production ceased, and until its complete removal by antennating and licking workers. In the latter case, the chemical must have a low volatility. Natural degradation or evaporation might also be involved in its fading.

Workers recognise queens from a short distance (cf. section IV.B.b.) and most of them avoid making prolonged contact with her or avoid contact altogether. It was nevertheless clear that workers antennate queen corpses more intensely than live queens. It is likely that the emission of the chemical responsible for the close range recognition of the queen is modified or interrupted after her death or that other chemicals, which overcome her repulsive effect, are released by the corpse. The perception distance of a queen corpse by workers was not measured and whether this substance is still emitted is not known. This observation suggests that the behaviour of workers gathering around the corpse of a queen is not comparable to the

retinue behaviour. Furthermore, workers also aggregate and inspect the fresh corpse of a nestmate worker, whereas live workers are usually ignored. This indicates that individuals can discriminate live from freshly killed individuals. Gathering of workers around a queen corpse might therefore be due to the effects of death rather than to persistence of the pheromone eliciting retinue. The use of queen corpses to exclude behavioural effects in retinue formation, as suggested by several authors (Coglitore and Cammaerts 1981, Velthuis 1972), has therefore to be realised with care. Characteristics of worker aggregation around queen corpses should also be controlled with live workers and worker corpses. Further significance of this observation is given in the section IV.C.d.

Queens of *M. gulosa* elicit an assembly of workers around them at all times. Retinue workers neither feed nor groom the queen frequently, indicating that their presence is not directly linked with queen tending. Furthermore, as oviposition activity of queens is cyclic (cf. section III.C.c.), the presence of workers around their queen is not exclusively due to egg-laying of the queen. They may play a role in the distribution of queen pheromones, as do retinue honeybee workers (Seeley 1979). This possibility will be examined in section IV.C.b.

IV.B.d. Source of the queen arrestant pheromone

Chemical analyses described in this section were realised in cooperation with the laboratory of Graeme Jones of Keele University in the frame of the European Community "Training and Mobility of Researchers" and "Improving Human Potential networks and particularly the "INSECTS" (INtegrated Studies of the EConomy of insecT Societies) project. It is mainly the work of Ph.D. student Richard Beard. Analyses of the chemical composition of the queen and queenright as well as orphaned worker (cf. section V.B.) major glands and cuticle of *M. gulosa* were conducted.

IV.B.d.i. Distribution of the arrestant pheromone on the queen's body

Introduction Researchers designed various simple experiments to locate the source of the pheromone responsible for the aggregation of workers around their queens. These consisted of restricting their access to different parts of the queens' bodies (Butler 1954 for bees, Passera 1980 for ants) or presenting them body parts of queens and monitoring the persistence of aggregation and tending by workers (Brian 1973, Passera 1980, Coglitore and Cammaerts

1981, Cariou-Etienne et al. 1992, Vargo and Hulsey 2000 for ants; Pain 1955, Darchen 1956, Verheijen-Voogd 1959 for bees). Ultimately, these experiments gave more information on the availability of pheromone on each body part that on their source. I used a similar methodology in order to check whether the chemical responsible for the aggregation of workers around *M. gulosa* queens could be obtained from a particular part of their body or if it was spread all over it. For this, access of workers to their queen was restricted to half of the body, that is, to her gaster (i.e. the abdomen without first segment, the latter being attached to the thorax) or her head plus mesosoma (thorax and first abdominal segment) with the legs.

Methods I separated a Petri dish (200Øx35mm) in two equal parts with a vertical plastic plate. In the middle of the plate a 6x1mm vertical slit was cut into which the queen's petiole was fitted, so that her gaster protruded in one half of the Petri dish and the head, mesosoma and legs (front body portion) in the other side, while she was in a natural resting position). Ten workers (five large foragers and five small individuals from a brood chamber) were introduced in each of the sides. The Petri dish was covered with a Plexiglas roof to allow observation and prevent the ants from escaping. A 25cm^2 area around the queen was videotaped for one hour. After a 15 minute habituation period, the aggregation of workers around the queen was monitored on replay by pausing the tape. The number of workers gathering around the queen's body parts was counted at 5 minute intervals for 45 minutes. Workers were then exchanged between parts to control for their preferences. After a further 15 minute habituation period, the videotaping and counting was resumed. Each 5 minute interval was treated independently from the preceding interval, with the result that a worker present at the queen's side could be counted several times. Counts were considered as independent for statistical treatment with a Wilcoxon test. This experiment was conducted three times with different colonies (C, D, E). The queen C was killed by freezing at -70°C for 15 minutes and the experiment was repeated with her corpse after 10 minutes thawing.

Results Gaster elicited aggregation of a significantly higher number of workers than the front body portion of queens in two colonies out of three (Wilcoxon test, T_C=16.5, p_C<0.01; T_D=10.0, p_D<0.01, T_E=43.0, p_E=0.33, figure IV.B.5.). The front portion of the dead queen elicited aggregation of an equal number of workers than her gaster ($T_{dead\ C}$=24.0, $p_{dead\ C}$= 0.24). Comparison of the aggregative power of the queen before and after death showed that there was an increase in the number of workers gathering around her front portion ($T_{live/dead}$=5.5, p<0.01). In contrast, the gaster kept similar properties ($T_{live/dead}$ =54.0, p=0.47).

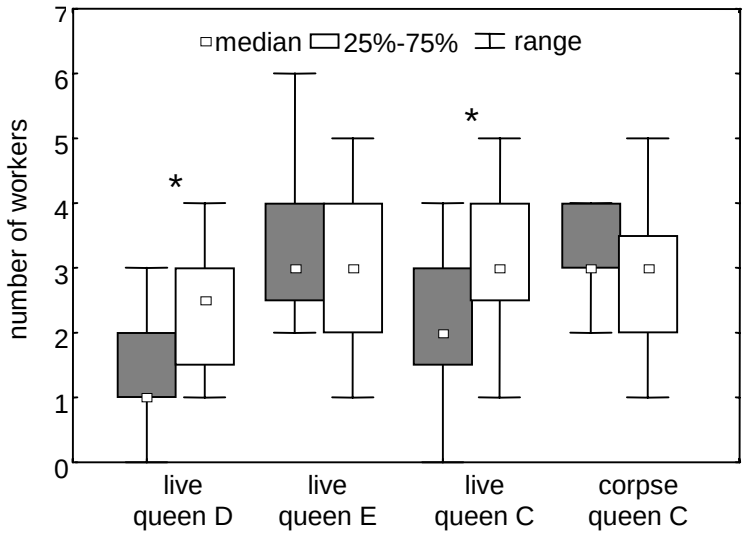


Figure IV.B.5.: cumulated number of workers gathering around the anterior part (head, thorax and legs, plain boxes) of their queen or around her gaster (open boxes). The experiment was realised with live queens (colonies C, D and E) and a dead queen (colony C). Workers had access to only one of the queen halves for 10 observations (counting for 45 minutes at 5 minutes intervals) and were then given access to the second half for 10 more observations. Thus, each bar represents 20 counts. * indicate significant differences (Wilcoxon test, $p<0.01$).

Discussion Both anterior and posterior body portions of the queen elicit retinue formation, indicating that the pheromone is spread over the whole body. Workers approaching the queen from any direction can therefore recognise her and be simulated to stay at her side. The test conducted with a freshly killed queen showed that the aggregative power of her gaster remained constant shortly after death, whereas the rest of the body elicited more aggregation. A behavioural effect (i.e. absence of behaviour) or a change in chemicals emitted could explain this difference and therefore be involved in combination with pheromones in the aggregation of workers around queens. The experiment should be repeated to verify the constancy of the phenomenon.

Among others, Coglitore and Cammaerts (1981) and Vargo and Hulsey (2000) proposed that the body part attracting the higher number of workers is likely to contain the source of the chemical responsible for the attraction. In *M. gulosa*, the number of workers aggregating around the head, mesosoma and legs was inferior or equal to the gaster. The gaster's surface area being markedly smaller than that of the head, mesosoma and legs together, it must be richer in arrestant pheromone and might contain its source. If it is assumed that the pheromone is produced in a gland and released from a single point, a

mechanism must exist to spread it over the cuticle. It is widely acknowledged that selfgrooming could serve this purpose. Naumann (1991) showed that grooming behaviour was partly responsible for the backward translocation of a queen mandibular pheromone component in honeybee workers exposed to lures treated with the substance. The same behaviour is likely to have the same effect in queens that produce the chemical.

According to recent studies, several other hypotheses have to be considered. The hypothesis proposed by Coglitore and Cammaerts (1981) and taken over by Cariou-Etienne et al. (1992), is that dispersed epithelial cells produce the attractant, so that each body part emits some pheromone. Alternatively, several glands located in different body parts were shown to produce arrestant substances in the fire ants and the honeybee queens (Slessor et al. 1998, Wossler and Crewe 1999a, Katzav-Gozansky et al. 2001, Vargo and Hulsey 2000). These glands might produce similar products or different substances having the same effect on worker behaviour. In this case, pheromone repartition on the body is dependant on the mode of release of the different gland products as well as on self-grooming. According to this view, the gaster of *M. gulosa* queens arrested more workers than the front body portion because of a more important pheromone production (if it contains more epithelial cells or a more productive gland), or because it is the source of the most efficient arrestant (if the products of different glands have different properties in eliciting worker aggregation).

Selfgrooming blurs the picture of pheromone emission due to the contamination it induces, and makes the identification of its source more difficult. Cariou-Etienne et al. (1992) showed that the mesosoma of *Iridomyrmex humilis* was the most attractive part of queens when presented to workers with the legs. Once the legs were removed, the mesosoma lost a considerable part of its power to elicit aggregation, thus showing the importance of the legs. The loss of interest for crushed legs did not satisfactorily prove the role of tactile cues and disprove the role of chemicals in eliciting aggregation. Contamination from internal products may have overridden the arrestant. As discussed by the authors themselves, it remains likely that the legs accumulate the pheromone during self-grooming.

Since self-grooming was impossible during the experiment realised here with *M. gulosa*, the mesosoma and head should have lost their “attractiveness” after some time if the gaster was the exclusive source and emitter of the chemical. The decrease in “attractiveness” of different body parts with time was rarely tested in studies on queen “attraction” (see Brian 1973 for an exception). This might represent a more reliable method to locate the source or at least the emission site of the pheromone in the body part that keeps its properties for the longest duration. Preventing active spreading of the pheromones (either by using severed

body parts or by preventing selfgrooming) should induce a faster depletion in pheromone on the parts normally contaminated during self-grooming and not emitting it. The duration of the experiment described here was too short for a depletion to be sufficiently pronounced and the "attractiveness" to disappear. The queen should be kept without possibility of self-grooming for at least 8 hours in order to test which of these hypotheses (discrete vs. diffuse pheromone source) is correct (queens perform self-grooming for 2min42±3min52 every 2h±1h40, range 1min40-8h40, n=144 hours time-lapse video-recording on colonies A and B). Unfortunately, this is too disturbing for the queens, as well as for the colonies deprived of their queen for the duration of the observations.

The best-studied species for pheromone production, transport and accumulation is the German cockroach (*Blattella germanica*). Radiolabeling technique was used to study the production and translocation of sexual pheromones and their hydrocarbon precursors from the production to the deposition sites. The compounds were produced by abdominal integument and appeared to be shuttled by lipophorins in the hemolymph to the epicuticle, among other deposition sites. The end result is a rather even distribution of the products on the whole cuticle of the insect differing dramatically from the internal distribution. Indeed, most of the compounds are located in the abdomen, which is their production site (Gu et al. 1995). Identification of the pheromones in ants has still to be achieved before one can elucidate their transport mechanisms.

Attempts to identify the source of queen recognition pheromones through sections of queens or differential access to body parts of live individuals have rarely been successful. These methods give a good picture of the distribution of the queen pheromones on her body and of their availability to workers, but do not allow locating their origin unless they are crushed (Vargo and Hulsey 2000). Ultimately, the most reliable way to identify precisely the source of a pheromone is to test individually the possible production organs or tissues (e.g. Vander Meer et al. 1980). In the following section, I examine the role of the CHCs and narrow down the search of the source of arrestant pheromones to the level of glands.

IV.B.d.ii. The role of the cuticular hydrocarbons (CHCs)

Introduction The identity, source and emission of arrestant pheromones have been characterised in only two Hymenoptera species so far: the honeybee and the fire ant. In honeybees, a blend of five non-volatile fatty acids and aromatic compounds is produced in the

mandibular glands and spread over the cuticle presumably by self-grooming (Slessor et al. 1998, Naumann 1991, Naumann et al. 1991, 1992). The tergal and Dufour glands produce other attractants, but their chemical nature is not known (Wossler and Crewe 1999b, Katzav-Gozansky et al. 2000). In the fire ants, the poison gland produces a blend of complex ketones released during egg laying, which have arrestant properties (Vander Meer et al. 1980, Rocca 1983a, 1983b, Obin et al. 1988). In the two last cases, whether workers obtain the pheromones from the cuticle of queens remains to be elucidated. Although nothing is known on the chemical identity of arrestant pheromones in other ants, cuticular extracts of queens of several species belonging to the formicine, myrmicine and ecitonine subfamilies have been tested for their arrestant properties. It was demonstrated that the chemicals extracted with various organic solvents elicited aggregation (Stumper 1956, Watkins and Cole 1966, Delage 1967, 1968). In some species, however, this method did not yield reproducible results (Stumper 1956, Cariou-Etienne et al. 1992) or yielded negative results (Carr 1962, Delage 1968, Coglitore and Cammaerts 1981).

Here, the possibility that chemicals present on the surface of *M. gulosa* queen's cuticle carry information about her identity and trigger worker aggregation was investigated. Cuticular extracts were realised with a nonpolar solvent (hexane), thereby preferentially collecting hydrocarbons. Hydrocarbons represent the most abundant class of epicuticular lipids in insects (Lockey 1988). They contribute to the protection of the insects against desiccation (Wigglesworth 1964, Hadley 1980) and also play a role in communication. In solitary insects, they serve as sex pheromones or anti-aphrodisiacs and presumably intervene in mate recognition (reviewed in Singer 1998, Howard and Blomquist 1982, Howard 1993). In several species of ants, it has recently been shown that cuticular hydrocarbon (CHC) compounds mediate nestmate recognition (Lahav et al. 1999, Thomas et al. 1999, Wagner et al. 1999). CHC blends of most the species investigated are complex mixtures of dozens of compounds of different classes. Enough variation to encode queen identity in addition to colony recognition can presumably be obtained by variations in quantity (absolute or relative) of the HCs present on the cuticle.

Correlation of CHC profiles with the reproductive status of individuals has been described in several solitary as well as social insects (Dillwith et al. 1983, Trabalon et al. 1990, Schal et al. 1994, Ayasse et al. 1995, Peeters et al. 1999, Liebig et al. 2000, Cuvillier-Hot et al. 2001, Sledge et al. 2001). The queen of *M. gulosa* is the sole reproductive individual in the colony (cf. chapter III.), a queen-specific signature encoded in CHC profile or a fertility signal (Liebig et al. 2000) could therefore contribute to queen recognition by

workers. Moreover, behaviours like trophallaxis and intensive grooming or licking of the queen, promoting the formation of a colony "Gestalt" (by distributing and mixing the genetic and environmental cues contributed by the individuals in a colony, Crozier and Dix 1979, Soroker et al. 1995) are absent or limited in *M. gulosa.* One can therefore expect differences in CHC profiles to occur between workers and functional queens.

Hydrocarbon, as well as other lipid constituents of *M. gulosa* workers collected in the Sydney region have already been identified by Cavill et al. (1970). The authors collected 200g of ants (presumably hundreds of workers from different nests) of which the head, thorax and gasters were separately extracted by the Soxhlet method. The principle of this extraction technique being to pool the hundreds of body parts obtained, interindividual variation disappears and all the internal and glands products are mixed with the cuticular compounds. In order to allow interindividual comparisons as well as separation of the products from difference sources (cuticle versus glands) the analysis had to be repeated with modern techniques. Gas chromatography coupled with mass spectrometry is sensitive enough to allow single individuals' cuticle or glands to be analysed and compared.

Workers of several ant species use the hydrocarbon fractions of cuticular extracts to discriminate nestmates from non-nestmates (see above). In contrast, honeybee workers could not be trained to differentiate between extracts of comb waxes of varying composition and nestmate of different castes on the basis of their HC fraction. In fact, bees used more polar compounds for discrimination (Fröhlich et al. 2000, 2001), suggesting potential variability in the cues used. The first step to verify the hypothesis that CHCs are used by *M. gulosa* workers to detect the presence of their queen is to demonstrate their ability to perceive HCs. Whether this was the case was tested by applying single synthetic hydrocarbons on live workers. The behaviour of nestmates facing these dummies was monitored. In order to test the response of workers to a queen specific mixture of HCs, a synthetic queen CHC blend was made up. The HCs that represented the major quantitative differences between queen and worker profiles were mixed together in appropriate quantities so as to transform a worker on which the blend is applied into a queen-like individual. Among the 93 compound peaks extracted from cuticles by SPME, 11 major HC compounds were synthesised by Richard Beard from Keele University and 4 purchased (corresponding to a total 13 compound peaks from the queens' CHC profiles).

Methods

Characterisation of queen and worker CHC profiles

Comparison of absolute quantities In order to estimate the difference in quantities of compounds extracted from queens and workers, hexane extracts were analysed by GC (cf. section II.C.b.). To establish the correlation of the quantity of CHCs extracted with body surface and with oenocytes density (cf. section V.E.), only gasters were extracted. Three queens (from colonies B, E and I) were extracted and the total peak areas averaged for three runs of each extracts. Workers from colonies (B, C, D, E, F and I) were extracted and the extracts run once each.

CHC profiles of *M. gulosa* contain numerous peaks. The number of variables to perform statistical analysis was reduced by selecting those peaks which represented more than 0.1% relative peak area, and which occurred in more than 80% of all individuals. The extraction method was standard; the total amount of cuticular compounds extracted from queens and workers, as well as the amount of each compound peak could therefore be compared (Mann-Whitney test).

Comparison of relative quantities The non-destructive technique of Solid Phase Microextraction (SPME) allowed extraction of queens without the need to sacrifice them. Sample size obtained was therefore compatible with statistical treatment. Twelve mated queens from the 12 colonies and 12 workers from 5 queenright colonies were extracted. The areas of 59 selected peaks were transformed with Reyment's formula (cf. section II.C.b.) and used as variables in an ANOVA. The Tukey HSD test for unequal samples was used for post-hoc comparisons.

Chemical identification (and quantification) of CHCs

Hexane extracts of CHCs of functional queens (n=2, from 2 colonies), queenright workers (n= 6, from one colony) were analysed in Keele, UK. For this analysis, the oven temperature was initially 60°C for 5 minutes, then increased at a rate of 4ºC/min to 300ºC and kept at this temperature for 10 minutes (run total duration = 75 minutes).

Bioassay of CHCs extract

Queens of *M. gulosa* are somewhat larger than workers (cf. section III.C.b.), and presumably more CHCs can be extracted from their cuticle. Amounts of CHCs assayed must therefore be controlled and matched. Although the ratio of CHC extracted from queen and workers was obtained from extraction of gasters only, it was assumed to be constant and thus generalised to the whole body. Queen extracts were therefore tested against an extract of several workers originating in the same colony as the queen in order to compensate for the difference in CHC they produce.

Whole body extracts of the queen and the workers in 1ml of hexane were obtained by the standard method already described (cf. section II.C.b.). Preliminary tests showed that a quantity of a hundredth of the queen extract presented to the workers gave representative results. This amount was subsequently used in all the assays. Extracts were applied on pentane rinsed glass cover slides (18x18mm). After the solvent had evaporated, two cover slides were simultaneously introduced in the nest of origin, among the workers. On one of these glass lures 10µl of queen cuticular extract were deposited, whereas on the other 10µl of the worker extract were applied. The experiments were conducted in the 24 hours following the queen's death. Glass lures on which the corresponding amount of hexane was deposited constituted the control for the solvent's action on worker aggregation. Responses of workers to CHCs of their queen and nestmates was tested again after 3d, one week and once workers had started laying male eggs, one month later (cf. section III.C.c.). The cuticular extracts were also tested in an alien queenright colony, as well as in an alien orphaned colony. The number of ants antennating the spot where the extracts were deposited was counted at one-minute intervals for ten minutes. Each count was treated independently, with the result that workers staying more than one minute at the cover slide, inspecting it, were counted several times. Six extract fractions were presented to the workers, with an interval of at least 10 minutes between two trials. Cumulated numbers of workers counted at the glass lure over ten minutes were compared with the Friedman two-way ANOVA and the Wilcoxon test.

Detection of single CHCs by workers

Synthetic alkanes, alkenes and methyl alkanes with a length of 25 and 29 carbon atoms were dissolved separately in hexane to obtain solutions of 20mg/ml. A volume of 4µl of solution

was spread with a microsyringe over the whole body (but the antennae) of previously marked workers. The amount of 40µg of compound thus applied corresponded to the estimated amount of the most abundant compound resolved and therefore represents the maximal biologically relevant quantity of HC testable. The great majority of ants were not affected by the treatment in a remarkable manner. Those showing abnormal behaviour following application were discarded and new workers were used. A period of 10 minutes was allowed for solvent evaporation, after which the worker was reintroduced in its nest. As a control, a worker on which 4µl of the solvent was applied was simultaneously reintroduced in the nest. The number of nestmates antennating test and control workers was monitored at 30 seconds intervals for 5 minutes. Each of the six compounds was tested six times in three queenright colonies. Data were log transformed. The attention elicited by test and control workers was compared with a t-test for paired samples. Whether the different compounds elicited different responses toward the workers on which it was applied was determined with a factorial ANOVA and t-tests after log-transformation of the data.

Synthetic queen CHC profile

In order to correlate peak area given by the GC analysis with absolute amounts of compound, the gas chromatograph had to be calibrated. This was realised by injecting twice 0.5µl of the same hexane solution of know concentration of each compound. Given the large range obtained, the calibration was calculated on the basis of each of the 3 classes of the chemicals selected rather than on the individual compounds; i.e. on n-alkanes (n-C25, n-C26, n-C27 and n-C29), alkenes (9-C25:1, 9-C27:1 and 9-C29:1), and on methyl branched alkanes (3-MeC25, 3-MeC27, 9-MeC25, 9-MeC27, 9-MeC29, 11-MeC25, 11-MeC27 and 13-MeC27).

The 15 compounds constituting the 13 major peaks of queen cuticles were either purchased or synthesised. The differences in amount of each of these compounds extracted from queens (colonies B and I) and from workers (colonies B, C, D, E, F and I) were calculated. The appropriate quantities of the synthetic compounds, corresponding to the differences calculated, were mixed together in hexane. In order to test the quality of the blend obtained, 3 aliquots of 0.5µl were analysed by GC and the quantity of each compound obtained was compared with the theoretical values. The solution obtained was evaporated under a stream of nitrogen and rediluted in hexane to obtain the expected concentration. This

concentration was calculated so as to transform a live worker in a queen-like individual by applying 4µl of the solution on its cuticle.

After 10 minutes allowance for solvent evaporation, workers on which the blend was applied were reintroduced to their colonies. The number of nestmates antennating or biting them was monitored at one-minute intervals for 10 minutes. As control, workers on which the corresponding volume of hexane was applied were simultaneously introduced in the nest. Nine pairs of workers were thus tested in their own colony (E, F, H, J and K).

To verify that the compounds in the blend were transferred in right amounts and proportions, 6 treated workers were extracted by SPME after 24h spent in the nest. The profile obtained after application of queen blend was compared with that of 12 queens from 12 colonies (cf. section characterisation of queen and worker CHC profiles). Relative proportions as well as peak area were statistically compared with an ANOVA after Reyment and logarithmic transformation of the data respectively (cf. section II.C.b.). SPME is generally not used for quantitative assessment. As the extraction method was standard and the variation among the profiles obtained remained reasonably low, the comparison of quantities extracted (expressed in peak area) seemed valid. To further confirm the similarity between queen and treated worker profiles, they were compared with those of workers. A PCA was run on the Reyment transformed relative proportions of compound peaks that occurred in more than 90% of the individuals of each group and that represented more than 0.3% of the total peak area. The selection of 18 peaks obtained included 9 compounds out of the 10 that constituted the synthetic queen blend. The 3 principal components were used as variables in a DA.

Results

Characterisation of queen and worker CHC profiles

Comparison of absolute quantities The quantity of compounds extracted from queens was four times that extracted from workers (figure IV.B.6.). The maximum ratio of interindividual variation in quantity (expressed in total peak area) of HCs was 2.6 and 2.8 among queens and among workers respectively. Close inspection of the chromatograms revealed that peaks that were not integrated for some individuals were nevertheless present in traces in their profile, indicating that there were no qualitative differences in the CHC profiles of queens and workers. However quantitative differences were marked. Eighty-four percent of the

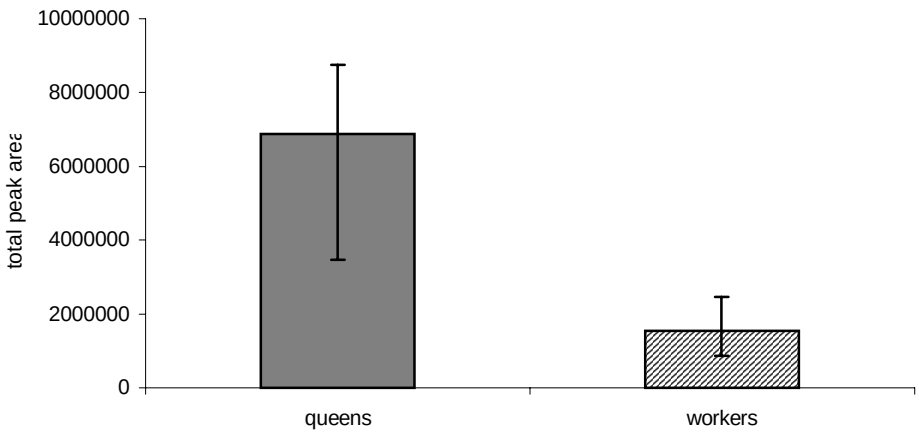


Figure IV.B.6.: average and range of the quantity of compounds extracted from queens (n=3) and workers (n=6) of *M. gulosa*, expressed in total peak area of the chromatograms.

compounds detected by GC were present in higher quantities in queens than in workers (figure IV.B.7a.). The compounds occurring in larger quantities in workers had a mean (±st.dev.) relative proportion of 0.60±0.53% of the total peak area and therefore represented minor compound peaks.

Comparison of relative quantities Eighty-eight percent (i.e. 52/59) of the peaks selected for the analysis were present in significantly different proportions in queens and in workers (Figure IV.A.7b.). Queens possess a larger proportion of short chain compounds (in the range C23-C27) than workers.

Chemical identification of CHCs

GC/MS of hexane extracts of the cuticle of *M. gulosa* revealed a complex mixture of linear alkanes and alkenes, methyl-branched alkanes and dimethyl-branched alkanes, belonging to the long chain hydrocarbon families (C23-C39). The cuticle of workers appeared poor in HCs compared to queens. This was an artefact due to a low sensitivity of the GC/MS at the time the samples were run. GC and SPME-GC confirmed the similar qualitative composition of cuticle in all the groups (cf. previous section). Composition of the cuticle of queens and workers are in fact qualitatively similar and the chemicals identified are listed in the table IV.B.1.

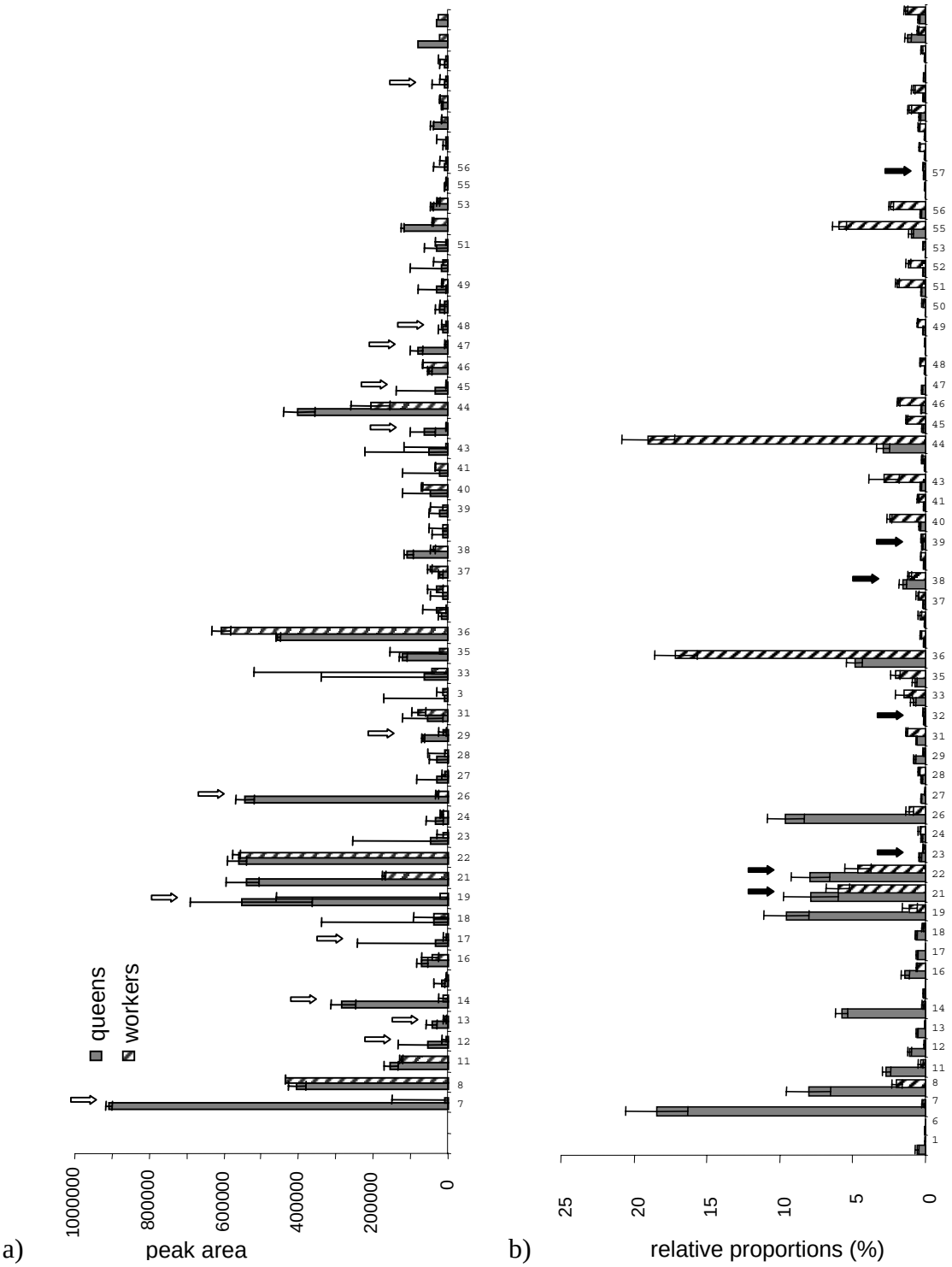


Figure IV.B.7.: CHC profiles of queens and workers of *M. gulosa*. Numbers on the x-axis correspond to the compounds listed in table IV.B.1. The peaks without numbers correspond to unknown compounds .a) absolute amounts of compound peaks (obtained from hexane extracts, $n_{queens}=3$, $n_{workers}=6$). Empty arrows designate significantly different peak areas (Mann-Whitney test, $p<0.05$), all other peaks were present in equal amounts. Error bars represent the range. b) relative amounts of the compound peaks (extracted by SPME technique, $n_{queens}=12$, $n_{workers}=12$). Plain arrows indicate equal proportions. All other peaks were present in significantly different proportions (ANOVA, Tukey post hoc test for unequal sample, $p<0.05$). Error bars represent standard deviation.

Table IV.B.1.: hydrocarbon composition of the cuticle of *M. gulosa* queens (n=2) and queenright (non-reproductive) workers (n=6).

peak	Assignment	Assignment Ions	ECL
1	n-C23	57 (100%)	23.00
2¤	9, 11-MeC23	57 (100%) 140/141, 168/169	23.37
2¤	7-MeC23	57 (100%) 112/113	23.44
3¤	3-MeC23	57 (100%) 56, 309	23.73
4¤	n-C24	57 (100%)	24.00
5¤	X-MeC24	57 (100%)	24.35
6	X,Y-DimeC24	57 (100%)	24.58
7	(Z9)-C25:1	43 (100%) 55, 69, 83, 97, 111 M+350 (173*,271*,M+444*)	24.81
8*	n-C25	57 (100%) M+ 352	25.00
11	9, 11, 13-MeC25	57 (100%) 140/141, 168/169, 196/197, 224/225, 252/253 (M+-15) 351	25.36
12	7-MeC25	57 (100%) 112/113, 280	25.41
13	5-MeC25	57 (100%) 84/85, (M+-57) 309	25.49
14	3-MeC25	57 (100%) 56, (M+-29) 337	25.73
14	(Z9)-C26	57 (100%) 83, 97, 111 (173*,285*)	25.76
15	5,Y-DimeC26	57 (100%) 84/85	25.82
16	n-C26	57 (100%)	26.00
17	X,Y-DimeC26	57 (100%)	26.07
18	8, 9, 10, 11, 12, 13-MethylC26	57 (100%) 126/127, 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225, 238/239	26.33
19	(*Z9*)-C27:1	57 (100%) 83, 97, 111 M+378 (173*,299*, M+472*)	26.74
20¤	(*E9*)-C27:1	57 (100%) 83, 97, 111	26.80
21*	n-C27	57 (100%) M+380	27.00
22*	9, 11, 13-MeC27	57 (100%) 140/141, 168/169, 196/197, 224/225 (M+-29) 365, (M+-15) 379	27.35
22	7-MeC27	57 (100%) 112/113, 309	27.40
23	5-MeC27	57 (100%) 84/85	27.50
24	9, Y-DimeC27	57 (100%) 140/141	27.64
26*	3-MeC27	57 (100%) 56, (M+-29) 365	27.74
27	5, 13-DimeC27	57 (100%) 84/85, 224	27.81
28*	n-C28	57 (100%)	28.00
29	Unknown branched alkane	57 (100%) 70/71, 196/197, 224/225	28.02
30¤	linear triterpene	69 (100%) 81	28.24
31	10, 11, 12, 13, 14-MeC28	57 (100%) 154/155, 168/169, 182/183, 196/197, 210/211, 224/225	28.31
32	linear triterpene	69 (100%)	28.53
33*	C29:1	57 (100%) 83, 97, 111	28.77
34¤	X,Y-DimeC28	57 (100%)	28.83
35*	n-C29	57 (100%)	29.00
36*	7, 9, 11, 13, 15-MeC29	57 (100%) 140/141, 168/169, 196/197, 224/225, 252/253, 280/281	29.33
36	7-MeC29	57 (100%) 112/113	29.39
37	9, 15; 11, 15-DimeC29	57 (100%) 140/141, 168/169, 224/225	29.63
38*	3-MeC29	57 (100%) 56, (M+-29) 393	29.73
39	n-C30	57 (100%)	30.00
40*	9, 10, 11, 13, 14, 15-MeC30	57 (100%) 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225, 238/239, 252/253, 266/267, 280/281	30.30
41	(Z)-C31:1	57 (100%) 83, 97, 111	30.75
42	(E)-C31:1	57 (100%) 83, 97, 111	30.88
43	C31	57 (100%)	31.00
44*	9, 11, 13, 15-MeC31	57 (100%) 140/141, 168/169, 196/197, 224/225, 280/281	
45	11, 17; 13, 17-DimeC31	57 (100%) 168/169, 196/197, 224/225	

46	9, 17-DimeC31	57 (100%) 140/141, 224/225
47	7, 17-DimeC31	57 (100%) 112/113, 224/225
48	Unknown branched alkane	57 (100%) 70/71, 154/155, 182/183, 224/225
49	8, 9, 10, 11, 12, 13, 14, 15-MeC32	57 (100%) 126/127, 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225
50	4-MeC32 - 8, 20; 8, 18; 8, 16-DimeC32	57 (100%) 70/71, 126/127, 196/197, 224/225, 252/253
51	9, 11, 13, 15, 17-MeC33	57 (100%) 140/141, 168/169, 196/197, 224/225, 252/253, 280/281, 308/309, 336/337
52	13, 19; 11, 19; 9, 19-DimeC33	57 (100%) 140/141, 168/169, 196/197, 224/225
53	9, 11, 13, 15-MeC35	57 (100%) 140/141, 168/169, 196/197, 224/225, 308/309, 336/337, 364/365
54	13, 21; 11, 21; 9, 21-MeC35	57 (100%) 140/141, 168/169, 196/197, 224/225
55**	9, 11, 13, 15-MeC37	57 (100%) 140/141, 168/169, 196/197, 224/225, 336/337, 364/365, 392/393
56**	13, 23; 11, 23; 9, 23-DimeC37	57 (100%) 140/141, 168/169, 196/197, 224/225
57	11, 13, 15-MeC39	57 (100%) 168/169, 196/197, 224/225, 364/365, 392/393
58¤	13, 25; 11, 25; 9, 25-DimeC39	57 (100%) 140/141, 168/169, 196/197, 224/225

Abbreviations are as follow: Cx = main chain length, MeCx = methyl-branched compound, DimeCx = dimethyl-branched alkane, Cx:1 = monoene.
¤ indicates the compounds not used in the ANOVA, 15 additional unidentified peaks (and therefore not listed in the table) were used.
* indicates the compounds used in the PCA and DA of section V.B.

Bioassay of CHCs extract

Queen and worker extracts tested 24, 72 hours, 1 week and 1 month after removal of the queen elicited different aggregation levels (Kruskal Wallis test, n=6, H=35.8, $p<0.01$). Workers gathered in significantly higher number around queen than around nestmate worker extracts. This was true in the 24h following queen removal (Wilcoxon test, n=6 in all cases, T=0.0, $p<0.05$), after 72h (T=0.0, $p<0.05$), as well as 1 week later (T=0.0, $p<0.05$). One month later, at the onset of worker reproduction, both extracts elicited similar aggregation (T=4.5, p=0.21). The interest workers showed in the queen extracts did not remain constant in time ($T_{24\text{-}72h}=1.0$, $p_{24\text{-}72h}<0.05$, $T_{72h\text{-}1w}=0.0$, $p_{72h\text{-}1w}<0.05$, $T_{1w\text{-}1m}=0.0$, $p_{1w\text{-}1m}<0.05$, figure IV.B.8.) and decreased steadily (number of workers attracted=77.1-2.6*days, r=-0.83). Interest toward worker extract stayed constant between 24 and 72h and between 1 week and one month ($T_{24\text{-}72h}=7.5$, $p_{24\text{-}72h}=0.53$,$T_{72h\text{-}1w}=0.0$, $p_{72h\text{-}1w}<0.05$, $T_{1w\text{-}1m}=3.0$, $p_{1w\text{-}1m}=0.23$, figure IV.B.8.), but the general trend also reflected a decrease in interest (number of workers attracted=11.1-0.3*days, r=-0.66). The general decrease in aggregation around the extracts of both queen and workers one week after queen sacrifice is, at least in part, due to the onset of agonistic interaction among workers. Indeed, many workers are involved in long lasting interactions (cf. section V.B.) and there are fewer opportunities for them to find and inspect

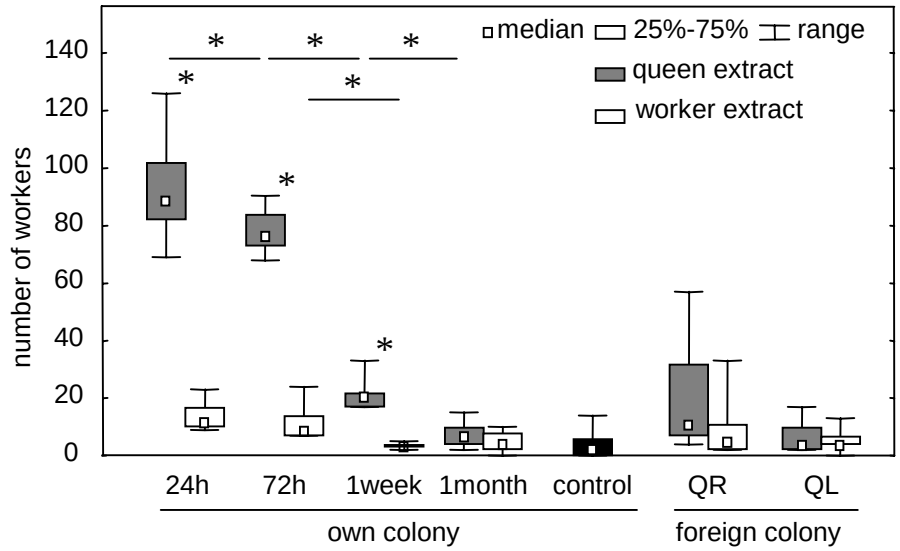
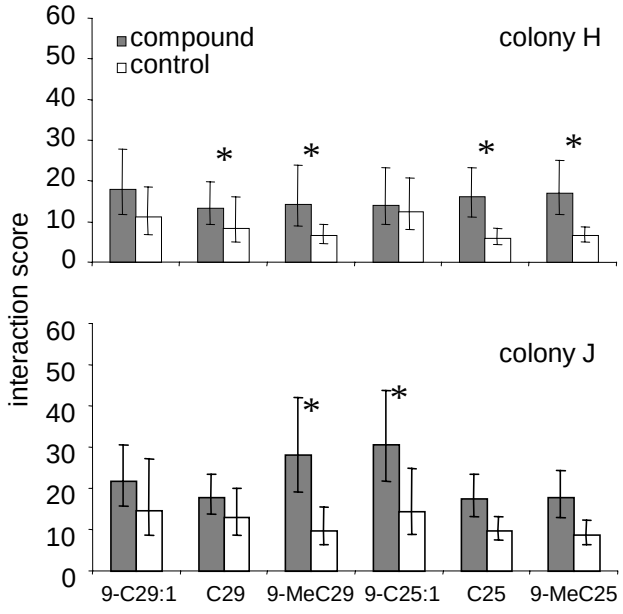


Figure IV.B.8.: number of workers aggregating around chemicals extracted from queen and worker cuticles and presented on glass slides. Extracts were presented to nestmate workers 24h, 72h, 1 week and 1 month after the queen was sacrificed One week after queen removal, the workers started to interact agonistically among each other; one month after orphanage, they started to lay reproductive eggs. Extracts were also presented to foreign queenright (QR) and queenless (QL) workers 24h after extraction. Solvent (hexane) was presented to workers as control. * indicate significant differences (Wilcoxon-test, $p<0.05$).

the glass lures. For the two last periods, the worker extracts did not elicit more aggregation than the solvent controls ($T_{1w}=2.5$, $p_{1w}=0.39$, $T_{1m}=3.0$, $p_{1m}=0.23$). When the queen and worker extracts were presented to alien queenright workers, preference for the queen extract was significant ($T=1.0$, $p<0.05$). However, the aggregation triggered was reduced compared to that displayed by nestmate workers shortly after the queen's death. On the other hand, orphaned alien workers showed no preference when exposed to the extracts ($T=11.0$, $p=0.17$; figure IV.B.8.). An explanation for the fact that queen extracts elicit less aggregation of nestmates after some time could be that the chemicals extracted are not stable and become denatured. To test this hypothesis, queen and worker cuticular extracts were tested again in a queenright nest 11 weeks after extraction. The level of interest shown by the foreign workers in the queen and worker cuticular extracts did not change with time ($T_{queen}=6.0$, $p_{queen}=0.35$; $T_{worker}=7.0$, $p_{worker}=0.89$).

Detection of CHCs by workers

Supplementation of single compounds on workers triggered a weak and uneven interest in nestmates. This interest was manifested by antennation and more rarely by biting. A factorial ANOVA (3 colonies*6 compounds) showed that colonies and the compounds applied on the dummies had significant effects, but that their was no interaction among both factors (colony: $F=11.1$, $p<0.01$, compound: $F=10.8$, $p<0.01$, colony*compound: $F=1.1$, $p=0.37$). As colonies differed in their response to compounds, the differences in attention that they triggered vs. the control were further compared with t-tests for paired sampled. The pattern obtained varied for each colony. Overall, treated workers attracted more attention than controls, but the differences were not always statistically different. A given compound could attract significantly more attention in one colony, but not in the others (figure IV.B.9.).



p-values	colony		
compound	H	J	K
9-C29:1	0.16	0.15	0.06
C29	0.02	0.13	0.52
9-MeC29	0.04	0.00	0.05
9-C25:1	0.87	0.03	0.09
C25	0.00	0.05	0.12
9-MeC25	0.03	0.05	0.06

Figure IV.B.9.: chemical supplementation: average number of workers antennating or biting (interaction score) nestmates on which single HC compounds have been applied. Each compound was applied on 6 workers in 3 colonies (H, J and K). Controls were workers on which solvent was applied. Error bars represent 95% confidence intervals. * represent significant differences (Wilcoxon test), p-values are given in the table. Abbreviations of HC compound names are as in table IV.B.1.

Synthetic queen CHC profile

The supplementation realised by application of the blend resulted in the compensation of 68% of the quantitative differences occurring between queens and workers. The accuracy of the mixture obtained is represented in the figure IV.B.10. (n=3 GC analyses). When applied on a worker, the blend neither elicited retinue formation nor did it trigger the characteristic crouching and antennae retraction shown by workers as they contact their queen. The number of workers antennating or biting the dummy individual was not significantly different from the control (15.1±14.1 vs. 9.9±6.7, n=9, T=13.0, p=0.26). Twenty-four hours after application of the blend, the quantities of compounds transferred in the blend and extracted from the treated workers (n=6) were not drastically different from those extracted from queens (ANOVA, F=785.6, p=0.03, figure IV.B.11.). A discriminant analysis (Wilk's Lambda=0.09, F[6, 50]=19.14, p<0.01) based on 18 compound peaks confirmed that the queen and treated workers were very similar (p=0.52, figure IV.B.12.).

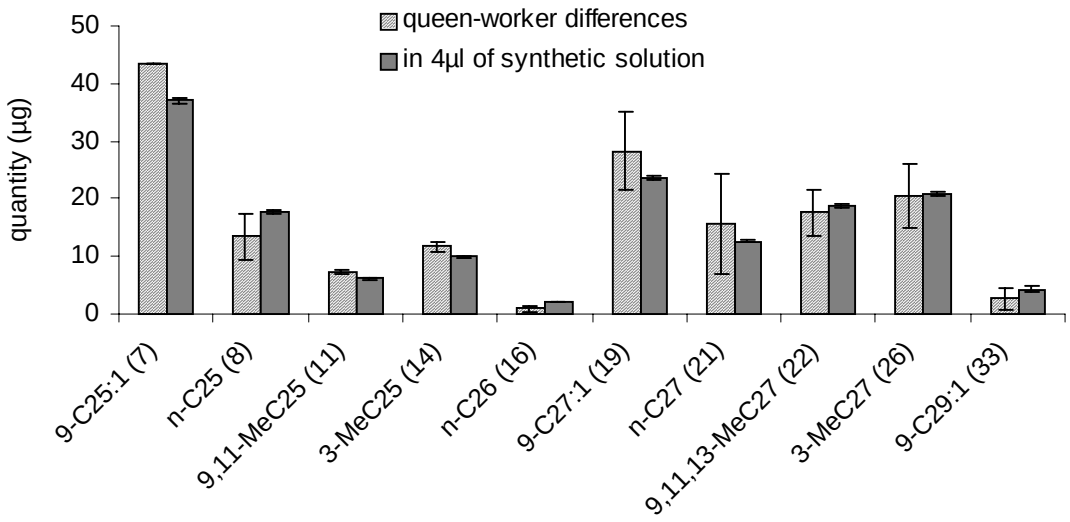


Figure IV.B.10.: accuracy of the synthetic queen blend. The difference in amount of compounds occurring between queens (n=2) and workers (n=6) was calculated from GC analysis of hexane extracts (shaded bars, 3 injections per queen, 1 per worker). The synthetic HCs were mixed in a solution, so that 4µl applied on a live worker made it "queen-like" regarding quantity and relative proportions of these compounds. Between parentheses is the number corresponding to the compounds in figure IV.B.7. The average quantities of chemicals obtained after GC analysis of the blend (n=3 injections) are represented with plain bars. Error bars represent the range.

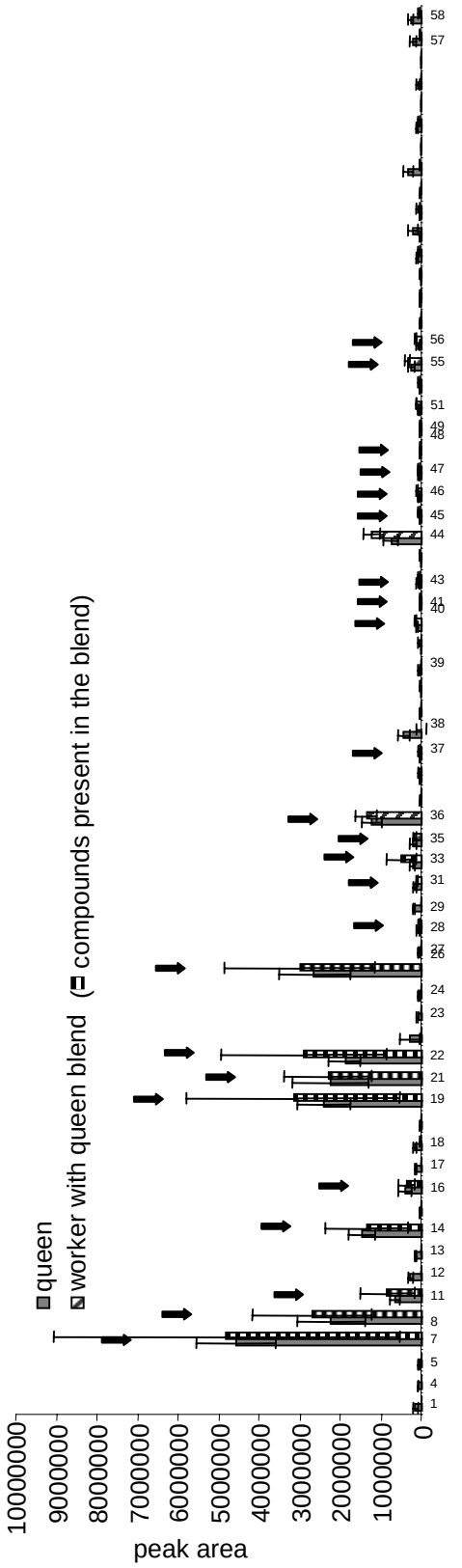


Figure IV.B.11.: comparison of peak areas of queens (n=12) and workers (n=6) on which the synthetic queen blend was applied. SPME method was used to extract the treated workers after a 24h stay in their nest. Arrows designate the peaks present in similar quantities in both groups (ANOVA, Tukey post hoc test for unequal sample). Error bars are standard deviation.

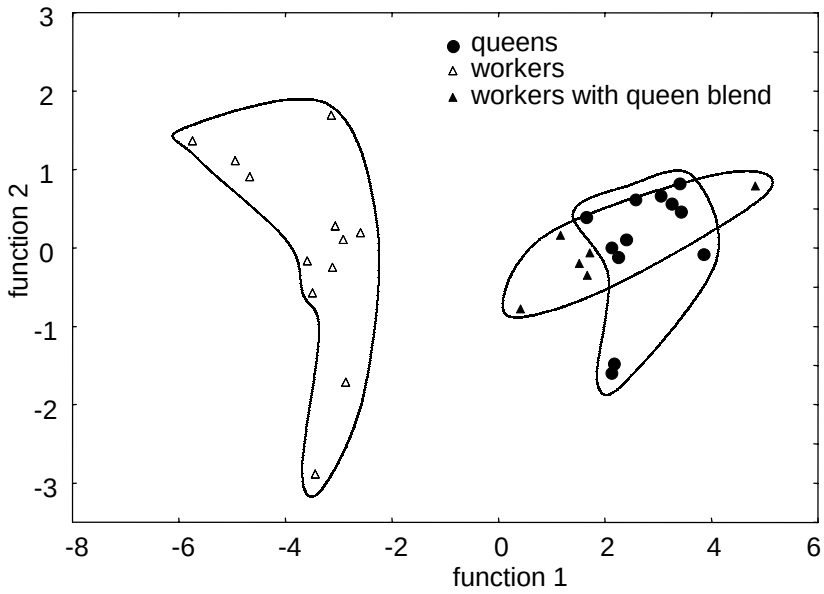


Figure IV.B.12.: discriminant analysis of 12 queens, 12 non-reproductive queenright workers and 6 workers on which the synthetic queen blend was applied. The analysis is based on 9 peaks from the blend and 9 other peaks that were common to at least 90% of the individuals in each group and that represented more than 0.3% of the total peak area. Profiles of treated workers and queens are not distinguished based on the 18 peaks selected.

Discussion

Characterisation and chemical identification of queen and workers CHC profiles

The compounds found in *M. gulosa* are characteristic for Hymenoptera and insect cuticle in general (Lockey 1988, Hefetz et al. 1992, Layton et al. 1994, Oldham et al. 1994, Butts et al. 1995, Monnin et al. 1998, Boulay et al. 2000, Liebig et al. 2000, Cuvillier-Hot et al. 2001, Lenoir et al. 2001, Liu et al. 2001). Queens and workers possess the same cuticular hydrocarbons. Although few compounds from queenright workers extracts were resolved by the GC-MS analysis, investigation with the more sensitive GC technique showed that they possessed the same compounds as queens. However, major differences in amount and relative proportion of the compound peaks distinguished both groups. Information about queen identity is therefore encoded in the CHCs. Chemical analyses of the polar constituents of cuticles were not completed yet, but compared across different groups of workers in section V.E. They represent 9 to 36% of the lipid epicuticular compounds that were extracted. There is no indication of which class of compounds or which compounds in a class the ants perceive and which of the perceived compounds are used for queen recognition. In a first attempt to

establish whether cuticular compounds can mediate queen identity, a queen hexane extract was tested for its properties as worker arrestant.

Bioassays of CHCs extracts

Workers showed aggregation preferentially around their own mother's cuticular extract. Indeed, queen cuticular chemicals presented to foreign queenright workers did not trigger as much interest as in nestmate workers. Such preference for nestmate vs. non-nestmate queen secretions was already described in a comparable experiment conducted with *Solenopsis invicta* (Watkins and Cole 1966, Jouvenaz et al. 1974, but see Lofgren et al. 1983 for an exception). Similarly, workers exposed to live queens preferred their mother versus a non-nestmate queen in several other species (Brian 1973, Keller and Passera 1989, Berton et al. 1991). The queen might therefore be recognised individually, or more likely, carry colony specific cues. Supporting this idea, when live queens (n=2) are introduced in foreign colonies, workers immediately surround them and antennate them "frenetically" or even bite them. This behaviour is more intense than the typical aggregation of workers in a retinue and suggests that workers discriminate between their mother and a foreign queen. These queens were not left for a long time in the foreign colony in order to avoid injuries; no definite answer can therefore be given concerning their fate and the signification of the aggregation they elicited. However, it indicates that queen pheromones and colonial cues act synergistically in retinue formation and that workers' behaviour facing a queen is determined by multiple cues.

Orphaned alien workers showed no preference at all for queen extracts. A possible factor affecting the response of workers exposed to queen extracts might be their "motivation". The freshly orphaned workers tested here were deprived of queen presence for the 2 hours necessary to prepare the extracts and experiment. They might already have detected the absence of their queen and searched for her. This state may have enhanced their response toward the extract presented. In contrast, the alien queenright workers exposed to the same extracts had continuously been in presence of their queen. On the other hand, the alien orphaned workers tested had been queenless for several weeks, so that queen extracts may have lost biological significance to them. This idea is supported by the fact that the interest of workers for their queens' cuticular extracts decreased with time, until they became indifferent. This decrease was clearly linked to the onset of agonistic interactions among workers and thus to their physiological response to orphanage.

The experiment reported here is still preliminary, however, the queen cuticular extract tested clearly elicited nestmate aggregation. This indicates that the cuticle of *M. gulosa* queens is the substrate of a recognition or attractant pheromone. Several studies produced results that support this idea (cf. introduction). In other investigations, where the extracts themselves were not tested or even in some cases of negative results for the extract testing, the extraction of queens in organic solvents deprived them of their "attractive" properties, again suggesting occurrence of a pheromone spread over the cuticle (Brian 1973, Berndt 1977, Berndt and Nitschmann 1979, Passera 1980, Coglitore and Cammaerts 1981). Hexane used here as solvent extracts preferentially non-polar hydrocarbons. The positive results obtained and the fact that profiles of queens and workers differ suggest a role of the CHCs in queen recognition. In order to verify whether workers can detect HCs when presented singly, supplementation of synthetic compounds on living ants was realised and the reaction of their nestmates was monitored.

Perception of single HCs by workers

Workers supplemented with n-pentacosane, 9-pentacosene, 9-methylpentacosane, n-nonacosane, 9-nonacosene and 9-methylnonacosane elicited slightly more attention than the control workers, indicating that the tested compounds were detected. However, the level of attention was much less than that triggered by a queen. This suggests that although the compounds are perceived, they have no arrestant function on their own. Noteworthy, even the major compound of queen profiles (9-pentacosene) did not elicit obvious interest in workers on which it was applied. Methodological problems might account for the absence of effect observed. However, it is more likely that compounds have to be presented in combination to mediate queen recognition. A synthetic queen blend was therefore made up of 15 compounds that represented the 13 major peaks of *M. gulosa* queens CHC profile. The quantity of compounds composing the mixture was calculated so as to compensate for the differences between queens and workers. Application of the blend on a living test worker therefore transformed it in a queen-like individual with respect to the compounds present in the blend.

Synthetic queen CHC profile

Ants interested in the dummy were slightly more numerous than those interested in the control worker. This again testifies from the perception of the HCs supplemented. However, none of the behaviours characteristically displayed by workers encountering their queen occurred when they were exposed to an individual carrying the queen blend. None of the HCs applied (alone or with the other compounds in the blend) is therefore responsible for retinue formation. The quality of the blend, i.e. its adjustment may have been too imperfect to mimic the queen's profile. Alternatively, one or several other HCs present in smaller amounts or chemicals from classes undetected by the GC analysis (more polar compounds) may be involved in combination with the compounds used or without.

In sum, queens and workers have distinct CHC profiles. The fact that the queen hexane extract elicits worker aggregation and that HCs are perceived by workers suggest that the latter function as attractant. Separating and testing the HC and non-HC fractions of total lipid extracts of queens remains necessary to verify this hypothesis. This problem will be addressed in section V.E, using workers as test individuals. The possibility that the arrestant pheromones originates one of the major glands (mandibular, post-pharyngeal and Dufour gland) and contaminates the cuticle was examined.

IV.B.d.iii. The role of major glands

Introduction Dufour glands composition of several species of *Myrmecia*, pygidial gland composition of *M. nigriceps*, as well as mandibular gland products of *M. gulosa* have already been analysed (Cavill and Williams 1967, Brophy and Nelson 1985, Jackson et al. 1989, 1990). These analyses focused on workers and some analyses were here repeated in order to compare gland products of queens and workers. The postpharyngeal (PPG) and Dufour glands were chemically analysed by Richard Beard at Keele University, Keele, UK. Poison glands were overlooked, as they were empty in all the queens dissected.

Congruency between the postpharyngeal gland (PPG) and cuticular hydrocarbons of individuals is expected due to its occurrence in several species of ants (Bagnères and Morgan 1991, Soroker et al. 1994, Soroker et al. 1998, Soroker and Hefetz 2000). It was demonstrated that PPGs do not synthesise the CHCs, but that they sequester them. They are collected from

the cuticle by self or allo-grooming and stored in the gland where they are presumably mixed in a colony specific "Gestalt" odour (Soroker et al. 1994, Hefetz and Soroker 2000). Whether the gland of *M. gulosa* contains CHCs is examined and its function is discussed.

It was recently demonstrated in honeybees and fire ants that recognition or arrestant pheromones could be produced in several glands or tissues of queens (Winston et al. 1998, Vargo and Hulsey 2000, Katzav-Gozansky et al. 2001). Testing products of the major glands of *M. gulosa* queens will reveal whether they possess arrestant properties and whether such queen signals are also redundant and complex in a "primitive" ant such as *M. gulosa*. In addition, comparison of CHCs and gland contents will verify the origin of the attractant substance extracted from the cuticle of *M. gulosa* queens.

Methods

Chemical analysis of gland contents

Gas chromatography/mass spectrometry (GC/MS) A solventless, solid injection method (Morgan and Wadhams 1972, Morgan 1990) was used for this analysis. This technique allows for the analysis of whole glands or reservoirs, including their volatile constituents. The ants were killed by freezing for 15 minutes at –70°C. After thawing, they were dissected in Ringer solution and the glands removed with tweezers cleaned in pentane. Contamination by other gland products was carefully avoided. Glands were kept in a soft glass capillary (1.5x15mm) sealed at both ends and frozen at –20°C, until analysis.

Dufour gland hydrocarbon content of queens (n=2, from 2 colonies) and queenright workers (n=10, from 6 colonies) were analysed. The oven temperature was initially 60°C for 5 minutes, then increased at a rate of 4ºC/min to 280ºC and kept at this temperature for 10 minutes (run total duration = 70 minutes). The hydrocarbon content of post-pharyngeal glands of queens (n=2, from 2 colonies), and queenright workers (n=9, from 6 colonies) was also analysed. The oven temperature was initially 60°C for 5 minutes, then increased at a rate of 20ºC/min to 140°C and from there to 300°C at a rate of 4°C/min. The temperature of 300°C was maintained for 20 minutes (total run duration = 69 minutes). Given the low sample size, quantitative differences in gland composition were not compared statistically.

Bioassays of gland extracts

Mandibular glands The queen was killed by freezing for a few minutes at –70°C. The mandibular glands of *M. gulosa* workers contain volatile compounds (Brophy and Nelson 1985). Instead of extracting the glands and losing the volatiles, the glands were dissected out and crushed on a live worker, which was immediately reintroduced in the nest and observed. The number of individuals antennating the dummy worker was counted at one-minute intervals for ten minutes. As a control, a gland from a worker was used.

Post-pharyngeal and Dufour glands The post-pharyngeal and Dufour glands were dissected out in Ringer solution, placed in a vial and frozen at –70°C for one hour. They were thawed for 30 minutes and 100µl of hexane were added into the vial. The extraction was improved by placing the preparation in an ultrasonic bath for 5 minutes in order to break up the reservoir walls and release the chemicals as completely as possible. Glands of queens and workers had a similar size and glands of single worker were therefore used as a control. Workers and queen extracted, as well as workers exposed to the extracts were nestmates. Extracts were presented in pairs on glass slides, following the method detailed in the previous section (IV.C.b.ii.). The assays were conducted in the 24 hours following the queen's removal.

Results

Chemical analysis of gland contents

GC/MS of entire Dufour glands of *M. gulosa* revealed a complex mixture of linear alkanes, alkenes, alkadienes and alkatrienes, methyl-branched alkanes, branched and unsaturated alcohols, saturated and unsaturated aldehydes, amides and terpenes of C13-C29 chain length. Quantities of queen and worker gland products were similar (figure IV.B.13.). Fourty-four percent of the compounds were common to both groups. The 56% compounds found in only one of the groups, had a relative area of 0.3% of the total peak area in average and might represent compounds in quantity at the limit of the detection threshold of the GC/MS. Qualitative differences in composition of the Dufour glands of queenright workers and queens were therefore represented by minor compounds. Queen and worker compounds are listed in the table IV.B.2. A single remarkable difference in the relative proportions of the

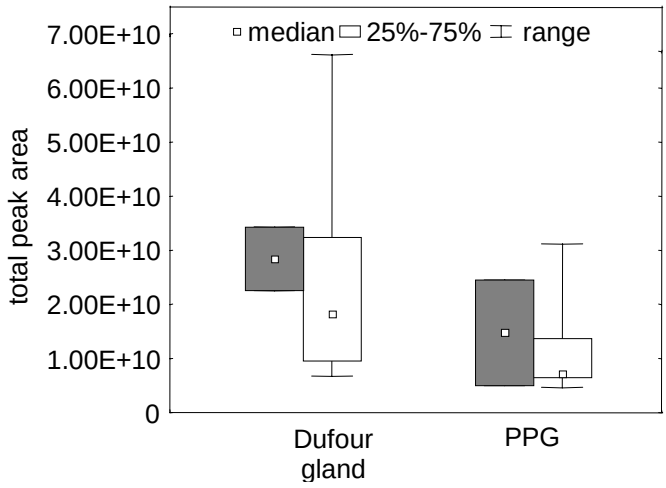


Figure IV.B.13.: quantity of products in the Dufour and post-pharyngeal gland of queens (n=2, plain bars) and workers (n=10 and 9 respectively, empty bars) of *M. gulosa*, expressed in total peak area of the chromatograms.

Table IV.B.2.: Dufour gland composition of *M. gulosa* queens (n=2) and queenright (non-reproductive) workers (n=10).

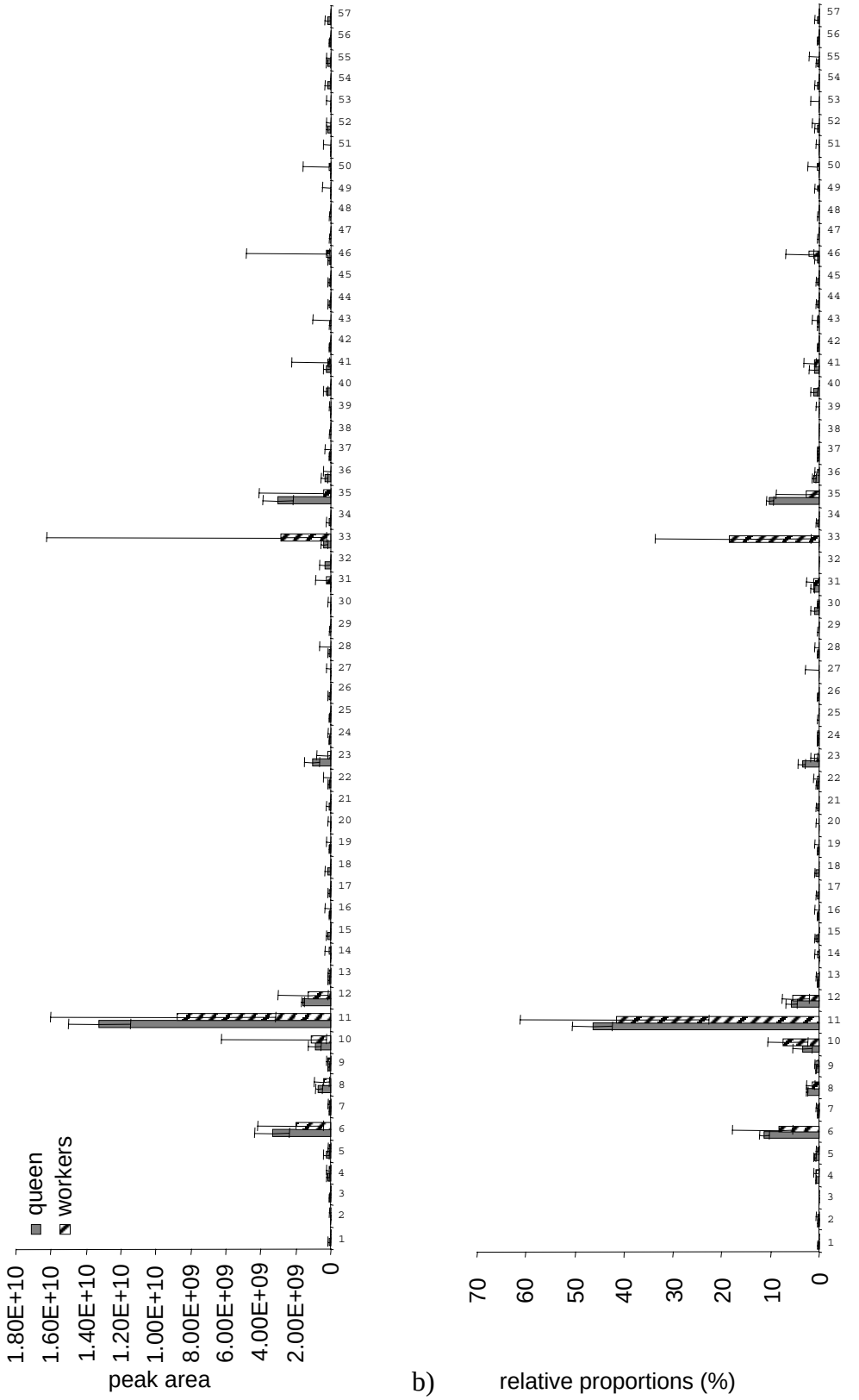
Peak	Assignment	Assignment ions	ECL
1	unknown	41 (100%) 70, 136	
2	n-C23	57 (100%), M+ 184	13.00
3	unknown	41 (100%) 70, 150	
4	n-C14	57 (100%) M+ 198	14.00
5	(*Z9*)-C15:1	41 (100%) 83, 97, 111, M+ 210	14.82
6	n-C15	43 (100%) M+ 212	15.00
7	C16:2	41 (100%) 55, 67, 81, 95, 110, M+ 222	15.78
8	(*Z9*)-C16:1	41 (100%) 83, 97, 111, M+ 224	15.84
9	n-C16	43 (100%) M+226	16.00
10	C17:2	41 (100%) 55, 67, 81, 109 M+ 236	16.79
11	(*Z8*)-C17:1	41 (100%) 83, 97, 111 M+ 238	16.91
12	n-C17	43 (100%) M+ 240	17.00
13	Hexadecadienal	41 (100%) 55, 67, 81, 109 M+236	17.17
14	trishomosesquiterpene	69 (100%)	17.79
15	C18:1	41(100%), 55, 69, 83, 97, 111 M+252	
16	Oxygenated trishomosesquiterpene	69 (100%) 41, M+ 262	17.92
17	Hexadecanal	41 (100%) 67, 82, 98 (M+-18) 222	
18	unknown	69 (100%) 55, 57, 41	
19	C19:4	79 (100%) M+260	18.54
20	C19:3	41 (100%) 79	18.61
21	unknown	57 (100%) 55, 69, 41	18.65
22	C19:2	41 (100%) 67 M+264	18.72
23	(Z)-C19:1	41 (100%) 83, 97, 111, M+ 266	18.81
24	n-C19	57 (100%) M+ 268	19.00
25	unknown	55 (100%)	
26	Unsaturated aldehyde	55 (100%) 55, 69, 81, 95, 111	22.09
27	Terpene	41 (100%) 69	20.14
28	Octadecanal	41 (100%) 67, 82, 98 (M+-18) 250	20.14
29	Octadecenol	41 (100%) 69, 82/83, 95/96 (M+-18) 250	20.31
30	Terpene	69 (100%) 41, 81	20.76

31	3,7,11,15-tetramethyl-2, 6,10,14-Hexadecatrien-1-ol (Geranylgeraniol)	69 (100%) 41 M+290	21.06
32	Unsaturated aldehyde	55 (100%) 55, 69, 81, 95, 111	
33	3,7,11,15-tetramethyl-6,10,14-Hexadecatrien-1-ol (Geranylcitronellol)	69 (100%) M+292	21.94
34	Unsaturated alcohol	41 (100%) 69, 82/83, 95/96 (M+-18) 264	
35	Eicosenal	41 (100%) M+294	22.20
36	Eicosenol	41 (100%) (M+-18) 278	22.78
37	n-C23	57 (100%) M+ 324	23.00
38	Tricosenal	41 (100%) 69, 82/83, 95/96	23.21
39	Octadecenamide	59 (100%) 72 M+281	23.90
40	C25:1	43 (100%), 83, 97, 111	24.82
41	n-C25	57 (100%), M+ 352	25.00
42	3-MeC25	57 (100%) M+352	25.71
43	n-C26	57 (100%), M+ 366	26.00
44	11-MeC26	57 (100%) 168	26.30
45	C27:1	43 (100%) 55, 69, 83, 97, 111 M+378	26.81
46	n-C27	57 (100%), M+ 380	27.00
47	9, 11, 13-MeC27	57 (100%) 140, 168, 196	27.26
48	3-MeC27	57 (100%), 56	27.75
49	n-C28	57 (100%), M+ 394	28.00
50	n-C29	57 (100%), M+ 408	29.00
51	9, 11-MeC29	57 (100%) 140/141, 168/169	
52	Terpene	69 (100%) 41, 81	
53	Terpene	69 (100%) 41, 81	
54	Unknown	55 (100%)	
55	Terpene	69 (100%)	
56	Unknown	55 (100%)	
57	Unknown	55 (100%)	

Abbreviations are as follow: Cx = main chain length, Me = methyl-branched compound, Cx:1 = monoene, Cx:2 = diene, Cx:3 = triene, Cx:4 = tetraene.

compounds identified could be found between workers and queens (figure IV.B.14.). It concerned the proportions of eicosenal and geranylcitronellol, the former dominating the latter in queens and inversely in workers.

The PPGs of *M. gulosa* contained a complex mixture of linear alkanes and alkenes, methyl-branched alkanes, dimethyl-branched alkanes, belonging to the long chain hydrocarbon families (C23-C43). Traces of alcohols, aldehydes and fatty acids were also detected. Quantity of products stored in PPGs of queens and workers were comparable (figure IV.B.13.). However, their composition differed. Only Fifty percent (35/70) of the compounds identified were common to queens and workers. The major qualitative difference concerned the 9-pentacosene, which is present in large quantity in queens (16% of total peak area), but absent in workers. The other differences corresponded to minor compounds (0.58% of the total peak area on average). The gland composition is listed in the table IV.B.3. Many differences were found in the quantity and relative proportions of the compounds between



a) b)

Figure IV.B.14.: Dufour gland content of queens and workers of *M. gulosa*. Numbers on the x-axis correspond to the compounds listed in table IV.B.2. a) median of the absolute amounts of compound peaks (obtained from hexane extracts); b) median of the relative amounts of the compound peaks. Error bars represent the range.

Table IV.B.3.: post-pharyngeal gland (PPG) composition of *M. gulosa* queens (n=2) and queenright (non-reproductive) workers (n=9). Qualitative differences are detailed in the text.

peak	Assignment	Assignment ions	ECL
1	Heptadecenal	41 (100%) (M+-18) 234	
1	Octadecenoic Acid methyl ester	55 (100%) 74, 264 M+296	21.12
2	Octadecanoic acid	55 (100%) 60, 264, M+312	21.75
3	Nonadecenol	55 (100%) M+278	
4	Tricosane	57 (100%), M+ 324	23.00
5	unsaturated amide	59 (100%) 41, 72	23.60
6	3-Methyltricosane	57 (100%), M+ 338	23.72
7	Octadecenamide	59 (100%) 72 M+281	23.94
8	Tetracosane	57 (100%)	24.00
9	N-Methyloctadecenamide	41 (100%) 55, 69, 81, 95, 111	24.22
10	(Z)-Pentacosene	41 (100%) M+ 350	24.83
11	Pentacosane	57 (100%) M+ 352	25.00
12	9, 11, 13-Methylpentacosane	57 (100%) 140/141, 168/169, 196/197, 224/225	25.36
13	7-Methylpentacosane	57 (100%) 112/113	25.46
14	5-Methylpentacosane	57 (100%) 84/85	25.49
15	3-Methylpentacosane (Hexacosene)	57 (100%) 56, 337, 83, 97, 111	25.79
16	5, 11; 5, 13-Dimethylpentacosane	57 (100%) 84/85, 196/197, 224/225	25.85
16	5, Y-Dimethylpentacosane	57 (100%) 84/85	25.91
17	Hexacosane	57 (100%)	26.00
18	Branched alkane	57 (100%) 70/71, 224/225, 252/253, 280/281	26.09
19	8, 9, 10, 11, 12, 13-Methylhexacosane	57 (100%) 126/127, 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225	26.34
20	(Z)-Heptacosene	43 (100%) 55, 69, 83, 97, 111, M+ 378	26.72
21	Heptacosane	57 (100%) M+ 380	27.00
22	7, 9, 11, 13-Methylheptacosane	57 (100%) 112/113, 140/141, 168/169, 196/197, 224/225, 252/253, 280/281	27.42
23	5-Methylheptacosane	57 (100%) 56, 337	27.51
24	9, 13-Dimethylheptacosane	57 (100%) 140/141, 224/225	27.64
25	3-Methylheptacosane	57 (100%) 56	27.73
26	5, 15; 5,13-Dimethylheptacosane	57 (100%) 84/85, 196/197, 224/225	27.78
27	Octacosane	57 (100%)	28.00
28	Branched alkane	57 (100%) 70/71, 126/127, 154/155	28.08
29	7, 8, 9, 10, 11, 12, 13, 14-Methyloctacosane	57 (100%) 112/113, 126/127 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225	28.30
30	X,Y-Dimethyloctacosane	57 (100%)	28.59
31	(Z)-Nonacosene	43 (100%) 55, 69, 83, 97, 111	28.74
32	Nonacosane	57 (100%)	29.00
33	7, 9, 11, 13, 15-Methylnonacosane	57 (100%) 112/113, 140/141, 168/169, 196/197, 224/225, 252/253, 280/281, 308/309, 407	29.33
34	5-Methylnonacosane	57 (100%) 84/85	29.48
35	13, 15; 11, 15-Dimethylnonacosane	57 (100%) 196/197, 168/169, 224/225	29.54
36	9, 15-dimethylnonacosane	57 (100%) 140/141, 224/225	29.59
37	7, 15-dimethylnonacosane	57 (100%) 112/113, 224/225	29.65
38	3-Methylnonacosane	57 (100%) 56	29.71
39	5, 15-dimethylnonacosane	57 (100%) 84/85, 224/225	29.76
40	Triacontane	57 (100%)	30.00
41	unknown branched hydrocarbon	57 (100%) 70/71, 196/197, 224/225	30.04
42	7, 8, 9, 10, 11, 13, 14, 15-Methyltriacontane	57 (100%) 112/113, 126/127, 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225	30.26
43	4-Methyltriacontane (X, Y-Dimethyltriacontane)	57 (100%) 70, 196/197, 224/225	30.58
44	(Z)-Hentriacontene	43 (100%) 55, 69, 83, 97, 111, M+434	30.84
45	7, 9, 11, 13, 15-Methylhentriacontane	57 (100%) 112/113, 140/141, 168/169, 196/197, 224/225, 252/253, 280/281, 308/309, 336/337	
46	11,17-Dimethylhentriacontane, 13,17-Dimethylhentriacontane	57 (100%) 168/169, 196/197, 224/225	
47	9, 17-Dimethylhentriacontane	57 (100%) 140/141, 224/225	
48	7, 17-Dimethylhentriacontane	57 (100%) 112/113, 224/225	
49	5, 17-Methylhentriacontane	57 (100%) 84/85, 224/225	

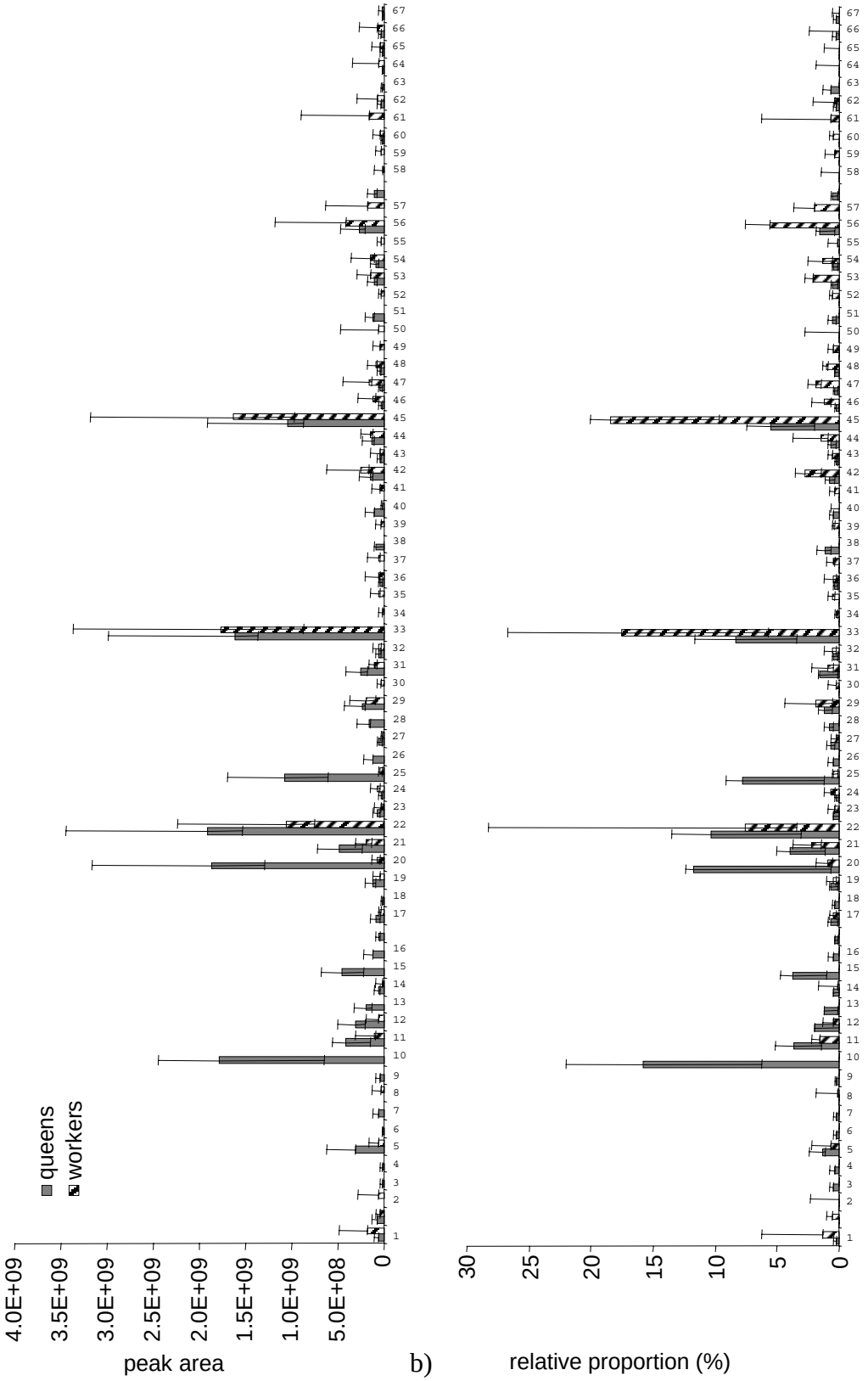
50	unknown branched hydrocarbon	57 (100%)
51	Dotriacontane	57 (100%)
52	unknown branched hydrocarbon	57 (100%) 70/71, 196/197, 224/225
53	8, 9, 10, 11, 12, 13, 14, 15, 16-Methyldotriacontane	57 (100%) 126/127, 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/225, 252/253, 280/281, 308/309
54	4-Methyldotriacontane, (8, 12; 8, 14)-Dimethyldotriacontane	57 (100%)70/71, 126/127, 196/197, 224/225, 252/253
55	(Z)-Tritriacontene	57 (100%) 83, 97, 111
56	7, 9, 11, 13, 15, 17, 19-Methyltritriacontane	57 (100%) 112/113, 140/141, 168/169, 196/197, 224/225, 252/253, 280/281, 308/309, 336/337
57	7, 19; 9,19; 11,21; 13, 19-Dimethyltritriacontane	57 (100%) 168/169, 196/197, 224/225, 112/113, 140/141, 224/225
57	9, 23-Dimethyltritriacontane	57 (100%) 140/141, 168/169
58	5, 19-Dimethyltritriacontane	57 (100%) 84/85, 224/225
59	5, 9, 19-Trimethyltritriacontane	57 (100%) 84/85, 224/225, 154/155
60	9, 10, 11, 12, 13, 14, 15-Methyltetratriacontane	57 (100%) 140/141, 154/155, 168/169, 182/183, 196/197, 210/211, 224/25
61	8, 12-Dimethyltetratriacontane	57 (100%) 126/127, 196/197
62	9, 11, 13, 15, 17-Methylpentriacontane	57 (100%) 140/141, 168/169, 196/197, 224/225, 252/253, 280/281, 308/309, 336/337
63	9, 11, 13, 15-Methylpentriacontane	57 (100%) 140/141, 168/169, 196/197, 224/225
64	11, 23; 13, 21; 9, 23-Dimethylpentatriacontane	57 (100%) 140/141, 168/169, 196/197, 224/225
65	9, 11, 13, 15-Methylheptatriacontane	57 (100%) 140/141, 168/169, 196/197, 224/225, 336/337, 364/365
66	11, 23; 13, 23-Dimethylheptatriacontane	57 (100%) 168/169, 196/197, 224/225
67	X,Y-dimethyloctatriacontane	57 (100%)

Abbreviations are as follow: Cx = main chain length, MeCx = methyl-branched compound, DimeCx = dimethyl-branched alkane, Cx:1 = alkene.

queens and workers (figure IV.B.15.). Comparison of PPGs with CHCs revealed a close match of the profiles in both the identity of the compounds present and in their quantity (figure IV.B.16.).

Bioassays of gland extracts

The worker on which a queen mandibular gland was crushed attracted distinctly more nestmates than an individual on which worker mandibular gland product was applied (101 vs. 75 workers over the 10 minutes). Similarly, workers exposed to PPG and Dufour gland hexane extracts of their queen or of nestmates gathered in significantly higher number around the queen extracts (T_{PPG}=0.0, p_{PPG}<0.05, T_{Dufour}=0.0, p_{Dufour}<0.05, figure IV.B.17.). They antennated and sometimes licked the spot where the extract was deposited. This preference was more pronounced for the PPG than for the Dufour gland extract (U=4.0, p<0.05). In an alien colony, PPG and Dufour gland queen extracts elicited equal attention (U=9.5, p=0.17).



a) b)

Figure IV.B.15.: post-pharyngeal gland content of queens and workers of *M. gulosa*. Numbers on the x-axis correspond to the compounds listed in table IV.B.3. a) median of the absolute amounts of compound peaks (obtained from hexane extracts). b) median of the relative amounts of the compound peaks. Error bars represent the range.

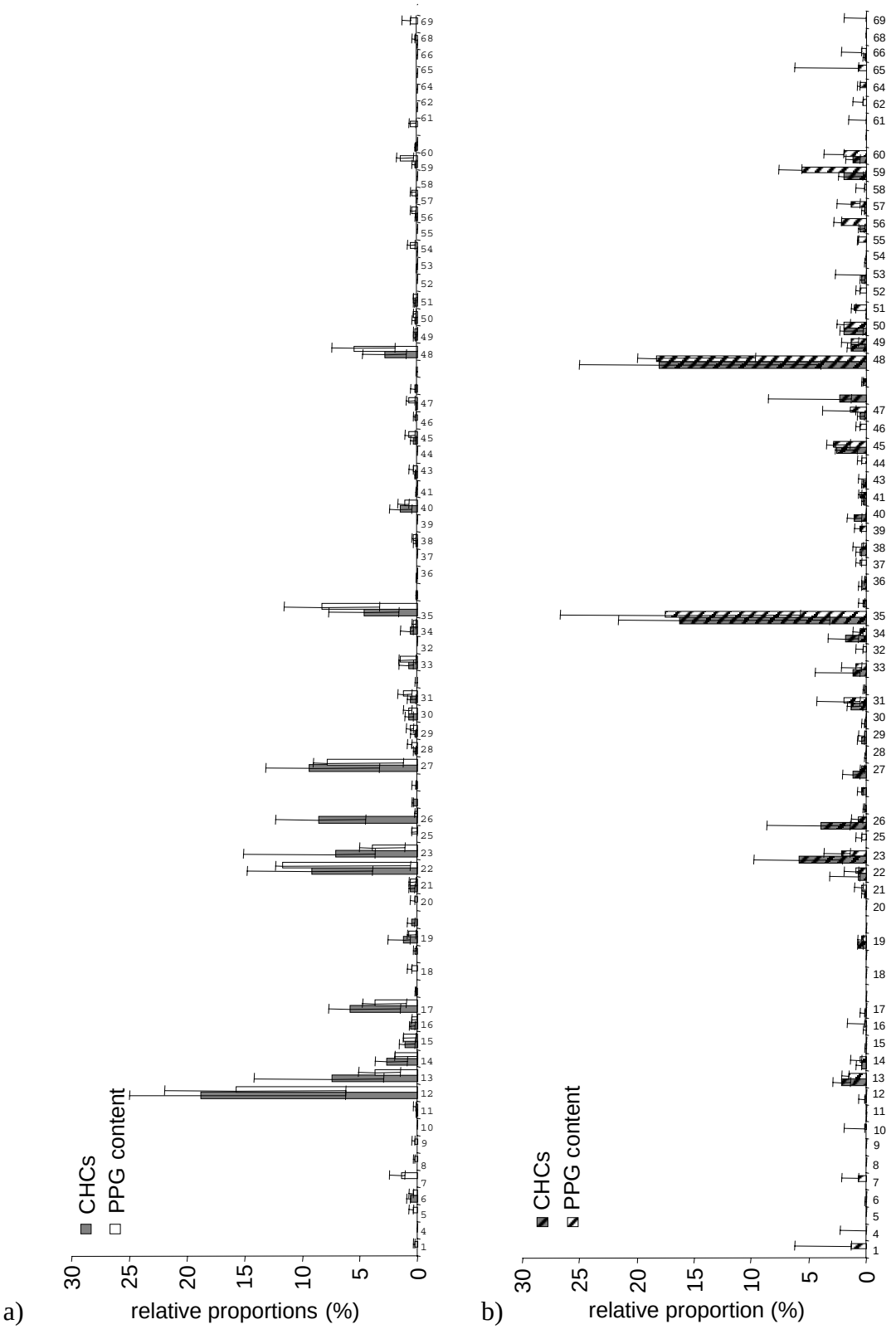


Figure IV.B.16.: congruency between CHCs and PPG content in a) queens and b) workers of *M. gulosa*. The cuticle of queens (n=3) and workers (n=6) were extracted in hexane and analysed by GC. Glands of queens (n=2) and workers (n=9) were dissected, extracted in hexane and analysed by GC/MS. Median values are given with range. The numbers on the x-axis correspond to the compounds listed in table IV.B.3.

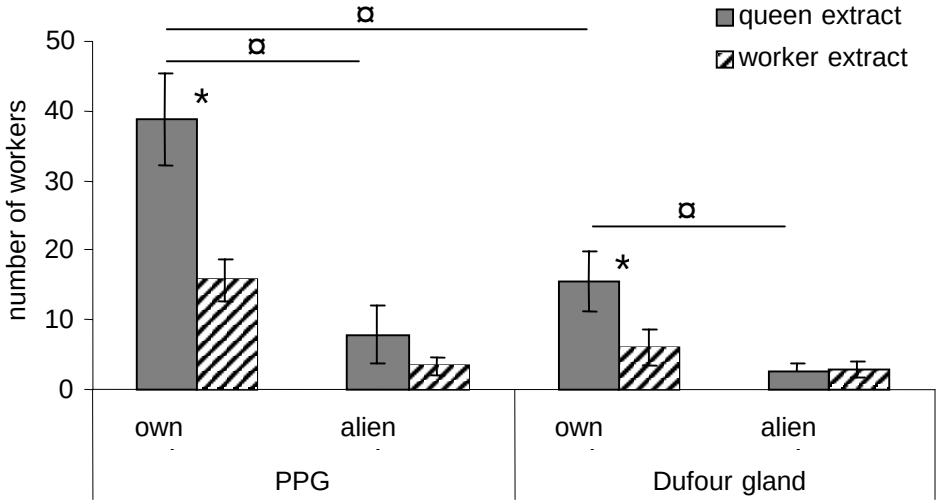


Figure IV.B.17.: average number of workers aggregating around PPG and Dufour gland extracts. Extracts of queens and workers were exposed to nestmates (own colony) and to queenright non-nestmates (alien colony). Six extract fractions (1/100) were tested in the 24h following queen sacrifice. * and ¤ represent differences statistically significant (Wilcoxon and Mann-Whitney test respectively, $p<0.05$).

Discussion Queen Dufour gland extracts were preferred to workers' in the bioassay. The major difference between queens and worker Dufour gland contents is constituted by two compounds, which are probably at the basis of the discrimination. Extraction of the gastral tergites by SPME showed that Dufour gland products do not contaminate the cuticle of individuals. In contrast, major compounds found on the cuticle were present in small amount in the gland. Their presence in minute quantity indicates that they were not produced in the gland, but most likely contaminated it.

As in the formicines, myrmicines and ponerines (cf. introduction), the same HCs can be found on the cuticle and in the PPGs of *M. gulosa*. PPG content closely matched the CHC composition in both queen and workers. Indeed, the important absolute as well as relative quantitative differences corresponded to those found between the cuticular profiles of both groups. Therefore, the fact that the chemicals stored in their PPG possess the same arrestant properties as those on the cuticle was expected. Similarly, one of the sources of worker "attractant" in *Solenopsis invicta* is the PPG (Vargo and Hulsey 2000). This strongly suggests that cuticular compounds, and presumably CHCs, are involved in queen recognition. The results of the above-mentioned study however, do not show in the first place that cuticular compounds are attractive for workers. Indeed, the authors tested crushed body parts for their arrestant properties, but not cuticular extracts alone.

In contrast to the results obtained for the composition of CHC blends, GC/MS analyses of PPGs of queen and workers showed qualitative differences in their gland content. The main difference corresponded to 9-pentacosene, which is absent in workers, while other discrepancies concerned minor compounds. It is likely that the latter were at the limit of the detection threshold of the GC/MS and thus were not integrated (cf. section IV.B.d.ii.). PPGs might also fail to sequester HC compounds present in small amounts on the cuticle, thus inducing qualitative differences when compounds are present in low quantity in one of the groups only.

Workers show more interest for the products of various queen glands compared to those of workers. Dufour and post-pharyngeal glands contained similar amount of products in queens and workers, indicating that the discrimination is based on their composition and not on the quantity of chemicals they contain. This interest is less important or absent when extracts are tested in foreign queenright or queenless colonies. Assuming that CHCs mediate nestmate recognition in *M. gulosa*, (as in the species were their involvement was demonstrated: Lahav et al. 1999, Thomas et al. 1999, Wagner et al. 1999), the fact that cuticular and PPG hexane extracts of queens elicit more interest in nestmates than in non-nestmates is well explained. In contrast, the greater aggregation elicited by nestmate queen Dufour extract compared to non-nestmates is unexpected. Unless the compounds constitute an additional colony signature, “motivation” (cf. section IV.B.d.ii.) of the workers could explain the difference in interest observed.

Composition of mandibular glands was not analysed, but the fact that workers showed more interest in the products of a queen’s gland compared to that of a worker suggests that their content differ.

IV.B.e. Discussion: the queen arrestant pheromones

The fact that the queens are immobile and that their shape alone does not elicit worker aggregation, whereas their corpses or cuticular extracts do, indicate that semiochemicals play the major role in mediating their recognition and in eliciting retinue formation. Workers form a retinue around their queen even during the periods when she is not ovipositing (i.e. during the 39 day long inactive periods of the egg-laying cycle, cf. section III.C.c.). The arrestant pheromone release is therefore continuous and not linked to oviposition, as in *Solenopsis invicta* (Obin et al. 1988). The fact that workers aggregate around both front and rear part

when access to queen is experimentally limited, demonstrates that arrestant pheromones are spread over the entire body of the queens.

These and other arguments support the hypothesis that CHCs are involved in queen recognition or play a role as arrestant pheromone. CHCs cover the whole body of insects, as do the arrestant pheromones of *M. gulosa*. The queens' CHC profiles remained constant, independent from their egg-laying cycle (results not shown), and were distinct from those of workers. Queens possess short-chained compounds in higher quantity and proportion than workers, providing a basis for discrimination. The fact that a hexane extract from a queen's cuticle elicited a marked aggregation of nestmate workers suggests that the latter are able to recognise a queen and her colony membership from her CHC bouquet. However, the presence of more polar substances in the extract that could mediate these effects cannot be excluded.

To determine the role of CHCs, synthetic compounds were presented to workers. Their behavior indicates that CHCs are detected, but that none of the compounds tested released aggregation behaviour. A synthetic queen blend also failed to elicit formation of a retinue. This does not however refute the hypothesis that HCs could function as pheromones. CHCs compounds on the epicuticle are numerous and one or several minor components not tested here, or a different blend of compounds than those assayed, could be involved. Confirmation of the role of this class of lipids could be obtained by the purification and testing of the HC fractions of queen cuticular extracts (containing exclusively HCs, but all the compounds in natural proportions). The number of workers available does not represent a limiting factor and allows for more experimental possibilities and higher sample size. The detection of worker CHCs and their role as a basis for behavioral regulation mechanisms of worker reproduction was thus tested (cf. section V.E.c.).

Workers aggregated around cuticular and gland extracts or gland products of their queen, indicating multiple signals and potentially complex queen pheromonal recognition and regulation mechanisms. Post-pharyngeal glands of workers were demonstrated to sequester CHCs and more polar cuticular lipids (cf. introduction, Soroker et al. 1998), and therefore contain a similar chemical blend as the cuticle. It was therefore expected that the pheromones spread over the queen's body were also present in her PPG and that its content would trigger aggregation. Volatility of the long-chained HCs found on insect cuticles is presumed to be weak, and whether they can account for the distance perception of queens in *M. gulosa* is doubtful. Yet another class of more volatile chemicals might be involved, adding to the complexity of the pheromonal queen recognition mechanism.

Queens of *M. gulosa* are not often groomed or fed by retinue workers. The arrestant pheromones they emit thus do not contribute remarkably to their tending by workers. By increasing the number of workers contacting the queen, these semiochemicals may ensure the distribution of other pheromones, notably of the primer type. Alternatively, they may themselves possess other pheromonal functions. To help understand the function of the arrestant pheromones and their possible interaction with primer semiochemicals, the mode of perception of the primer pheromones regulating worker reproduction, as well as their origin, was investigated.

IV.C. Queen primer pheromone: regulation of worker reproduction

IV.C.a. Introduction

In most, if not all the "highly" eusocial hymenoptera species, queens do not perform any particular behaviour that could account for their regulative effect on worker reproduction or gyne rearing. That is, they do not interact agonistically with workers or larvae to inhibit their reproduction or the rearing of gynes. It is therefore widely acknowledged that these regulation mechanisms involve pheromones. A method generally used to exclude the queen's behaviour in these mechanisms consists of showing that queen corpses remain active (Carr 1962, Brian 1973, Fletcher and Blum 1981a, Hölldobler and Wilson 1983, Vargo and Fletcher 1986, Vargo and Passera 1991 for ants; Velthuis 1972 for bees). Strong evidence for pheromonal regulation mechanisms also comes from the fact that when active corpses are washed with a solvent, they lose their regulative power (Brian 1973, Berndt 1977, Passera 1980, Fletcher and Blum 1981a, Vargo and Passera 1991). When pheromones of the primer type are considered, the time lag (days or even weeks) until the physiology of individuals responds to experimental manipulation complicates bioassaying and explains the fact that only one primer pheromone has been chemically identified in the social Hymenoptera until now: the mandibular gland pheromone of the honeybee queen. In ants, the best model species for the study of primer pheromones found so far is the fire ant, *Solenopsis invicta*. However, despite the advantage conferred by relatively fast responses to bioassays, only the site of storage and of production of a primer pheromone could be located in the poison sac and gland complex of the queens (Vargo 1997). The chemical identity of the pheromone remains unknown and as in all the other species, primer pheromones must be examined indirectly through their effects on individuals.

In *M. gulosa* and in the genus as a whole, the factors influencing production of new gynes are only partly known (cf. chapter III.); the role of overwintering, for example, has not been studied. Therefore, only the primer pheromone regulating worker reproduction was examined in detail here. The onset of reproduction by workers and the triggering of agonistic interactions that is associated to it are used as indicators of the lack of perception of the queen primer pheromone. The aim of the following sections is to determine how this pheromone is distributed among colony members and what is its chemical nature. For the reasons already evoked in section IV.B., special attention is paid to the role of CHCs and their putative primer pheromonal function.

IV.C.b. Is the primer pheromone regulating worker reproduction volatile or is direct contact necessary?

Introduction In most ant species workers still possess functional ovaries and can lay male-destined eggs (see Bourke and Franks 1995, p.228 for exceptions). This usually happens when the colonies lose their queen (Bourke 1988, Choe 1988). Similarly, virgin queens of *Solenopsis invicta* shed their wings and start reproducing in the absence of their queen (Fletcher and Blum 1981b). In all the species studied so far, queen pheromones regulating reproduction in workers or virgin queens proved to have low volatility. Workers or virgin queens behave as if they were orphaned when they are separated from their queen by a double mesh that prevents physical contacts but not passage of volatile pheromones between the two parts (Butler 1954, Naumann et al. 1991, Visscher and Dukas 1995 for bees; Akre and Reed 1983 for wasps; Passera 1980, Fletcher and Blum 1981b, Fletcher and Ross unpublished cited in Willer and Fletcher 1986, Liebig et al. 1999, Gobin et al. 1999, Tsuji et al. 1999, Dietemann et al. 2000, Sledge et al. 2001 for ants). Depending on the extent to which individuals had access to each other through a single mesh or cage, queen pheromones may be partly transmitted across the barrier or not at all (Akre and Reed 1983 for wasps; Passera 1980, Fletcher and Blum 1981b, Vargo 1988, Gobin et al. 1999, Tsuji et al. 1999, Sledge et al. 2001 for ants). Physical contact with the queen herself or with nestmates carrying the queen pheromones was necessary in each of these experiments for individuals to perceive the pheromones. The question of whether direct (queen-worker) contacts are necessary or if indirect contacts (via other workers) are enough to transmit the queen pheromones cannot be satisfactorily answered with single mesh experiments. Although they were successfully used in some cases (Velthuis 1972 for bees; Fletcher and Blum 1981b for ants), it disturbs the movement of individuals in the nests and restricts their possibility of interactions with the body parts that fit through the gaps of the mesh. A negative result therefore does not exclude the possibility of indirect pheromone transmission, but could simply be due to an insufficient quantity or rate of pheromone transferred. Furthermore, there is possibility of direct contact between the separated workers and the queen on the other side (Sledge et al. 2001). A way to circumvent these problems was found by Tsuji et al. (1999). Their experimental design was adapted to *M. gulosa* in order to investigate whether the queen pheromone regulating worker reproduction is volatile or whether it is transmitted by direct or indirect contacts with the queen.

Methods The experimental nest consisted of a single chamber (420x200mm) moulded in Plaster of Paris and covered with glass plates to allow for observation. *M. gulosa* is a species showing bimodal worker size frequency distribution (cf. section III.C.b.). Colony size was reduced to a hundred large and a hundred small workers, together with the queen. The workers were selected from various nest chambers (queen chamber, brood chamber, chamber without brood, foraging arena), in order to avoid biasing the composition of the groups in favour of a particular age or task class. All of the brood was removed. A copper wire (0.2mm diameter) was tied up around the thorax of each individual (queen included), perpendicularly to its body axe, passing between the coxae of the first and second pairs of legs. In order to maintain the wire on top of the thorax, a droplet of bee wax mixed with colophonium was used to glue it on the mesonotum. The wire was bent upward on each side of the ant's body so that it did not interfere with leg movement. The wire span was shorter (12mm) for small workers than for large workers (14mm) in order to reduce the bias of a longer wire compared to body size. Individuals equipped with the wire and the mixture used to glue it behaved and were treated normally by their nestmates and their mortality was low.

The workers equipped with the wire were left one week to accommodate in the nest. Control observations were realised during the following week. On the third week, a 15mm wide barrier drilled with 51 holes (8mm in diameter) was then placed in the middle of the chamber. In one of the sides, a compartment was created by placing a double mesh. Three nest parts (A, B and C, figure IV.C.1.) were thus delimited. A tube (25mm internal diameter)

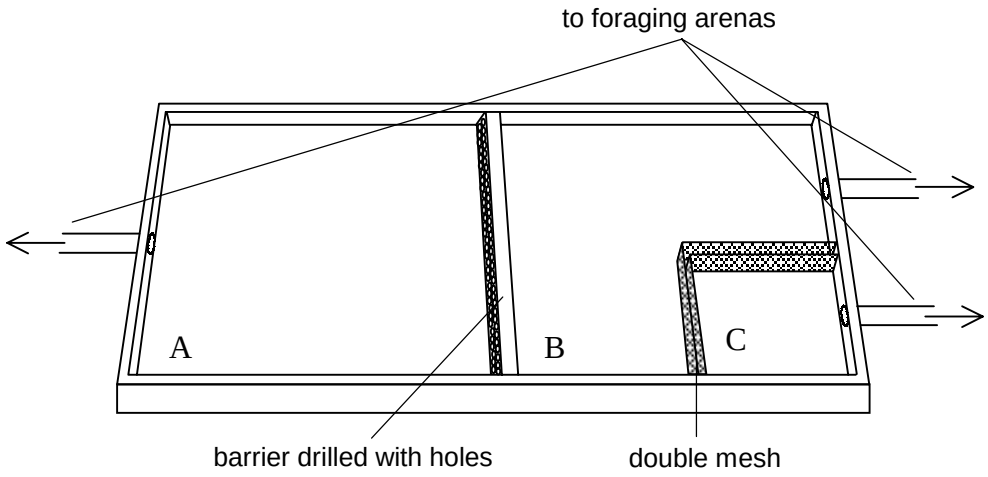


Figure IV.C.1.: experimental nest adapted from Tsuji et al. (1999). The double mesh prevented direct as well as indirect contact between nestmates, whereas the barrier drilled with holes (8mmØ) prevented those workers equipped with a 12 or 14mm long wire to pass and change nest part.

connected each of these parts to its own foraging area (185x185x90mm). Four groups of 50 workers (25 large and 25 small individuals) were created. Three of them (a, b and c) were respectively placed in the nest parts A, B and C (nest parts are designated by capital letters, whereas workers placed in these parts are designated with small letters). The wire tied up around the workers' thoraces prevented them from passing through the holes of the barrier. The barrier was wide enough to prevent the workers from antennating individuals from the opposite side, unless the latter also tried to do so in the same hole, which rarely occurred. The wire of the workers assigned to the fourth group (d) was cut so that it was short enough to allow them to cross the barrier. They therefore had access to both the nest parts A and B. The queen was placed together with the group of workers (b), in the nest part B. The group (b) served as control for the effect of the copper wire on the workers' behaviour and ovarian development. The double mesh prevented any worker movement between nest parts B and C. This group served as control for the behaviour and ovarian development of workers deprived of direct as well as indirect (via other workers) contact with their queen. Only workers from the groups (b) and (d) could make direct contact with the queen. The experiment was repeated 4 times with 3 colonies (D, H and I). A colony containing 2 queens (H) was split into 2 parts (Ha and Hb) and each of these colony halves was used for an experiment. Twenty-seven days after the division of colony Hb, workers killed their queen while forcing her to pass the barrier. Only data collected until the 25th day were used in the analysis. This happened earlier in one further repetition of this experiment (colony G), so that results could not be obtained.

In order to exclude the possibility of too few workers being able to cross the barrier to carry enough queen pheromone to prevent the workers (a) from reproducing, the experiment was repeated with 2 large colonies (E and F), where at least 200 workers could cross the barrier. Experimental nests consisted only of the parts A and B and only the workers blocked away from the queen had copper wires fixed on their thorax.

Workers of *M. gulosa* interact agonistically among each other and lay reproductive eggs when orphaned (cf. chapter III.). Occurrence of fights among workers and accumulation of reproductive eggs were therefore monitored. The agonistic interactions were unidirectional with the aggressor performing antennal boxing on the head, or biting an appendage (leg, mandible, antenna or petiole) of the victim. Observation bouts of 1 to 3 hours were scheduled daily between 9h00 and 19h00 for both the control (workers equipped with the wire, but free to move in the whole nest, 6-11 hours over 5-6 days) and experimental periods (divided nest, 22-31 hours over 13-31 days). At the end of the study, all the surviving workers were dissected. The experiments were terminated 2-13 days after eggs started to accumulate in the

nest parts C, to ensure that workers had enough time to become reproductive. However; in one of these colonies (D), no eggs were laid and the experiment was put to an end 31 days after its start.

Dissections were done in Ringer solution. Ovaries were checked for the presence of trophic or reproductive oocytes (cf. chapter III. for the characters allowing the distinction between the 2 types of eggs). Some individuals had oocytes that were considered as intermediate between trophic and reproductive. The latter had a size characteristic for reproductive oocytes and had a dense yolk, but lacked a thick layer of follicular cells. Workers were classified in two categories: non-reproductive individuals possessed empty ovaries or ovaries containing trophic eggs, reproductive ones had intermediate or reproductive oocytes. During 10 minute periods, the number of workers passing or trying to pass the barrier was monitored. The same impeded individual trying to pass the barrier repeatedly during a ten-minute session was counted only once.

Results Agonistic interactions rarely occurred during the control period, when workers all had wires tied up around the thorax and were free to move in the nest. Once the barrier and double mesh were installed, workers in nest parts A and C were unable to make direct contact with their queen. From the day 6.8$\pm$2.5 days (range 4-10) and 7.8$\pm$1.0 (range 7-9) after nest division, the number of agonistic interactions occurring per hour of observation increased steadily in the nest parts A and C respectively. Individuals performed antennal boxing and bit each other. At the end of the experiment, the number of agonistic interactions that occurred per hour of observation during control periods across the 4 experiments was compared. The comparison was repeated for each of the nest parts. Differences were significant in all the cases (Kruskal-Wallis test, $n_{control}=35$, $H_{control}=26.7$, $p_{control}<0.01$, $n_A=72$, $H_A=14.6$, $p_A<0.01$, $n_B=72$, $H_B=10.2$, $p_B=0.02$, $n_C=72$, $H_C=10.0$, $p_C=0.02$). Data could therefore not be pooled and the number of agonistic interactions had to be compared separately for each experiment. The comparison of the number of agonistic interactions occurring in control periods and in the nest parts in each experiment yielded significant differences (Kruskal Wallis test, $n_D=71$, $H_D=16.4$, $p_D<0.01$, $n_{Ha}=45$, $H_{Ha}=21.7$, $p_{Ha}<0.01$, $n_{Hb}=65$, $H_{Hb}=33.6$, $p_{Hb}<0.01$, $n_I=70$, $H_I=51.1$, $p_I<0.01$). The behaviour of individuals in nest parts B remained unchanged compared to the control in only two experiments. In the other two, the number of fights was significantly higher during the control period. The occurrence of a few interactions during the control periods in colony D and Ha, but their quasi absence in parts B, was enough to produce the

difference. It is however not reflective of the reality of the phenomenon, as there were much fewer interactions in both control period and in part B than in parts A and C. Overall, the differences in number of fights is due to the aggressiveness of individuals in nest parts A and C (table IV.C.1., figure IV.C.2.).

Reproductive eggs started to accumulate in part C 18±3.6 (mean±st.dev., range 15-22) days after double mesh separation. Whether workers (a) laid reproductive eggs is not known

Table IV.C.1.: the number of agonistic interactions that occurred in the control period and in the different nest parts was compared in each experiment (Mann-Whitney test and Fisher combined probability).

nest part			B		A		C	
colony or group	$n_{control}$	$n_{A, B, C}$	$U_{B/control}$	$p_{B/control}$	$U_{A/control}$	$p_{A/control}$	$U_{C/control}$	$p_{C/control}$
D	8	14	54.0	0.04	14.0	<0.01	14.0	<0.01
Ha	7	13	20.0	0.01	12.0	<0.01	19.0	0.03
Hb	11	18	82.5	0.16	22	<0.01	22.0	<0.01
I	10	20	100.0	1.00	10	<0.01	5.0	<0.01
Fisher combined probability				0.01<p<0.02		<0.001		<0.001

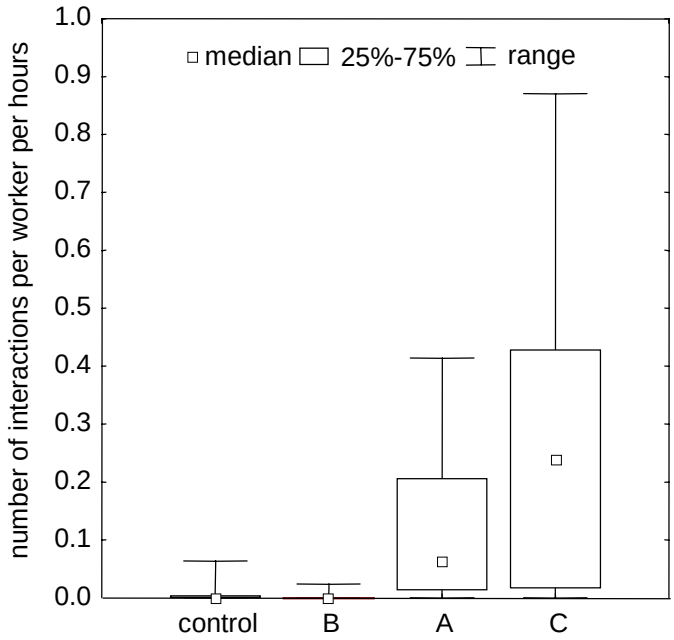


Figure IV.C.2.: level of agonistic interactions (per individual per hour) during the control periods and in each part (A, B and C) after nest division. Although the differences in the frequency of agonistic interactions among control periods and among each nest parts were significant across experiments (see text), the trend in the 4 experiments was comparable and the data were pooled for the box-whisker plot.

because workers rarely assume an egg-laying posture (cf. chapter III.). Furthermore, accumulation of worker-laid eggs could not be monitored, as these could not be distinguished from those laid by the queen.

However, dissections confirmed that some workers (c) as well as (a) were producing reproductive oocytes. Workers of groups (b) and (d) had empty ovarioles or trophic oocytes.

Unimpeded workers (d) crossed the barrier in both directions as frequently (7.2±3.2 and 6.2±1.6 ants per 10 minutes passing form A to B and B to A respectively). As the same workers (d) could pass the barrier in both directions, differences in the frequency of crossing in each direction could not be tested statistically. In contrast, workers (a) and (b) represent two distinct groups and the frequency of their attempts to pass the barrier can be compared. The frequency of attempts for workers (a) to pass the barrier toward nest part B were significantly different in each experiment, as was that of workers (b) that tried to pass in part A (Kruskal-Wallis test, $n_{(a)\ to\ B}=50$, $H_{(a)\ to\ B}=18.4$, $p_{(a)\ to\ B}<0.01$, $n_{(b)\ to\ A}=50$, $H_{(b)\ to\ A}=16.2$, $p_{(b)\ to\ A}<0.01$). Data could therefore not be pooled and frequencies of attempts had to be compared separately for each experiment. Significantly more impeded workers (a) from the queenless side of the nest were observed trying to cross the barrier than workers (b) blocked in the queenright side in each experiment (Wilcoxon test, $n_D=12$, $T_D=0.0$, $p_D<0.01$; $n_{Ha}=14$, $T_{Ha}=0.0$, $p_{Ha}<0.01$; $n_{Hb}=10$, $T_{Hb}=8.0$, $p_{Hb}<0.05$, $n_I=14$, $T_I=0.0$, $p_I<0.01$, Fisher combined probability $p<0.01$, figure IV.C.3.). Their attempts were also more frequent and intense, as they continuously tried to pass for several minutes before giving up. This behaviour started in the 24h after the barrier was installed in the nest.

When the experiment was repeated with larger colonies (n=2) and therefore many more workers could cross the barrier, agonistic interactions in part A could again be observed.

Discussion When workers are separated from their queen by a double mesh, agonistic interactions are triggered and some start to reproduce. This shows that, as in most the species of social Hymenoptera studied until now, the queen pheromone regulating the reproduction of workers in *M. gulosa* is not volatile. Furthermore, some of the workers that had only indirect contact with their queen (via other workers) started to interact agonistically and some produced reproductive oocytes. Whether they actually laid eggs is not known. Workers ([b] and [d]) that had access to the queen did not behave as orphans. The fact that workers (b) did not fight or produce reproductive eggs shows that the wire used to restrict workers' movements was not responsible for the triggering of these effects. Direct contact with the

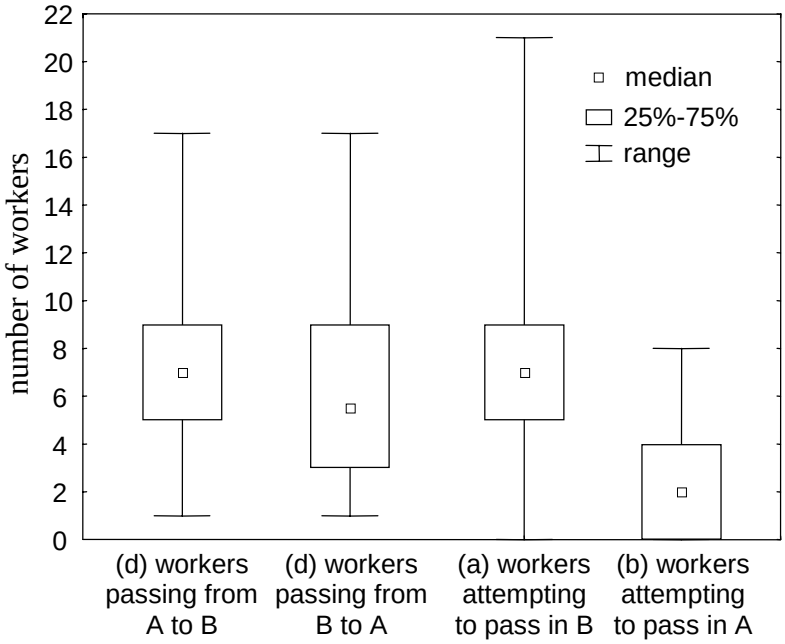


Figure IV.C.3.: number of ants crossing or attempting to cross the barrier separating the nest in the queenless (A) and queenright parts (B). The direction of passage is given with the group of workers involved. (d) workers are free to move between A and B, (b) workers are blocked with the queen, (a) workers are blocked away from her. Although the frequencies of crossing attempts were different across colonies, the pattern was comparable for each experiment and the data were pooled for the box-whisker plot. The number of individuals attempting to cross the barrier is significantly higher for group (a) than for group (b) in each experiment (Fisher combined probability $p<0.01$).

queen or at least close proximity is therefore necessary for workers to perceive the pheromone that regulate their reproduction. This demonstrates that workers cannot transfer the information about the presence of the queen to other workers. This occurs in the honeybee where workers become contaminated with pheromones and function as “messengers” (Seeley 1979, Naumann et al. 1991, 1992).

The fact that more workers that were blocked away from their queen tried to pass the barrier than individuals blocked with the queen, and the fact that they did so more intensely, suggests that they seek the queen. This behaviour was also observed in a preliminary experiment without the nest part C, indicating that the presence of workers isolated behind the double mesh on the other side of the barrier was not its cause. Thus, the major difference between the nests parts A and B that could explain the workers’ attempts to pass the barrier was the presence of the queen in the latter part. Whether workers simply reacted to her absence on their side or detected her presence on the other side of the barrier could not be verified. Two hypotheses can be proposed to explain this putative searching behaviour. First,

as queens feed exclusively on trophic eggs laid by workers, the latter may have been selected to regularly check the hunger state of their queen and feed her when necessary. On the other hand, in the frame of the signalling hypothesis (Keller and Nonacs 1993), workers might be looking for the queen to verify her presence or monitor her health in order to behave accordingly. In the case where they cannot find her or if her health is declining (thus reducing her fertility), it is in their interest to start reproducing.

On two occasions (at the end of the study of colony Hb and after several weeks of separation in the large colony F), workers tried to force their queens to pass the barrier by grasping one of their antennae and walking backward through a hole of the Plexiglas fence. They thus pulled on the queens and finally tore both the antennae away. This soon resulted in the death of the two queens. In one of these cases, a single worker was responsible for this. The significance of this behaviour is not clear. It suggests that workers either detect the absence of a queen from some parts of the nest or the effect of her absence on individuals in this part, and that they try to remedy to it. Hölldobler and Carlin (1989) described a comparable phenomenon in *Aphaenogaster cockerelli*. In this case however, workers carried other workers (with more developed ovaries) to the vicinity of the queen. As queens feed on trophic eggs, the authors suggested a trophic role for this behaviour: the workers brought next to the queen might feed her by laying eggs. Alternatively, they proposed that these workers are brought next to the queen in order to expose them to queen pheromones and prevent them from laying viable eggs. In the experiment described here, *M. gulosa* workers were never forced nestmates to pass the barrier.

No evidence of worker reproduction in queenright situation could be obtained. Thus, workers must become exposed to queen pheromones regularly, suggesting that they come in close proximity to the queen on a regular basis. This must be reflected in their movement in the nest or in the queens'. The spatial and temporal patterns of queen-workers contacts are examined in the next section.

IV.C.c. Queen-worker encounter pattern

Introduction Direct physical contact or presence in close proximity is needed for the workers of *M. gulosa* to perceive the presence of the queen. The regulation of their behaviour is therefore depending on how frequently the workers come into contact with their queen. They must be exposed to a sufficient quantity of pheromones in order to detect the queen's

presence and this at least once in 3 days. This period corresponded to the shortest delay needed for the physiological changes induced by orphaning to affect the workers' behaviour in an observable fashion (cf. chapter III.). It is not possible to follow individuals continuously over such a long period to determine its frequency of contact with the queen. A study of the pattern of queen-worker contacts over shorter periods can nevertheless give information about their contact rate. In this study, attention was also paid to who initiated contacts and which part of the queen was touched, in order to determine whether workers seek contact with the queen and whether they prefer a particular body part.

Queens occasionally move around the nest and visit several chambers before coming back where they spend most of their time, next to the eggs. These visits could have a role in pheromone distribution either by allowing an increase in the number of workers contacted or by a pheromonal marking of the nest, as occurs in the honeybee (Juška 1978, Seeley 1979, Lensky and Slabezki 1981, Naumann et al. 1991, 1992). The frequency and duration of the queen's visits of her nest were measured. The substances deposited by queens confined in glass Petri dishes were collected, analysed chemically and tested for their arrestant properties, in order to verify the occurrence of pheromonal marking of the nest. Finally, whether the visits or marking of their nests by queens participate to pheromone distribution and are involved in the regulation of worker reproduction was investigated by preventing them from moving around and by monitoring the behaviour of workers.

Methods

Queen-worker contacts Three colonies (A, B and C) were used to study the proportion of workers in a colony that met and had physical contact with their queen. An interaction was considered as a contact when an individual touched any part of another ant with its antennae. This gives good indication that the individual touching a nestmate detected its presence. Data were collected during the observations already described in section IV.B.c. (frequency of presence in the retinue). The observation bouts, scheduled between 9h00 and 19h00, lasted 1, 2 or 3 consecutive hours. All the individuals were individually marked. Analysis of the proportion of individuals in a colony encountering either their queen or a control worker (large immobile worker sitting close to brood, cf. section IV.B.c.) was realised on an hourly basis in order to increase sample size for statistical comparison. Given the high frequency of contacts, contacts with the queen and with the control workers were not monitored simultaneously, but during different observation sessions. Influence of density on the rate of

queen-workers contacts was monitored in colony A. Upon completion of the observations of queen-worker contacts, the colony was transferred to a smaller nest of similar structure (770 to 380cm^2, therefore doubling the density). The ants were left to accommodate in the new nest for two weeks before the observations were repeated. The table IV.C.2. gives the observation duration for each colony.

The proportion of workers contacting the focal individuals were compared across observation periods for the colony A with Friedman's ANOVA after arcsine transformation (Sokal and Rohlf 1995). The differences in proportions of workers (transformed data) contacting either queens or control workers were compared with a Mann-Whitney test. In order to follow the evolution of interindividual contacts in time, the cumulated proportion of workers that had contact with their queen or a control worker was plotted each half hour during periods of 3 and 2 consecutive hours respectively. Regression lines were calculated from the arcsine transformation of the proportions and statistically compared. The proportion of contacts initiated by retinue workers (cf. section IV.B.c.) was determined by identifying the individuals at the queen's side at 10 minute intervals. During these observations, the individual initiating each contact, as well as the body part touched during the interaction was identified (with the exception of colony C where body part contacted was not monitored). Thus, three colonies were observed during several sessions of 2-8 consecutive days, separated by 1-4 months (detailed in table IV.C.3.). Queen-workers contacts were monitored for 4h daily on 2 consecutive days. Contacts between control workers and their nestmates were monitored for 3h, on 2 consecutive days as well. The perimeter of an imaginary circle enclosing the queen was calculated in order to test whether the frequency of contacts correlated with the dimensions of the body parts or if workers were attracted to a particular part. Nine dorsal view pictures of 3 queens (colonies C, F and G) in natural positions were scanned. The circle, the diameter of which corresponded to the length (l) of the individual (tip of mandibles to tip of gaster, perimeter = πl), was fitted on each queen. Four radii passing by the tip of both front legs and both hind legs were drawn to delimit the areas where the legs, head or gaster would be touched first by an approaching individual. The arc length s1 and s2 were calculated with the formula $s=\theta/r$ (with $r=l/2$, figure IV.C.4.). The approximate percentage of bi-dimensional space (as average arc length of the circles) available for worker to establish physical contact with the different body parts was therefore known and could be compared with the observed values of contact frequency of each part.

Table IV.C.2.: colonies used to monitor queen-worker contacts together with duration of observations.

colony code and observation session	colony size	hours of observation	
		queen-worker contacts	worker-control worker contacts
A1	250	8	3
A2	250	8	3
A3*	250	8	3
B	150	8	3
C	420	24	4

*: colony transferred into a smaller nest, increasing the density twofold.

Table IV.C.3.: observation sessions of queen-workers physical contacts (focussing on the initiator of the contact and on the body part touched). Session durations are given in hours. Colonies A, B and C were observed for 1 to 3 sessions.

colony code and observation session	queen-workers contact	worker-worker contacts
A1	8	3
A2	8	3
A3*	8	3
B1	8	3
B2	16	-
C	24	4

*: colony transferred into a smaller nest, increasing the density twofold.

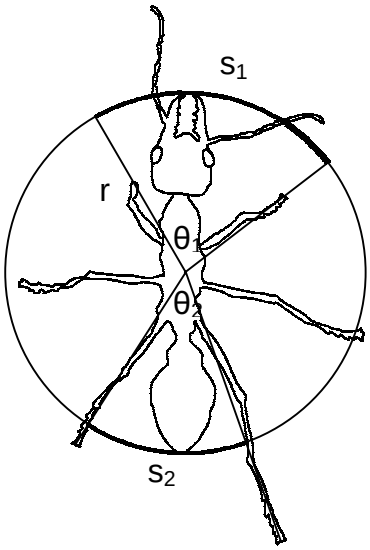


Figure IV.C.4.: arc length corresponding to head (s_1), gaster (s_2) and legs dimensions, expressed in percentage of the perimeter of a circle enclosing the queen.

Queen movements in the nest

Queen visits in the nest The four colonies (A, B, C and K) used for these observations contained 200 to 800 individuals. Nest chambers were moulded in Plaster of Paris nests and organised so as to recreate the architecture of a natural nest. Chambers were arranged in a branching pattern from a central tunnel. Each chamber had only one entrance connected to the tunnel. To change chamber, the ants had therefore to pass through the main tunnel. A video camera was placed so that the whole nest could be videotaped. The time-lapse mode was used to record the movement of queens in the nests for 72 consecutive hours. To facilitate reading of the tapes, queens were marked with a large dot of white paint on the thorax. On playback, the tapes were paused when the queen started to move away from her resting position and the time noted. She sometimes moved inside the brood chamber she sat in. However, only her movements when she changed chamber were taken into account and the duration of the subsequent visit was noted. A visit was considered over when the queen became immobile again and this, at any place in the nest. Visits were treated as two distinct events if they were separated by an interval of at least 10 minutes. Colonies A, B, C were videotaped for three-72h period each and colony K was videotaped for a single 72h period. Data were analysed per 24h period, so that sufficient samples were available to compare statistically the visiting activity of each queen (Wilcoxon test). In two 72h video records for colonies A and B, attention was paid to the duration of the queens' movements inside the chamber where they spent most of their time.

On two occasions in colony B and C, queens started to visit their nests during the monitoring of queen-workers contacts by direct observation. This allowed checking the rate of contacts of workers with their queen for 10 minute intervals during which the queen walked and visited the nest. These rates of contacts could be compared with those that occurred during the four 10 minutes periods directly preceding the visit. As one of the visits of queen C started as soon as 20 minutes after the start of the observations, it was not used in the calculation of the encounter rate.

Queen marking of the nest Queens of 7 colonies (C, D, E, F, H, J and K) were taken out of their nests and isolated in a Petri dish previously cleaned with hexane. Isolation duration of 4 hours was chosen in order to maximise the quantity of substance collected and to minimise the disturbance of the colonies deprived of their queens for this period of time. The colourless oily deposit visible on the bottom of the dish was washed with 1 ml of hexane.

Solvent was left to evaporate overnight under a fume hood and the secretions were redissolved in 20µl of hexane. A volume of 0.5µl of the solution was analysed by GC (see section II.C.b. for the methodology). As a control, large as well as small workers of the same colonies were isolated individually in the same conditions and the wash analysed.

The cuticular extracts of the 2 queens (colonies B and I) and 6 large workers (from colonies B, C, D, E, F and I) analysed in section IV.B.d.ii (characterisation of queen and worker profiles) were used here for qualitative comparison with the substances deposited on the substrate. In order to increase sample size, extracts of one queen (colony E) and 6 small workers (colonies D, E, F, H, J and K) obtained following the same methodology were included in the comparison. The statistical comparison of the relative proportions of the compound peaks obtained from washes of the substrate and from cuticle extraction was performed using a multivariate analysis. Selecting the peaks that accounted for more than 1% of relative peak area, and which occurred in more than 90% of all individuals permitted the reduction of the number of variables. The relative areas of the 12 selected peaks were restandardised to 100% and transformed following Reyment's formula (1989) (cf. section II.C.b.) and were used as variables in a principal component analysis (PCA). The principal components were then used as variables for a discriminant analysis (DA). In order to assess the release rate of the secretions deposited, the quantity of HCs collected was estimated. The calibration values obtained for the three major compound classes found on the cuticle of *M. gulosa* (section IV.B.d.ii.) were averaged in order to obtain a rough estimate of the total quantity of HCs deposited on the substrate by the different groups.

Detection of queen marking by workers To test whether the substances deposited by queens on the substrate elicit worker aggregation, filter paper on which the queen sat for 4 hours was exposed to workers in absence of the queen. Their presence on that spot or on a control spot of equal surface, where nestmate workers were confined for the same duration, was monitored. The number of workers confined to the control area was matched with the ratio of quantities of hydrocarbons collected from Petri dishes where queens and workers were confined during the previous experiment. Individuals were placed on filter paper covering the bottom of large Petri dishes (200Øx35mm) and confined under small plastic Petri dishes (68Øx12mm). These were distant from 40mm (their centres were 100mm apart). The area occupied by the dish was delineated on the filter paper. Queen and workers, as well as the small dishes under which they were confined were removed after the 4 hour periods.

Small workers collected near the egg pile of the colony were then introduced in the large Petri dish. These workers usually sit close to the queen in the nest and are likely to respond to queen substances in this experiment. The walls of the test arena (large Petri dish) were coated with Fluon to force the workers to stay on the filter paper. After 10 minutes habituation, the number of workers with at least the head above the test and control areas was counted at 5 minute intervals for 45 minutes. Each 5-minute period was considered as independent, with the result that the same worker could be counted several times on the same area. Twenty hours later, the experiment was repeated with the same filter paper in order to monitor the persistence of the effect of the substances deposited. Queens and nestmate workers from 3 colonies (H, J and K) were used. Intercolonial differences in the gathering response on worker and queen deposits were compared with the Kruskal-Wallis test. The cumulated numbers of ants gathering on each area for the 10 counts were compared with a Wilcoxon test.

Queen caging A piece of Plexiglas (110x26x3mm) drilled with holes (diameter 8mm) was placed in a chamber of the nest, so as to delimit a blind area (110x45mm) representing 6.4% of the nest surface. The queen was equipped with a wire twisted around her thorax, as in the experiment of section IV.C.b. The wire's extremities pointed outward, perpendicularly to the queen's body axis, and formed a rigid appendix of 14mm length that prevented the queen from passing through the holes of the Plexiglas barrier. She was therefore confined to the small area, whereas workers could freely move in the whole nest, pass the barrier in both directions and make contact with their caged queen. The experiment was repeated four times with colonies B, C, I and K (300-800 workers per nest). Consequences of caging on the queen-worker contact pattern were monitored in colonies B and C, following the methodology already described for non-manipulated colonies. Over 3 days, for 2 (colony B) and 4 (colony C) consecutive hours, the identity of workers making contact with a queen equipped with a wire but free to move in the nest (i.e. not confined behind the barrier) were noted. This period allowed controlling for the effect of the wire on contact rate and on the proportion of the colony that had contact. Three days after the queen was caged, the observations resumed for another 3 periods of 2 and 4 consecutive hours. The observation sessions (non-manipulated [section queen-worker contacts], free manipulated queen and caged manipulated queen) started at 7 days interval. One more observation session of 2 and 4 consecutive hours was realised 20 and 19 days after queen caging in each colony (B and C respectively). Longer-term changes induced by the manipulation could thereby be tracked. Occurrence of agonistic

interactions among workers of the 4 experimental colonies was regularly monitored for 2-4 months after caging, in order to test the role of the queen's visit and putative pheromonal marking in the nest on the regulation of reproduction in workers. Sporadic observations were adequate to detect the occurrence of agonistic interactions as these often last for minutes, hours or even days and involve many workers.

Results

Queen-worker contacts On average, 9 to 20% of the workers of a colony had physical contact with their queen or with a control worker within an hour. The proportion of individuals making contact with the focus individual in colony A was similar across observation sessions (n_{queen}=8, Chi^2_{queen}=0.3, p_{queen}<0.88, n_{worker}=3, Chi^2_{worker}=2.0, p_{worker}<0.37). The doubling of density in colony A therefore did not affect the proportion of workers making contact with the queen or with a control worker. The data sets were therefore pooled for the analysis on the one-hour basis. The proportion of workers contacting the queens was significantly lower than that contacting the control workers in colony A, but significantly higher in colony B (n_{Aqueen}=24, $n_{Aworker}$=9, U_A=22.0, p_A<0.05, n_{Bqueen}=8, $n_{Bworker}$=3, U_B=2.0, p_B<0.05). Both proportions were similar in colony C (n_{Cqueen}=24, $n_{Cworker}$=4, U_C=42.5, p_C=0.72; figure IV.C.5.). The number of workers that met the focus

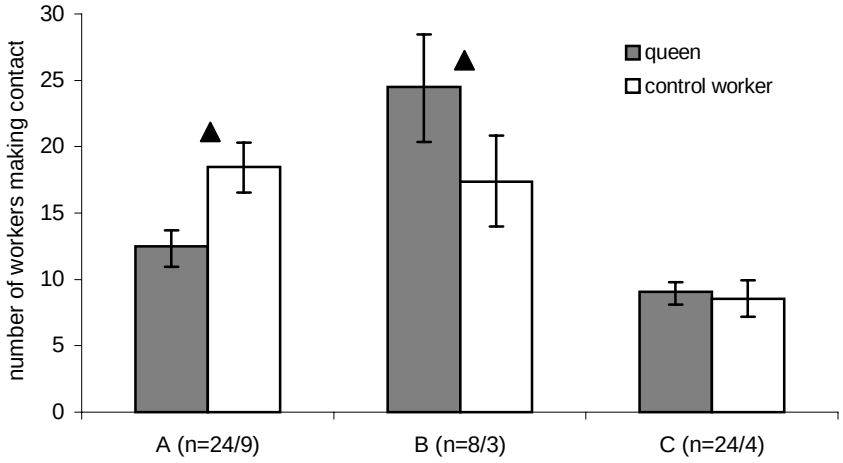


Figure IV.C.5.: proportion of workers in a colony that made physical contact with either the queen or a control worker during one hour. Duration of observations (in hours) are given in parentheses for contacts with queen/control workers respectively. Error bars represent 95% confidence intervals calculated from the arcsine transformed data. ▲ represent significant difference (Mann-Whitney test, p<0.05).

individuals in an hour was not correlated with colony size (r_{queen}=0.40, r_{worker}=0.23).

During 3 hours periods, 18 to 38% of the workers had contact with their queen (figure IV.C.6.). The sample size was not sufficient to perform a statistical comparison of the proportion of workers that had contact with their queen and with a worker after 2 hours periods. However, it can be deduced from figure IV.C.6. that these proportions are very similar in colony C, but less so in A and B. In these latter colonies, the differences had opposite directions. However, regression lines of the cumulated proportions of workers contacting the queen or a worker were similar. Only their intersects differed significantly in colony A (table IV.C.4.). A difference in intersect reflects a lower number of workers that had contact with an individual for the first half hour of observation and is correlated to the number of nestmates staying close to this individual. The rates of contacts and proportion of workers having contacts with the focus individuals varied considerably between colonies and were inversely correlated with colony size (r_{queen}=-0.94, r_{worker}=-0.66).

As workers recognise the queen from a distance (cf. section IV.B.b.), a minority (0.2-13.8%) of the physical contacts occurring between them were fortuitous (72 hours of observation). Fortuitous contact is defined as an individual colliding into another as it antennates the ground while walking in the nest. The interest of the former was clearly not in the nestmate it ran into. Most of the contacts (mean=71%, range 50.4-84.4) were initiated by workers. Although queens stayed motionless for most of the time (cf. next section), they continuously antennated the workers close to them. They were thus responsible for 15.0-45.1% of the contacts (figure IV.C.7a.). As workers recognise the queen from a distance (cf. section IV.B.b.), a minority (0.2-13.8%) of the physical contacts occurring between them were fortuitous (72 hours of observation). Fortuitous contact is defined as an individual colliding into another as it antennates the ground while walking in the nest. The interest of the former was clearly not in the nestmate it ran into. Most of the contacts (mean=71%, range 50.7-84.4) were initiated by workers. Although queens stayed motionless for most of the time (cf. next section), they continuously antennated the workers close enough to them. They were thus responsible for 15.0-45.1% of the contacts (figure IV.C.7a.).

The legs are the first limbs exposed to workers as they approach their queen from the side, or to small retinue workers that remain crouched at the queen's side, lower than the rest of her body. Head and gaster were touched first as workers arrived from the front or the rear respectively. They never reached the thorax in the first place. Workers touched the legs of the queen in 50 to 74% of the contacts they initiated (figure V.C.8.). This high contact frequency was not due to a particular attraction to the legs. The space they occupy represent on average

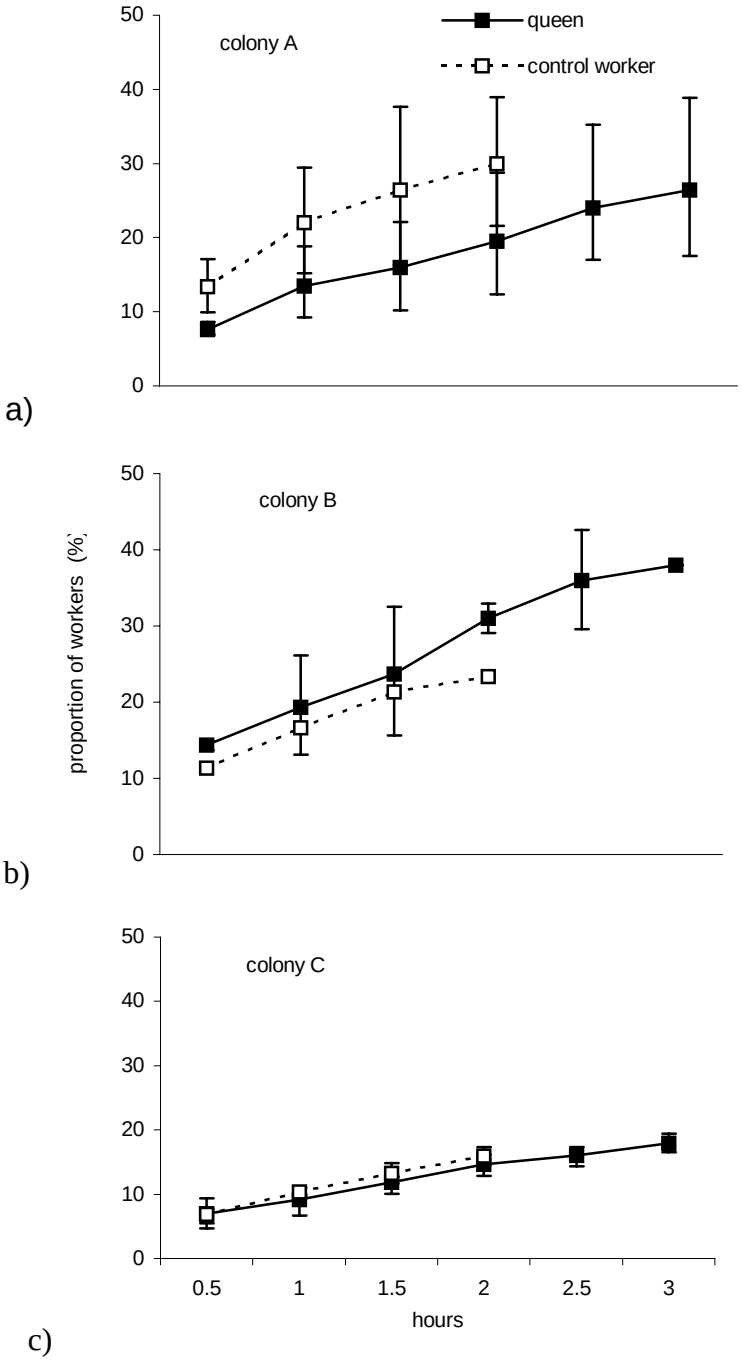
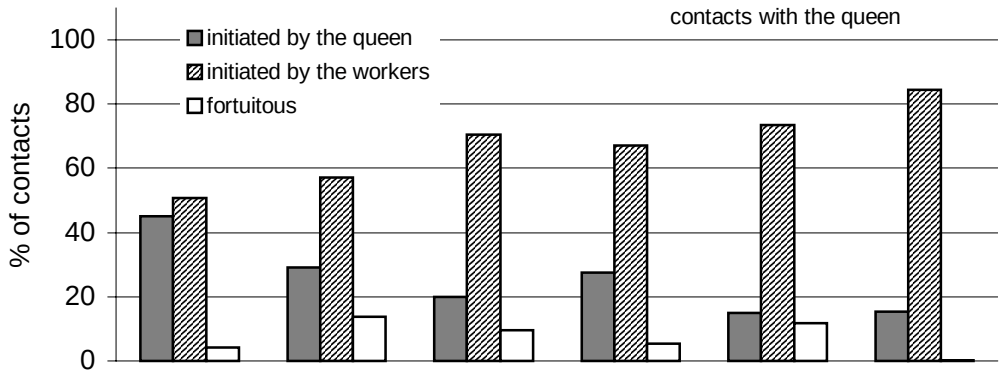


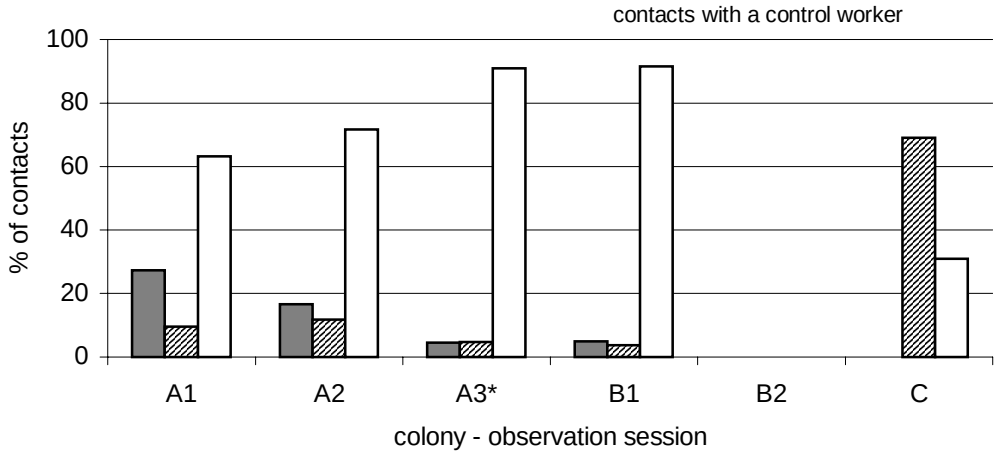
Figure IV.B.6.: proportion of workers having had contact with their queen or with a control worker in colonies A, B and C for 3 and 2 hours periods respectively. Sample size is as follows: colony A four 3h periods for queen contacts and two 2h periods for control worker contacts; colony B two 3h and one 2h periods for queen and worker contact respectively; colony C eight 3h and two 2h periods for queen and worker contact respectively. Only observation sessions with similar worker density in the nest were used. Error bars represent 95% confidence interval calculated on the transformed data.

Table IV.C.4.: regression lines corresponding to the cumulated proportion (arcsine transformed) of workers that had physical contact with their queen or with a control worker in 3 colonies (cf. figure IV.C.6.).

colony	A	B	C
queen/worker contacts	$y_1 = 14.6 + 5.2\ x_1$	$y_1 = 19.7 + 7.8\ x_1$	$y_1 = 14.4 + 3.6\ x_1$
coefficient of determination r^2=	0.60	0.91	0.84
p	<0.01	<0.01	<0.01
observations, n=	6x3h	2x3h	8x4h
worker-control/worker contacts	$y_2 = 18.9 + 7.6\ x_2$	$y_2 = 18.2 + 6.6\ x_2$	$y_2 = 12.8 + 5.5\ x_2$
r^2=	0.87	0.95	0.92
p	0.01	0.02	<0.01
n=	3x2h	2h	2x2h
comparison of slopes	p=0.21	p=0.6	p=0.09
comparison of intersects	p<0.01	p=0.04	p=0.22



a)



b)

Figure IV.C.7.: queens or workers may initiate interactions. Contacts may also occur fortuitously, as two individuals collide into one another. How contacts between a) queen and workers or b) between two workers arose is represented for 3 colonies over several observation sessions. *: the worker density was increased in this colony by decreasing nest surface. Data on control worker contacts were not collected for the second observation session in colony B.

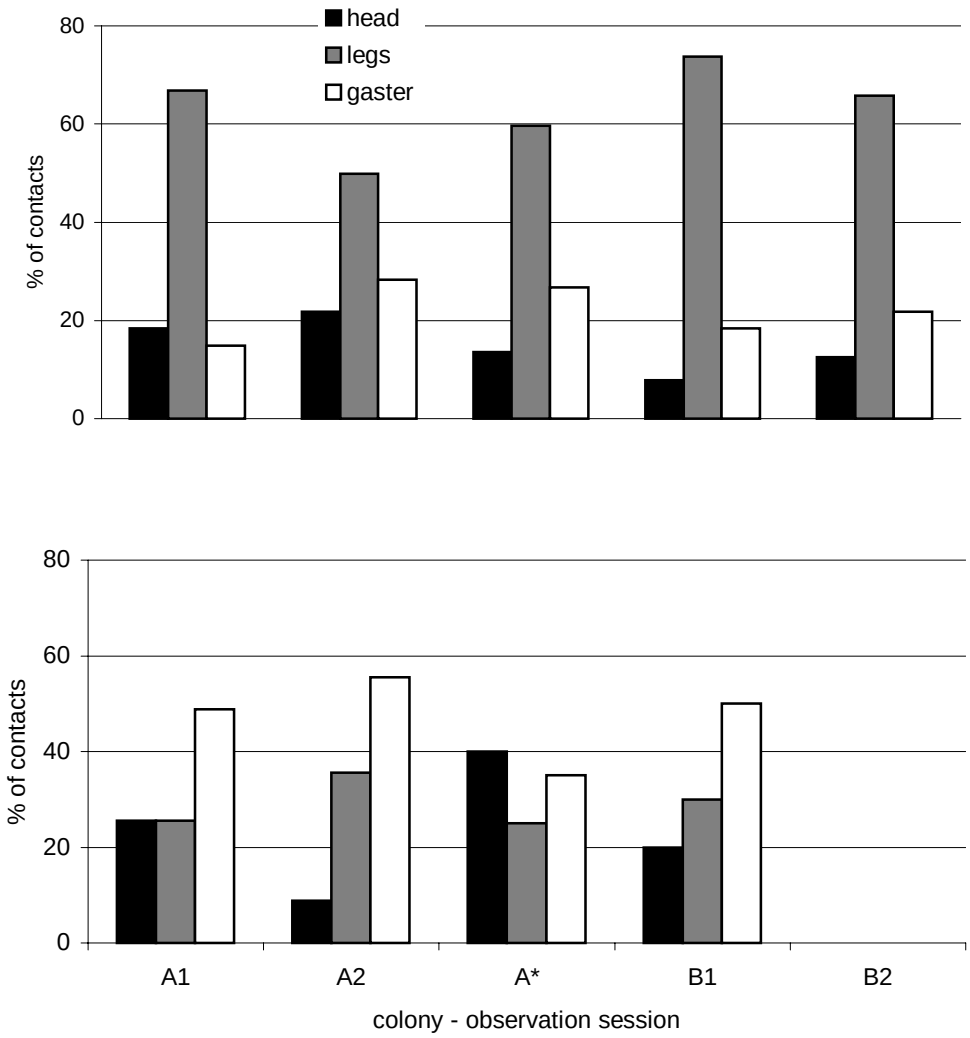


Figure IV.C.8.: body parts touched by workers when they initiated physical contact with a nestmate queen (a) or immobile worker (b). Data on control worker contacts were not collected for the second observation session in colony B.

66.3% of the perimeter of a circle enclosing the queen, the probability for individuals to touch the legs first when approaching their queen is therefore higher than for the head or gaster. This closely matches the observed frequency (on average: 65.7% of contacts with the legs). Difference between predicted and observed frequency of contacts with the head and gaster diverged more (19.2 vs. 12.2 and 14.5 vs. 22.2 respectively, Chi^2=6.55, p=0.04), but were from the same order of magnitude.

Most of the contacts between workers were fortuitous, occurring as a moving individual collided into another (mean=69.7%, range 63.2-91.4, figure IV.C.7b.). Colony C, in which most of the contacts among nestmate workers (69.0%) occurred on purpose, represented an exception to this rule. As control workers were chosen according their position

next to brood, they were sometimes subject to contacts by the queen. Indeed, she sporadically moved around in the chamber (cf. next section). Workers touched other workers more often on the gaster (47% of the cases) compared to legs and head (23 and 29%of the cases respectively, figure IV.C.8b.).

Queen visits in the nest Queens of *M. gulosa* spent 1.6±1.0% (mean±st.dev., n=10 seventy-two hours video recording periods) of their time visiting their nest. The queens' visits occurred every day to every three days, and each of them invested comparable time in this activity per day (Kruskal-Wallis ANOVA, n=30 visits for 4 queens, H=3.29, p=0.35). During the visits, queens entered one or several nest chambers before coming back to their starting point. Sporadic direct observations of these visits (n=3) showed that queens met 1.5 times more workers during 10 minutes periods when they moved in the nest, compared to when they did not leave their chambers (figure IV.C.9.). During their visits, the queens met some workers they had never encountered before. They represented a proportion of 10 to 30% of the total number of workers encountered during the 50 minute observations (40 minute control periods and 10 minute periods of queen movement). Without leaving the chamber where they usually stayed, the queens made short range moves for 16.3±1.8% of their time (mean±st.dev., n=144 hours video recording of colonies A and B).

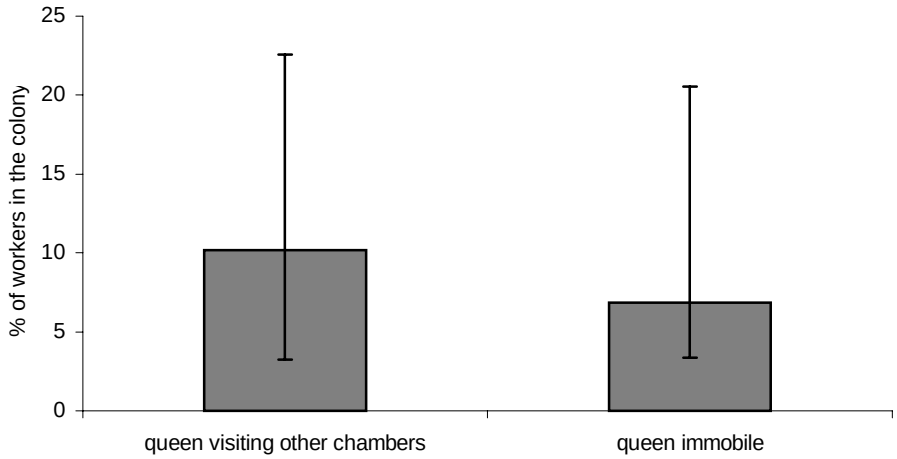


Figure IV.C.9.: average proportions of the workers that have physical contact with their queens (n=2, colonies B and C) for 10 minutes when they visit their nest and when they were immobile. The control periods (queens immobile) directly preceded the visits (n=3) by the queens. Error bars represent the range.

Queen marking of the nest HCs could be collected from the Petri dishes where queens and workers were confined. Queens deposited approximately twice more HCs on the substrate than workers. However, the differences among groups were not significant (Kruskal-Wallis test, n=20, H=2.98, p=0.23, figure IV.C.10.) because of important interindividual variations in release rates. A crude estimate of the quantity of substance collected in the Petri dishes indicate that individuals deposit hydrocarbons on the substrate at a rate of 0.2-0.5µg per hour. A principal component analysis of the transformed area of 12 peaks produced 3 principal components that explained 77% of the variability observed. They constituted the basis of the DA (Wilk's Lambda=0.06, F[15,88]=10.3, p<0.01, figure IV.C.11.), which showed that the HCs queens deposit on the substrate match their CHCs (posterior classification probability, p=0.94). Both their CHC profile and their footprint HC deposits are distinct from the workers' (table IV.C.5.). CHC profiles of large and small workers are not significantly segregated (p=0.20). In contrast, each group could be distinguished by its HC deposit profile. However, worker groups' HC deposit profiles overlapped with each other's cuticular profiles and only partly overlapped with their own cuticular profiles (table IV.C.6.).

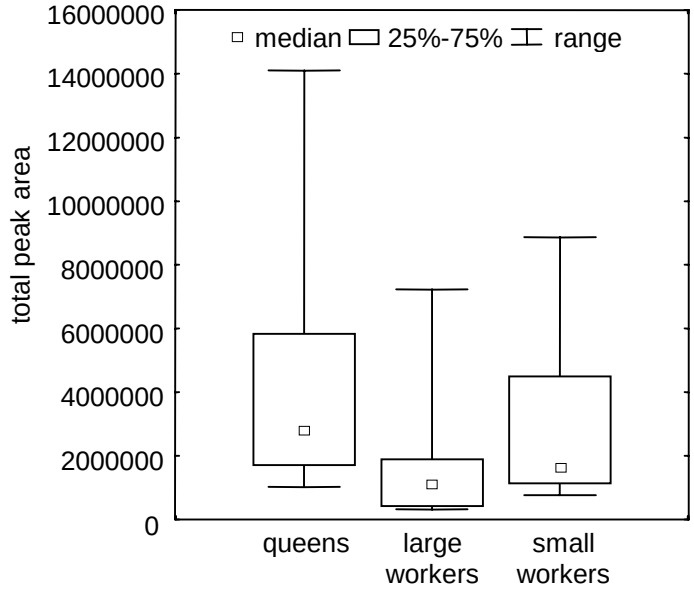


Figure IV.C.10.: quantity (expressed in total peak area) of HCs deposited by individuals on the bottom of a Petri dish where they have been confined for 4 hours. Queens and workers deposit between 0.2 and 0.5µg of hydrocarbons per hour on their substrate.

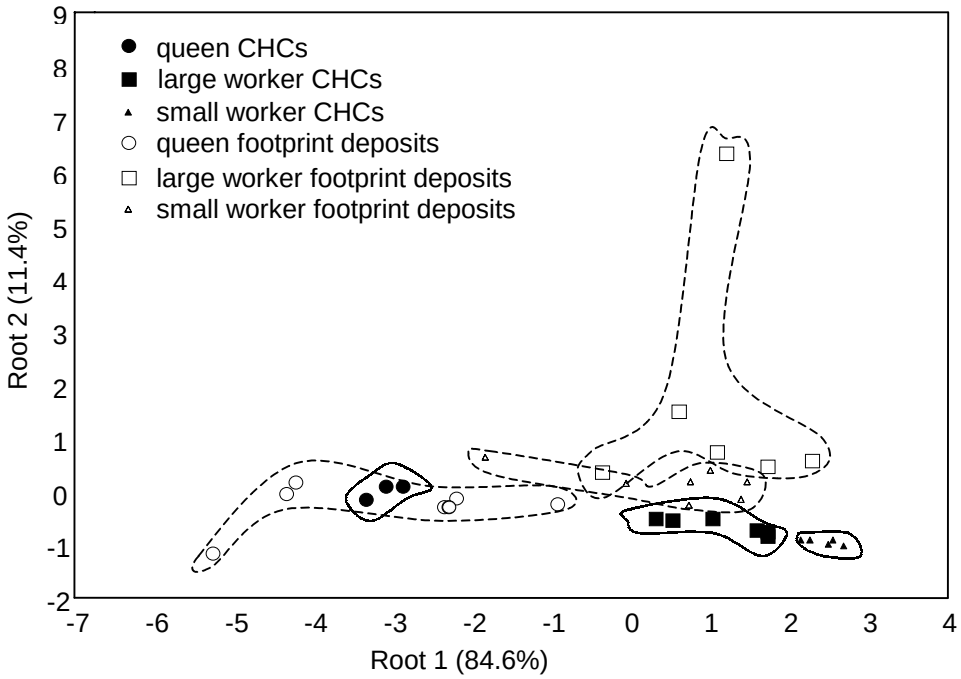


Figure IV.C.11.: discriminant analysis (DA) of CHCs (solid lines and plain forms) and foot print deposits (dotted lines and open forms) of queens, large and small workers. Twelve peaks common to all groups and with a relative area of more than 1% were used as variables for a principal component analysis. The DA was based on the 3 principal components obtained. Percentages of variance explained by the two main roots are given between parentheses. Posterior probabilities and classification matrix are given in tables IV.B.5 and 6.

Table IV.C.5.: posterior classification probabilities of the groups compared by DA (CHCs and foot print deposits of queens, large and small workers).

group	QCHCs	LCHCs	SCHCs	Qfoot	Lfoot	Sfoot
QCHCs		0.00	0.00	0.94	0.00	0.00
LCHCs	0.00		0.20	0.00	0.00	0.17
SCHCs	0.00	0.20		0.00	0.00	0.00
Qfoot	0.94	0.00	0.00		0.00	0.00
Lfoot	0.00	0.00	0.00	0.00		0.01
Sfoot	0.00	0.17	0.00	0.00	0.01	

abbreviations are as follow: - QCHCs, LCHCs, SCHCs: cuticular hydrocarbons of queens, large and small workers respectively;
- Qfoot, Lfoot, Sfoot: footprint deposits of queens, large and small workers respectively.

Table IV.C.6.: classification matrix of the discriminant analysis comparing CHCs and deposit HCs of queens, large and small workers.

		predicted group					
group	% correct classification	QCHCs	LCHCs	SCHCs	Qfoot	Lfoot	Sfoot
QCHCs	62.0%	5	0	0	3	0	0
LCHCs	100.3%	0	6	0	0	0	0
SCHCs	100%	0	0	6	0	0	0
Qfoot	57.1%	2	0	0	4	0	1
Lfoot	33.3%	0	1	1	0	2	2
Sfoot	57.1%	1	2	0	0	0	4
total	67.5%	8	9	7	7	2	7

abbreviations as for table IV.C.5.

Detection of queen marking by workers Approximately two times more HCs could be collected from Petri dishes where queens had been confined. Therefore, the attractiveness of the area where the queen had been sitting was compared with an area of equal surface where 2 workers had been confined. Test workers aggregated preferentially on an area previously occupied by the queen (Wilcoxon test, n=10 in all cases, T_H=0.0, p_H=0.02, T_J=0.0, p_J=0.02, T_K=0.0, p_K<0.01, figure IV.C.12a.). The substances deposited by the different queens did not elicit aggregation of comparable number of workers (Kruskal-Wallis ANOVA, n=30, H=12.4, p<0.01). Worker deposits were equally poorly "attractive" (n=30, H=0.6, p=0.76). An equal

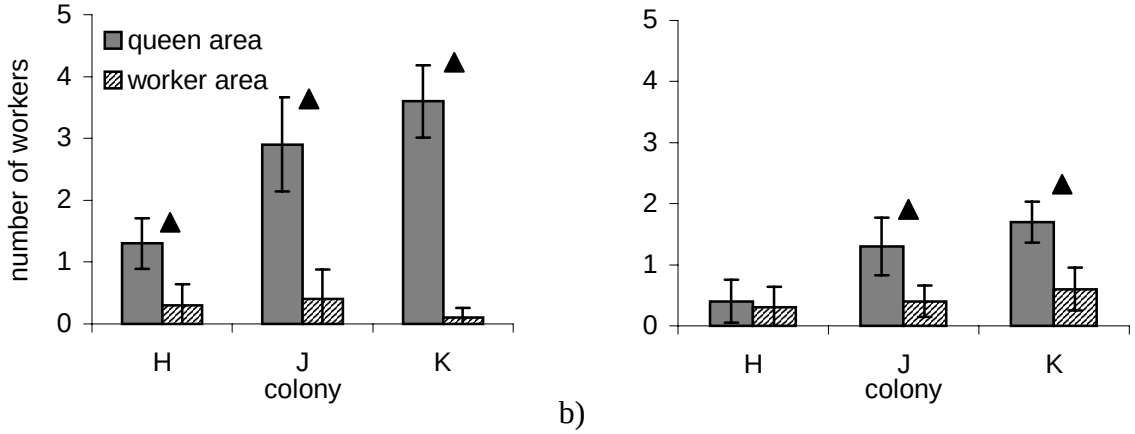


Figure IV.C.12.: attractiveness of filter paper areas on which queen and workers form 3 colonies (H, J, K) were confined for 4 hours. Ten small nestmate workers collected near the egg pile were introduced in the test arena a) immediately after queen and workers removal, b) 20 hours later. Their number on queen and worker area was monitored at one minute intervals for 10 minutes. ▲ indicate significant differences (Wilcoxon test, p<0.05). Error bars are standard deviation.

or inferior number of workers could be counted on the area where workers had been confined compared to the rest of the Petri dish surface minus the queen area (Chi^2_H=5.0, p_H=0.03, Chi^2_J=2.1, p_J=0.15, Chi^2_K=5.9, p_K=0.02). After 20 hours, the filter paper area on which queens were confined elicited less aggregation. The differences were significant in two cases (Wilcoxon test, n=10 in all cases, on T_H=3.5, p_H=0.04, T_J=9.0, p_J=0.06 NS, T_K=0.0, p_K=0.02). The number of individuals counted the queen area was significantly higher for the queens that deposited the most attractive substances on the previous day (n=6 in all cases, T_H=4.0, p_H=0.72 NS, T_J=0.0, p_J=0.01, T_K=0.0, p_K=0.04, figure IV.C.12b.).

Queen caging The average proportion of workers that had physical contact with their queen in non-manipulated and experimental situations was plotted along time in figure IV.C.13. The proportion of workers contacting their queen equipped with a wire but free to move in the nest was less than in the non-manipulated situation in colony B, but similar in colony C. Contact

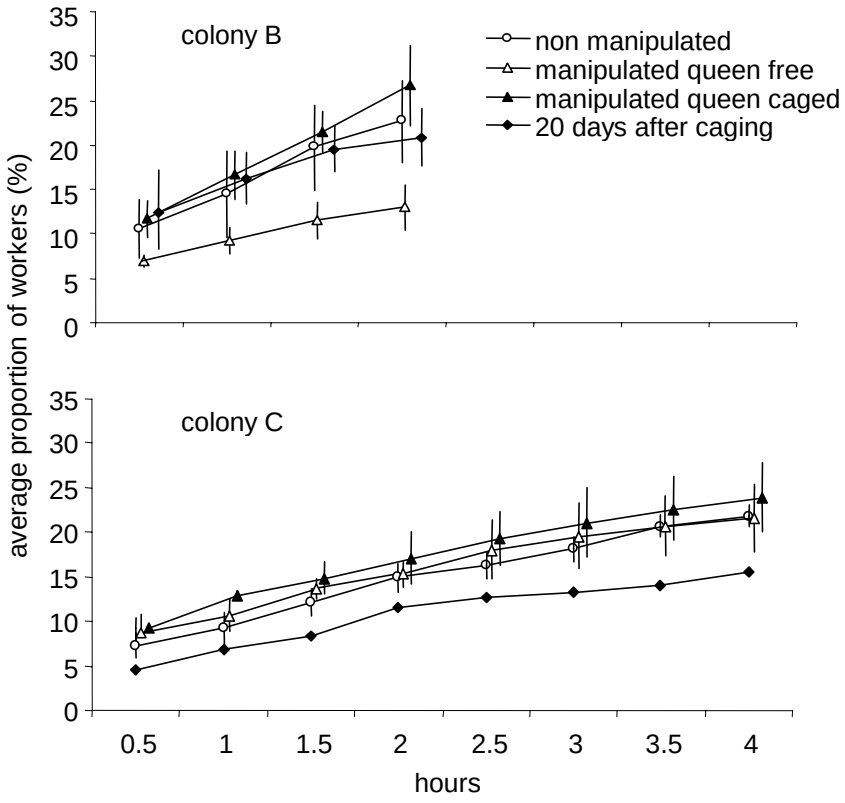


Figure IV.C.13.: Average proportion of workers in colonies B and C having physical contact with their queen. Contacts with queens were observed under four situations: non-manipulated control, manipulated queen (with wire), but free to move around, caged queen (with wire) and caged queen after 19 or 20 days (colony C and B respectively). Error bars represent 95% confidence limits calculated on the transformed data.

rate with the caged queens was identical with the control period in B, indicating that the decrease in proportion of workers having contact with the manipulated but free queen was likely to be an artefact. In colony C, contact rate with the caged queen remained constant, but the proportion of workers having contact decreased significantly (figure IV.C.13., table IV.C.7.). Three weeks after caging, as many workers met their queen as in the non-manipulated period in colony B. In contrast, in colony C, the number of workers visiting her was smaller. The number of queen-worker physical interactions established followed this general pattern. There were as many contacts in the non-manipulated and caged periods of colony B, but significantly less during the control period (queen with wire but free, repeated measures ANOVA, $F=7.2$, $p<0.01$). In C, the number of contacts was lower in the non-manipulated period and similar in the experimental periods ($F=3.9$, $p=0.03$). The number of contacts per hour adjusted for colony size was different in these colonies (0.67 vs. 0.28 contacts per hour, $t=19.31$, $p<0.01$) is noteworthy.

Table IV.C.7.: regression on time of the cumulated proportion of workers (arcsine transformed) that had direct physical contact with their queen in colonies B and C (cf. figure IV.C.13.). Slopes and intersects obtained for each experimental condition are compared with the non-manipulated situation. Sample size in hours of observations is given.

colony	B	C
non-manipulated situation	$y_1 = 15.6 + 6.6\ x_1$	$y_1 = 14.4 + 3.6\ x_1$
coefficient of determination r^2=	0.42	0.84
p-value	<0.01	<0.01
observations n=	7x2h	8x4h
free queen with wire	$y_2 = 13.6 + 3.8\ x_2$	$y_2 = 16.4 + 3.0\ x_2$
r^2=	0.51	0.75
p-value	<0.01	<0.01
n=	7x2h	4x4h
comparison of slopes	p=0.11	p=0.13
comparison of intersects	p<0.01	p=0.06
caged queen with wire	$y_3 = 16.6 + 6.9\ x_3$	$y_3 = 17.5 + 3.1\ x_3$
r^2=	0.8	0.83
p-value	<0.01	<0.01
n=	6x2h	3x4h
comparison of slopes	p=0.58	p=0.24
comparison of intersects	p=0.1	p<0.01
caged queen after 3 weeks	$y_4 = 18.8 + 4.5\ x_4$	$y_4 = 12.2 + 3.0\ x_4$
r^2=	0.74	0.92
p-value	<0.01	<0.01
n=	2x2h	4h
comparison of slopes	p=0.62	p=0.8
comparison of intersects	p=0.60	p<0.01

Agonistic interactions among workers could not be observed in the 2 to 4 months following queen caging in the four colonies studied. No sexuals were reared in these colonies.

Discussion

Queen-worker contacts In laboratory nests, a large proportion of workers meet their queen in a short time interval. Comparable proportions of individuals in the colonies make contact with their queen and with a control worker. Furthermore, the contact rates with both queen and other workers are similar. Workers therefore seem to encounter their queen while performing their usual tasks (brood care, nest maintenance). As workers move around the nest and the queen remains immobile, the former initiate most the contacts as they approach the queen. They recognise the queen from a distance and move slowly toward her. They typically back away as soon as a contact is established (cf. section IV.B.b.). Thus, the body part they touch depends on their angle of approach and on the latter's spatial dimension. As the legs occupy more space, they are the appendages most often touched. In contrast, workers are not "attractive", and physical contacts among them arise fortuitously as they run into each other. Their approach is fast and random; they are therefore less likely to touch the thin legs first.

The repulsive effect of the queen on certain individuals tends to reduce the proportion of workers that establish physical contact. Her "attractiveness" for retinue workers has not much influence on this proportion as the same group of individuals stays at her side for long periods (cf. section IV.B.c.). Workers usually ignore each other and only the congestion inside the nest affects the proportion of nestmates contacting a random individual.

The density of workers determines congestion in the nest. In colony A, congestion did not influence the proportion of workers having contact with their queen during one-hour periods. However, a decrease became obvious if longer, three-hour periods were considered. This difference is explained by the higher relative importance of the retinue workers in the one-hour sessions counts. As mentioned above, they stay at the queen's side and are exposed to frequent queen contacts, but their low number does not induce an increase in the proportion of workers that establish contact with the queen. For longer time periods, the proportion of retinue workers compared to the total number of workers that encountered their queen decreased, whereas that of non-retinue workers increased. Variation in the number of the latter thus became more significant. Density also varied between the colonies studied and the importance of its effect on the contact pattern was confirmed by the negative correlation between colony size and proportion of workers contacting their queen. Colony congestion

reduces the turnover of workers in the queen chamber and therefore reduces the possibilities of queen contact for workers. In crowded honeybee hives, the quantity of 9-ODA (a component of the queen mandibular pheromone) distributed and its circulation in the nest were not altered compared to those of a non-crowded hive. However, similarly to what was observed here, congestion reduced the proportion of workers that were contaminated with the pheromone component (Naumann et al. 1993). Importantly in the honeybee case, even in populous colonies, a worker could receive the same quantity of pheromone as in an unpopulous one, given the opportunity to be in contact with an individual carrying it. Colony congestion could similarly affect the modalities of distribution of the queen pheromones regulating worker reproduction in *M. gulosa*.

Overall, the pattern of queen-worker contacts appeared variable. The interplay of the above-mentioned factors certainly contributed to this variability. Likewise, in the fire ants, Sorensen et al. (1985) reported varying proportions of individuals contaminated by a radiolabel applied on queens. Its distribution, being directly linked with interindividual contacts, suggests that pattern of contacts can vary according to uncontrolled conditions. These variations however, do not affect colony organisation as long as they allow individuals to detect the queens' pheromones before physiological reactions consequent to orphanage are triggered.

Queen visits Although moving in the nest increased the number of workers a queen can encounter by 50% per unit time, the short duration of visits (1.6% of their time-budget on average) make it a relatively inefficient mechanism to distribute queen pheromones. Queens spend more time (16.3% of their time-budget) moving within the brood chamber where they usually sit. But here again, the efficiency of pheromone propagation is limited, as they only encounter the workers already present in the chamber. While performing their usual tasks, workers frequently move in the nest; they therefore have multiple opportunities to encounter even an immobile queen once they have entered her chamber. In *P. apicalis*, Dietemann and Peeters (2000) observed that the queens moved in their nests up to 30% of their time. The importance of the visit in pheromone distribution might therefore be more important in this species. Seeley (1979) reports high movement activity of honeybee queens and suggest that it plays an important role in pheromone distribution. Juška (1978) and Lensky and Slabezki (1981) showed that honeybee queens deposit secretions on an area they have spent time on or walked on. These secretions elicited worker aggregation in absence of the queen and their

application on bottom edges of a comb hindered queen rearing cup constructions in conditions when it normally occurred (i.e. in crowded hives, where congestion prevents the queens from reaching the edges of the comb and depositing their pheromones). Whether these secretions originate in the queens' tarsal and/or the mandibular glands is not clear. Whether queens of *M. gulosa* secrete "trail substances" or "footprint pheromones" while moving in their nest was investigated.

Queen marking of the nest and detection by workers Queens and workers contaminate their substrate with the HCs they secrete. The congruency of the CHCs with the HCs collected from the Petri dishes where they had been confined suggests that the substrate becomes contaminated with CHCs. Given this similarity, no ultrastructural studies were envisaged to look for other glandular sources situated in the legs. No particular behaviour was observed in the individuals confined in the Petri dishes and no other body parts than the legs and antennae tips were frequently in contact with the substrate. This contamination is therefore likely to be passive and to occur via the legs. The CHCs collected by the tarsal brush during self-grooming (Hefetz et al. 2001) and not ingested by licking, could leak and be dispersed over the substrate while walking. Alternatively, the CHCs contained in the PPG could be spread on the brushes during self-grooming and be involved in the contamination of the substrate. Relative proportions in CHCs distinguished queens from workers (cf. section IV.B.d.ii.). Likewise, the profiles obtained from the HC deposits allowed distinguishing queen from workers. As the cuticular extracts of queens (cf. section IV.B.d.ii.), the deposits of queen have a releaser effect, in that they trigger worker aggregation. In contrast, worker deposits do not trigger much interest in nestmates. These substances could therefore represent a queen "footprint" pheromone. This is comparable to the phenomenon described in honeybees, where queen mandibular gland pheromones, normally spread over the body of queens, are deposited onto comb wax (Naumann et al. 1991, 1992).

The necessity of direct physical contacts or close proximity with the queen for a transfer of primer pheromone in *M. gulosa*, suggests that these "footprint pheromones" do not constitute a central factor in the regulation of worker reproduction. However, the experiment designed by Tsuji et al. (1999) does not permit for the exclusion of such a role. Indeed, the queen is prevented from visiting the part of the nest where "queenless" workers are confined and the putative "footprint pheromone" cannot be refreshed. Workers could therefore have reacted to the absence of "footprint pheromone" rather than to the absence of the queen. The

idea that workers could detect an absence of marking of the nest is supported by the fact that they tried to force the queen to pass the barrier in two colonies during the above-mentioned experiments (cf. section IV.C.b.). To test the importance of the pheromonal marking, queens were confined in a small portion of the nests and workers were left free to move in the whole nest and had free access to her.

Queen caging Caging queens for several months did not trigger agonistic interactions among workers. Although the experimental treatment slightly affected the proportion of workers making contact with their queen in the short term, regulation of worker reproduction was not disturbed. Thus, neither the extra number of contacts a queen makes during her visits in the nest (compared to when she is motionless), nor pheromonal marking of the nest play a major role in the regulation mechanism of worker reproduction. The substances deposited by the queen on the nest surface could go undetected by workers due to low quantities or too infrequent renewal, or they may have another role, undetected by the present observations. The latter possibility is suggested by the fact that workers apparently detected the absence of the queens in nest part A and tried to force them to pass the barrier (cf. section IV.C.b.).

As already mentioned, field colonies are larger (in size and worker population) and the contact rate with queens might be inferior to those observed here. Queen visits and/or pheromonal marking could play a more important role in regulating worker reproduction and gyne rearing by slightly enhancing the distribution of queen pheromones by increasing contact frequency with workers or by marking the nest.

VI.C.d. Effect of queen corpses

Introduction Researchers used queen corpses to test whether the effect of a queen on worker behaviour or physiology is mediated by her behaviour or exclusively by the pheromones she emits. It appeared that the presence of the queen's corpse in colonies accelerated the apparition of the symptoms of orphanage, which is contrary to most the reports (cf. section IV.C.a.). Preliminary results concerning this phenomenon are described and their signification is discussed.

Methods The queens from two colonies (C and D) were killed by freezing and reintroduced in their nest after thawing (cf. section IV.B.c.). Seven groups of at least 100 workers were deprived of their queens and the queen died naturally in two further colonies. The onset of agonistic interactions among the orphaned workers was monitored.

Results Agonistic interactions appeared in the hour following reintroduction of the dead queens in colonies C and D. In contrast, in the absence of a queen corpse, the workers started to fight after 8.7±3.8 days (mean±st.dev., range 7-17, n=7). The time of queen death could not be known with precision in the cases where it remained unnoticed for a few days (colonies A and G). These queens were still alive three days before they were found inanimate, but workers may have detected her failing health earlier. They thus started fighting 3 days after the queen was seen alive for the last time. As the physiological reactions linked to orphanage may have been induced before the actual death of the queen, these 2 colonies were not included in this comparison.

Discussion Workers' interactions with a corpse differ in quality and quantity from their interactions with a live queen. Indeed, workers seem to avoid physical contacts and stay away from a live queen, whereas they show more interest in her corpse. This suggests that they detect a change (absence of behaviour of the queen or a change in the chemicals emitted) and perform closer antennal inspection than usual (section IV.B.d.i.). Workers of the fire ants similarly respond to fresh corpses with vigorous and extended inspection (Howard and Tschinkel 1976).

The changes in worker behaviour when exposed to the corpse of their queen can be explained on the basis of only one chemical of which quantity emitted is different for a live or a dead individual. Different thresholds perceived by the workers might alter their behaviour. However, in *M. gulosa* (section IV.B.d.ii.), as well as in the honeybee (Naumann et al. 1991), the quantity of chemicals present on queens varies widely between individuals that are equally potent regarding regulation of colony organisation. This suggests that quality of the cues rather than their quantity is important. If the existence of several semiochemicals interacting to produce a queen signal (Keller and Nonacs 1993) is considered, variations in their ratio might give workers additional information on their queen's health or fertility state, that are relevant for the regulation of their behaviour. Occurrence of several substances that mediate queen recognition has already been reported in honeybees and in the fire ant (Winston and Slessor 1998, Wossler and Crewe 1999b, Vargo and Hulsey 2000, Katzav-Godzansky et al.

2001). Workers are likely to detect the queen's presence using singly each of the components of her chemical signal, but their association might be necessary to mediate regulatory effects (Slessor et al. 1998).

The idea that workers could compare different chemicals emitted by the queen in order to evaluate her health status is supported by the preliminary results that agonistic interactions are triggered earlier when queen corpses were available (n=2), compared to nests where the queen was simply removed (n=7). The observation that the queen corpse is recognised even after 1-2 days and that the workers can interact agonistically before this delay (as soon as 20 minutes after queen killing) is not compatible with a regulation of worker behaviour based on the simple detection of the queen's presence or absence. Information about her health state has to be conveyed by the chemicals she produces or by her behaviour.

Assuming that queen replacement does not occur in *M. gulosa*, an orphaned colony is condemned to death (Dietemann and Fresneau submitted). An early triggering of reproduction in presence of a queen corpse would thus be adaptive as it is in the workers' interest to start producing new female sexuals and to lay males-destined eggs as soon as possible before the colony perishes. On the other hand, the fact that workers cannot find their queen does not mean that she is dead or lost and that the situation is irreversible. Workers should not start to reproduce immediately in the case the queen is again located.

In contradiction with these arguments, some studies showed that queen corpses retain a regulative power for several weeks or even months (Pain 1955, Verheijen-Voogt 1959 for bees, Fletcher and Blum 1981a, Hölldobler and Wilson 1983) and that fresh corpses renewed periodically prevent female sexuals rearing and worker reproduction (Carr 1962, Brian 1973, Vargo and Passera 1991). In two cases only did dead queens lose their regulative power after death (Passera 1980, Edwards 1987). Whether production of males and gyne rearing in these studies occurred faster in orphaned worker groups with corpse than in those without corpse is not described. The interspecific differences might result from the mode of release of the pheromones involved. Loss of regulative activity by queen corpses is expected when pheromone release is bound to behaviour (e.g. to egg-laying: Edwards 1987, Obin et al. 1988). In *Solenopsis invicta*, queen corpses delay dealation by virgin queens although oviposition was demonstrated to be necessary for the release of the "inhibitory" pheromone (Fletcher and Blum 1981a, Obin et al. 1988). These paradoxical results could be explained by the interaction of several pheromones emitted by queens, as suggest the results obtained by Vargo and Hulsey (2000). Further studies on the mode of release and action of queen primer

pheromones are needed to understand interspecific differences observed and the interaction of multiple queen pheromones in a complex regulation system.

IV.C.e. Synthetic CHC profile

Introduction In *M. gulosa*, the pheromones acting on worker reproduction are not volatile and are perceived during direct physical contact with the queen. As already mentioned, CHCs possess the characteristics one attributes to queen pheromones or signals. They have low volatility, are spread over the cuticle of queens, are linked with ovarian status and are specific to queens. The availability of the synthetic queen blend of major hydrocarbons (cf. section IV.B.d.ii.) allowed for the preliminary testing of their role as primer pheromone involved in the regulation of worker reproduction.

Methods Two groups of 20 workers from one colony were isolated in small experimental nests. Individuals staying in the queen chamber (large and small workers) as well as foragers were chosen. The synthetic queen blend corresponding to the difference in quantity of compounds between queen and workers was applied on a worker from the original mother nest. This worker was introduced in the first experimental nest. A worker on which the corresponding volume of solvent (4µl) was applied was introduced in the second group, as a control. Both the workers were replaced every day for one month. Occurrence of agonistic interactions and presence of reproductive eggs were monitored daily in both experimental and control nests.

Results and Discussion Agonistic interactions were observed in the experimental group on the 14^{th} day. No fights could be observed in the control group. Reproductive eggs appeared in both groups simultaneously, on the 30th day. The blend of CHCs tested therefore did not delay the physiological responses characteristic of orphanage in the group tested. Given the technical difficulties of such assay (identity, proportion and quantity of compounds used, frequency of replacement, high response latency, inter-group variation in reaction to orphanage), no further attempts were made to repeat the experiment.

IV.C.f. Discussion: queen primer pheromone regulating worker reproduction

Workers were demonstrated to be unable to transfer the queen pheromones regulating worker reproduction to their nestmates. An indirect distribution mechanism of queen pheromones has to be based on a contamination of the workers that lick and antennate the queen, as it occurs in the honeybee (Naumann et al. 1991, 1992). Given the lack of tending of their queens by workers of *M. gulosa* (already noticed by Haskins and Haskins 1950), it is unlikely that such contamination can occur. Moreover, the brevity of the queen-worker contacts and the slow turnover of retinue workers make the existence of "messenger" workers (Seeley 1979) unlikely. In contrast, evidence was obtained for the necessity of direct physical contact between queen and workers for the pheromone's detection. Whether the perception of the queen at a short distance (cf. section IV.B.b.) is enough to maintain the regulation is not known. To verify this point, queens should be kept immobile behind a mesh through which workers could pass their antennae and reach the active space of this volatile pheromone, without however, being able to touch the queen. The experiment was not realised due to technical difficulty and to the disturbance engendered for the queens.

Workers detect the "footprint pheromone" deposited by a queen on a substrate she was confined on for several hours. They thus certainly can identify the place where the queen spends most of her time in the nest, even in her absence. However, the short periods during which she visits the nest may not allow enough chemicals to accumulate for the workers to detect it. This could explain why nest marking by the queen does not constitute a central factor in the regulation of worker reproduction. It could nevertheless constitute a component of a complex queen signal: although its absence does not trigger reproduction in workers, it may represent information about the queen's location in the nest. Complexity of the queen signalling was further suggested by the preliminary observation that the presence of the corpse of a queen might trigger faster physiological responses in orphaned workers.

Workers' movements in the nest often brought them in proximity to the queen, and the frequency of contact established did not require her to move around the nest and become accessible to a larger proportion of workers. However, natural nests of *M. gulosa* are larger and host a larger worker population than those kept in the laboratory for the present study. These results may therefore represent an overestimate of the queen contact rate for a given individual. The proportion of worker population establishing physical contact with the queen is nevertheless in accordance with that measured in other social Hymenoptera. In the honeybee, 35% of the broodnest workers in a colony of 15,000 individuals (i.e. approximately

5,000) directly contact their queen in 10 hours (Seeley 1979). In the fire ant, 50% of the 400 virgin queens placed in a nest of 40,000 workers become contaminated by a radiolabel applied on the body of the single queen within 2 hours (Sorensen et al. 1984). Considering the size of the honeybee and fire ant colonies and that of *M. gulosa* (up to 2000, cf. section II.B.), the results obtained here are consistent with the hypothesis that in natural colonies direct queen-worker contacts occur often enough for a majority of workers to meet their queen before the physiological changes induced by orphanage take place. Extending the regression line of the cumulated number of workers having had contact with their queen in the largest colony studied (colony C, n=420 individuals, figure IV.C.7c.), shows that, assuming a constant contact rate, 100% of the workers would have contacted the queen after 23.8 hours. This is well within the minimum of three days necessary for the workers to show the first syndromes of orphanage.

Given the low number of queens available, it was not possible to bioassay gland products or cuticular extracts in order to identify the source of the primer pheromone regulating worker reproduction. Alternatively, the role of CHCs was tested by exposing a group of orphaned nestmates to dummy workers on which synthetic queen blend had been applied. This treatment did not delay the onset of reproduction in workers. The hypothesis of a pheromonal role for the major HCs, as single compounds or as the blend tested, is not supported. It does however not exclude the possibility that other HC compounds or a different blend constitute the pheromone. Whether the purified HC fraction of queen cuticular extracts prevent or delay fighting among workers and their reproduction remains to be verified.

IV.D. Discussion: pheromonal mechanisms affecting worker reproduction

The pheromone regulating worker reproduction in *M. gulosa* workers is perceived by direct contact with the queen. No evidence for long-range attraction pheromones was found and workers seem to meet the queen during their usual activity. However, they might search for her when she is missing for approximately a day. The short-range arrestant substances thus do not contribute to the distribution of queen-produced pheromones in the nest. They are better interpreted in terms of recognition pheromone.

As in the honeybee (Seeley 1979), some workers of *M. gulosa* establish physical contacts with the queen whereas others back away from her proximity. This suggests that the need to contact the queen is not the same for all individuals. Several hypotheses can be

proposed to explain this observation. The repulsive effect of a queen is most easily explained by a need to avoid the effects of her pheromones. This could allow selfish workers to reproduce in her presence, which occurs at low frequency in honeybees (Visscher 1989, 1996, Ratnieks 1993). Worker reproduction in queenright situation has not been shown in the present study, but is unlikely. Although workers possessed information on the localisation of the queen in the nest, they did not take advantage of it to start reproducing away from her (cf. sections IV.C.b. and IV.C.c.). Tsuji et al. (1999) reached the same conclusion for *Diacamma* sp. from Japan. Without reproducing, workers that avoid the queen could keep a physiological head start in becoming reproductive once orphaned (Moritz et al. 2001, 2002). These authors indeed found more queen-like mandibular blends in workers that avoided the queen than in others. However, this implies that workers must be able to modulate their exposition to the queen pheromones in order not to develop their ovaries to the point they become egg-layers and can be identified as such. This would expose them to attacks by their nestmates (Sakagami 1954, Visscher and Dukas 1995) and to destruction of their eggs (Ratnieks and Visscher 1989, Ratnieks 1993), thus annihilating their investment in reproduction. Avoidance behaviours are intuitively expected under a queen control mechanism, i.e. if queens produce coercive pheromones suppressing the workers' reproduction against their interest (Keller and Nonacs 1993). However, potential reproductive workers could stay away from a queen signal for the same purpose, by "ignoring" the queen instead of avoiding her.

In a queen signaling system, workers are expected to verify the queen's presence and fertility. Although the queen's presence is recognised at a distance, information reflecting the queen's reproductive activity may not be encoded in this slightly volatile pheromone, but in less volatile cuticular compounds (like the HCs) that can be perceived only by contact. It is known in honeybees that important interindividual differences in reproductive potential occur (e.g. Velthuis 1970). Similarly, it was obvious throughout this study that only some workers start reproducing when freed from queen pheromones. Most of them continue to produce trophic eggs to sustain the colony. Thus, only workers that have the potential to become reproductives would need to physically contact the queen in order to determine when it becomes in their interest to produce sons instead of rearing brothers. Consistent with this hypothesis, retinue workers of *M. gulosa* possess more reproductive-like CHC profiles compared to randomly chosen individuals, and this is not due to contamination from queen substances (results not shown). This however, does not explain her repulsive effect on other individuals.

Although they can differ in their interpretation, the patterns of queen contact or avoidance obtained here and by Moritz et al. (2001) suggest the existence of a link between

behavior toward the queen and reproductive potential of workers. The avoidance or "ignoring" strategy could represent a good trade-off between giving up direct reproduction in the presence of the queen and increasing ones chances of future reproduction as orphan worker (Moritz et al. 2001, 2002) and needs further study. To follow the behaviour (frequency and type of contacts with the queen, spatial location) of individually marked workers before queen removal and their reproductive activity afterwards, could help verify this link, and might give insights to whether the queen is signalling her presence to workers or whether she is suppressing their reproduction.

Although *M. gulosa* belongs to a "primitive" taxon, pheromonal mechanisms affecting worker reproduction appear to be complex. Workers detect a slightly volatile queen pheromone at a short range that either repulse or attract them, they recognise the queen on the basis of several gland products, they can detect local information about her presence and presumably her absence, they seem to search for her when she is wanting and can distinguish dead from live queens. These effects are most probably mediated by several semiochemicals. Supporting this hypothesis, products of several glands elicit releaser responses in workers. Their chemical identification is necessary to test their individual role and understand their interactions in the regulatory functions of queens.

V. Worker reproduction, worker policing and the role of CHCs

V.A. Introduction

Ants and Hymenoptera societies in general are characterised by the altruistic behaviour of group members. Some individuals forego direct reproduction and help in rearing the offspring of one or a few individuals. This fact, which perplexed Darwin and made him doubt the validity of his theory of evolution, found a theoretical basis in Hamilton's theory of kin selection (1964a, 1964b). To give up its own reproduction is an evolutionary stable strategy if helpers contribute significantly to the rearing of individuals that are sufficiently related to them, thereby overcoming the cost of helping. They are then said to gain inclusive instead of direct fitness. In the Hymenoptera, the haplodiploid sex determination system engenders unusually high relatedness among female offspring. However, members of Hymenopteran societies are no clones and relatedness asymmetries generated by haplodiploidy can lead to diverging interests, which can paradoxically hinder the evolution of sociality. Trivers and Hare (1976) formulated the existence of conflicts of interests within societies. Divergence in interests arises regarding the ratio of male to female sexuals produced and the parentage of males in a colony. Figure V.A.1. shows that in a haplodiploid system, workers of monogynous and monoandrous colonies are more related to female than to male sexuals produced by the queen. They would therefore gain more fitness by rearing a higher number of

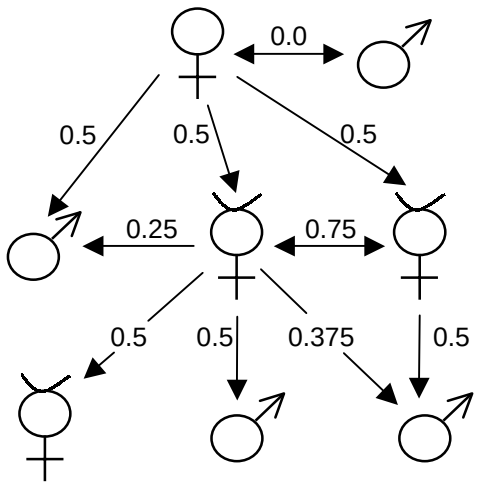


Figure V.A.1.: relatedness coefficients of nestmates in colony of Hymenoptera headed by a single, once-mated, outbred queen. ♀: queen, ♂: males, >O+: workers.

females. On the other hand, the queen is equally related to both her female and male offspring and her optimum ratio would be one female for one male. The conflict over parentage of males arises because workers in most species possess functional ovaries and are able to lay haploid, male-destined eggs. As they are more closely related to their own sons and nephews (coefficient of relatedness 0.5 and 0.375) than to their brothers (0.25, figure V.A.1.), they should rear the former instead of the latter. However, reproduction by workers is rare in queenright colonies and mainly occurs after death of the queen (Choe 1988, Bourke 1988a, 1988b, Bourke and Franks 1995, p. 235). A reason why reproduction by queenright workers could be counter selected is its cost to colony productivity. The more workers reproduce, the smaller is the number of individuals available to work and sustain the colony (Ratnieks 1988). Eventually colony output decreases, inducing a loss of fitness for all colony members (e.g. Martin et al. 2002). To avoid the burden of extra brood due to their own reproduction (if colony productivity is assumed to be fixed), fertile workers should replace the less valuable males eggs laid by the queen by their own. If they cannot distinguish between male and female queen produced brood early enough in development, they should not attempt replacement. The cost of mistakenly destroying sisters or of destroying advanced brood would exceed the gain in direct reproduction (Nonacs 1993, Bourke and Franks 1995, p. 240). Workers are therefore expected to refrain from reproducing or to prevent each other to do so (Cole 1986, Ratnieks 1988, Pamilo 1991, Frank 1995). This is also expected if the queens mate twice or more than twice. In this situation, a workers' average relatedness with a worker-produced male (0.125-0.25) falls below that of a brother (0.25). The queen-worker conflict over male parentage therefore disappears, but is replaced by a conflict among workers. If workers prefer brothers to nephews, it is still more advantageous for them to produce sons. Assuming that dominance hierarchy cannot be established (in large colonies for example), a given worker cannot impose rearing its offspring on all her nestmates and in this "one against many" dilemma, workers should prevent each other from reproducing, i.e. police each other. Efficient worker policing ultimately leads to self-restraint (Ratnieks 1988).

The definition of worker policing given by Ratnieks (1988) was formulated as the mechanisms through which "workers reduc[e] by whatever means or amount the fraction of worker produced males [...] in favour of sons of the queen". It has recently been broadened to include mechanisms settling more general reproductive conflicts among individuals of equivalent reproductive potential (Monnin and Ratnieks 2001). In this study, however, only regulation of male production is studied and the term "worker policing" fits its original definition. Proximate mechanisms of worker policing can take several forms. Among these

are the destruction of worker-laid reproductive eggs and the attack of egg-laying workers (Ratnieks 1988). Evidence for both mechanisms has been given in ants, bees and wasps (Ratnieks and Visscher 1989, Liebig et al. 1999, Gobin et al. 1999, Kikuta and Tsuji 1999, Foster and Ratnieks 2000a, Halling et al. 2001).

Although they posses functional ovaries and are able to lay male eggs *M. gulosa* workers do not reproduce in the queen's presence (cf. chapter III.). Nothing is yet known of the genetic structure of the colonies and mating status of queens in *Myrmecia*. It has however been established that in the closely related species *Nothomyrmecia macrops*, the mating frequency of queens varied from 1 to 3, with an effective number of male mates of 1.37 (Sanetra and Crozier 2001). In *M. gulosa*, as in *N. macrops*, mating frequency might be below the level favouring the spread of a "police allele". Productivity effects alone might nevertheless favour selection of policing behaviours. Workers do not seem to be able to establish structured dominance hierarchies when orphan (cf. chapter III.) and it is therefore unlikely that they can control the number of putative egg-layers. The cost of having many workers reproducing in a colony instead of laying trophic eggs and feeding nestmates and brood is thus likely to be high. Thus, the probability of a worker policing mechanism to occur in this species is high.

For worker policing to take place, workers have to be able to discriminate worker from queen-laid eggs and reproductive from non-reproductive workers. Occurrence of these two forms of worker policing was investigated in *M. gulosa*. The cues triggering policing remain elusive and it has been proposed that CHCs could function as fertility signals on which policing behaviour could be based (Peeters et al. 1999, Liebig et al. 2000, Cuvillier-Hot et al. 2001). The presumption of a correlation of CHCs with ovarian status in both queen and workers following the results of the previous chapter was verified. The role of the CHCs in the recognition of reproductive workers and therefore their involvement in policing mechanisms were tested. The origin of CHCs in oenocytes (Diehl 1975, Lockey 1988, de Renobales et al. 1991) was examined through histological technique.

V.B. Recognition of reproductive status and CHCs

V.B.a. Introduction

Dissections of a large number of workers showed that they never produced reproductive oocytes in presence of the queen (cf. chapter III.). Furthermore, males were never produced in queenright laboratory colonies of *M. gulosa*. Reproduction of workers in queenright conditions is presumably not part of these ants' lifecycle. As reproductive workers never occurred in the colonies, it is not known whether they can be recognised by their nestmates. To answer this question, one must therefore experimentally create the situation where queenright workers are exposed to reproductive sisters. This approach was already used to examine the problem in the honeybee (Visscher and Dukas 1995) and in the ants *Aphaenogaster cockerelli*, *Rhytidoponera confusa*, *Gnamptogenys menadensis* and *Harpegnathos saltator* (Hölldobler and Carlin 1989, Crosland 1990, Gobin et al. 1999, Liebig et al. 1999) and was repeated here with *M. gulosa*. Workers' physiological reactions to orphanage take several days. In order to verify whether workers can recognise nestmates that start to produce reproductive oocytes, they were exposed to individuals isolated from the nest for increasing durations. In parallel, the CHCs of the test individuals were extracted to examine whether changes in CHCs correlate with physiological status and can constitute a basis for the discrimination of reproductive against non-reproductive individuals.

V.B.b. Methods

Transfer of reproductive workers in queenright colonies Groups of 100 to 150 workers from 5 colonies were isolated from their mother nest. After several weeks, reproductive eggs accumulated in the nest, showing that some individuals had started to reproduce. As workers do not bend their gaster forward during oviposition (cf. section III.C.c.), it was not possible to identify egg-layers by direct observations. Some workers present near the egg pile had a protruding sting sheath and stood high on their legs, suggesting they were about to oviposit. They were taken out of the nest and individually isolated overnight in a plastic box containing a moist cotton ball. On the next day, the presence of eggs in the box was checked in order to identify those who laid reproductive eggs. Orphaned trophic egg-layers could be identified by direct observation of oviposition events. Reproductive and trophic egg-layers were marker

with paint and were returned to their nest until the experiment was realised. They were immediately re-accepted by their nestmates.

Orphan trophic and reproductive egg-layers were introduced in queenright colonies (n=7). Because of a low number of reproductive workers available, each worker was tested in up to 3 colonies, at intervals of at least 20 minutes. They were thus reintroduced in their mother colony, as well as in foreign colonies. Workers were always introduced in pairs (1 reproductive and 1 trophic egg-layer), in order to compare the discrimination ability of different colonies between these two types of individuals. Workers in a pair were always nestmates. When a colony was tested several times, successive introductions of the test workers were spaced at 10 minutes intervals. This interval allowed for the colonies to recover from the disturbance created by opening the nest to remove the introduced ants. Introduction of ants could be done without disturbing the nests. A total of 126 transfers were realised.

Numbers of worker performing antennal inspection or biting the introduced trophic or reproductive orphaned egg-layers were counted at one-minute intervals for 10 minutes. The cumulated number of interactions was calculated for each worker in the pair and these "interaction scores" were averaged for each colony. Increasing scores represent increased interest triggered by the transferred worker in nestmates, ranging from antennal inspection to attacks (score>29). The larger the difference between the interaction scores for reproductive and trophic orphan workers, the greater the discriminative ability of the colony was. The number of interactions directed toward the two types of individuals was compared with a Wilcoxon test. Colonies were used as cases and scores for trophic and reproductive egg-layers as variables.

The effect of orphanage and colony membership on the attention triggered by introduced non-reproductive individuals was examined. The interaction scores of queenright workers were compared with those of orphaned workers when both types of individuals were introduced in a foreign colony. Responses of queenright individuals exposed to orphaned nestmates or non-nestmates and interaction with their reproductive status were analysed with an ANOVA. Due to important intercolonial variations in the interaction scores obtained (tested with the Kruskal-Wallis statistic), the data could not be pooled for comparison, the data had to be compared on single colony basis. The low number of reproductive individuals available did not permit performing the analysis with all colonies; only 3 of them could be used.

Cuticular hydrocarbon profiles, gland products and reproduction The SPME technique (cf. section II.C.b.) was used to extract CHCs of several groups of individuals. The purpose was to investigate the correlation of CHC profiles with reproductive status. Functional mated queens (n=12, from 12 colonies) were therefore compared with virgin queens (n=12, from 2 colonies) and orphaned reproductive workers (n=12, from 5 colonies). Non-reproductive workers belonging to orphan and queenright colonies were included in the analysis (n=12 and n=10 from 4 and 2 colonies respectively). This allowed controlling for the effect of orphanage on the CHC profile. Profiles of males (n=11 from 2 colonies) were also examined. Transformed relative proportions of 16 peaks were used as variables in a PCA of which the 5 principal components constituted the basis of a DA.

GC/MS analysis of cuticular extracts (n=7) and whole glands (PPG, n=7; Dufour gland, n=6) of reproductive workers was performed by Richard Beard in Keele, UK (cf. section IV.B.d. for the methodology).

Evolution of CHCs profiles in orphaned workers and of their effect on queenright nestmates' behaviour Six groups of 17-22 workers (from 4 colonies) were isolated from their queen and nestmate workers in small rearing nests. Workers were reintroduced for 6 minutes, once a week, in their mother colony and were then returned to their queenless group. The experiment lasted for 8 weeks. As in the previous experiment, the number of antennating and biting workers was noted every minute of their stay in the mother nest. According to the cumulated number of interactions they triggered over the 6 minutes (their score), the reintroduced workers were classified in 3 groups: those eliciting low interest, mild attention or intense attacks; their scores were comprised between 0 and 5, 16 and 25 and above 29 respectively. In parallel, CHCs of selected workers were extracted by SPME technique at least one hour after the individuals' reintroduction. From 1 to 19 workers from each group were extracted weekly. Ovarian status of the introduced ants was checked at the end of the 7 weeks.

The CHC profiles of the individuals belonging to the 3 groups were compared by multivariate statistical analysis with those of queens (n=9), established reproductive orphaned workers (not from this experiment, n=8) and non-reproductive queenright workers (n=12). Peaks occurring in more than 80% of the individuals and representing more than 1% of the total peak area were chosen. The restandardised and Reyment transformed (cf. section II.C.b.) areas of the 19 peaks selected were used as variable in a principal component analysis (PCA)

and the resulting 5 first principal components constituted the basis of a discriminant analysis (DA).

V.B.c. Results

Transfer of reproductive workers in queenright colonies Orphaned workers did not behave aggressively when introduced in queenright colonies. They typically ran around the nest until they were stopped by the resident workers that inspected or bit them. Orphan reproductive egg-laying workers triggered significantly more antennation and biting than trophic egg-layers from orphaned colonies (Wilcoxon test, n=7, T=0.0, p<0.02, figure V.B.1.).

A subset of these data was used to examine the intercolonial differences in the responses to the same pair of workers. Pairs of colonies (n=3: C/D, E/F and H/K) were tested with the same pairs of workers (1 trophic + 1 reproductive egg-layer). Differences appeared in the attention elicited by these workers in the two colonies they were tested in. In contrast to reproductive egg-layers, trophic egg-layers were treated similarly in each pair of colonies (t-test for paired samples, $n_{trophicC/D}=12$, $t_{trophicC/D}=0.9$, $p_{trophicC/D}=0.39$, $n_{reproductiveC/D}=18$, $t_{reproductiveC/D}=-8.5$, $p_{reproductiveC/D}<0.01$; $n_{trophicE/F}=8$, $t_{trophicE/F}=-1.8$, $p_{trophicE/F}=0.11$, $n_{reproductiveC/D}=7$, $t_{reproductiveC/D}=0.8$, $p_{reproductiveC/D}<0.47$; $n_{trophicH/K}=6$, $t_{trophicH/K}=1.8$, $p_{trophicH/K}=0.14$, $n_{reproductiveH/K}=6$, $t_{reproductiveH/K}=3.7$, $p_{reproductiveH/K}<0.01$, figure V.B.2.).

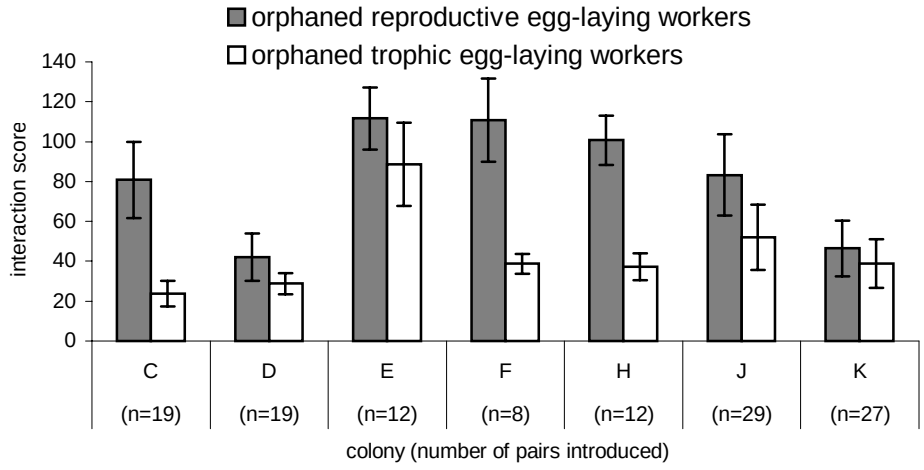


Figure V.B.1.: number of antennating and biting individuals counted at one-minute intervals for ten minutes (interaction score) for orphaned reproductive and trophic egg-layers. The workers were introduced simultaneously either in alien or mother colonies. Error bars represent standard deviation.

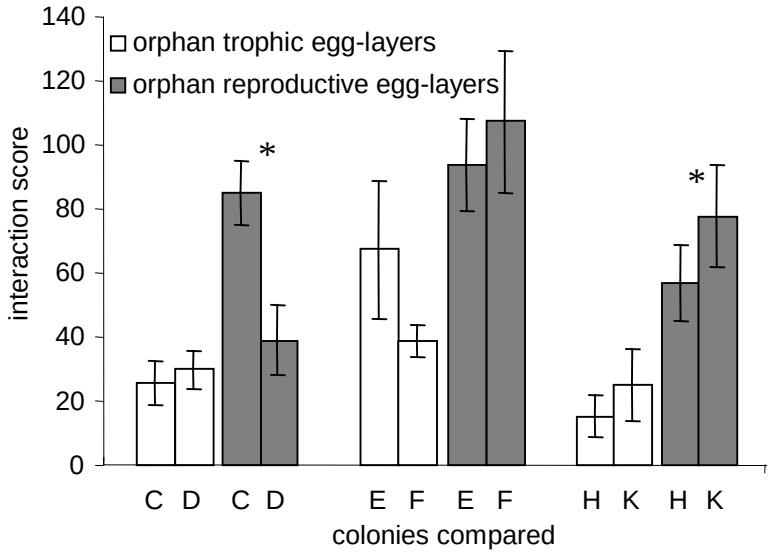


Figure V.B.2.: pairs of workers were introduced in two colonies in order to determine whether colonies vary in their treatment when exposed to the same individuals. Successive introduction of the same pair of test workers (1 trophic + 1 reproductive egg-layer) were realised at intervals of at least 20 minutes. Pairs of colonies tested were C/D, E/F and H/K. Sample size is given in the text. Error bars represent standard deviation. * designates significant differences (t-test for paired samples, $p<0.05$).

Reproductive status and colony membership (nestmate vs. non-nestmate) had a significant effect on interaction scores in all and 2 colonies respectively (ANOVA, reproductive status: $F_H=20.3$, $p_H<0.01$, $F_J=5.0$, $p_J<0.03$, $F_K=12.4$, $p_K<0.01$; colony membership: $F_H=6.5$, $p_H=0.02$, $F_J=0.1$, $p_J=0.85$, $F_K=7.0$, $p_K=0.01$). A post-hoc Tukey HSD test for unequal sample sizes showed important intercolonial variations in the responses (figure V.B.3.). Overall, reproductive workers triggered more attacks than non-reproductive individuals. An exception occurred in colony K, where nestmate orphan trophic egg-layers were attacked more (although not significantly so) than their reproductive counterparts. This response was consistent when these workers were introduced in other colonies.

Non-nestmates (both reproductive and trophic egg-layers) triggered more interest than nestmates orphaned workers. In all three colonies, nestmate orphan trophic egg-layers were attacked more intensely than non-nestmate queenright workers (figure V.B.3.).

Cuticular hydrocarbon profiles, gland products and reproduction The DA (Wilk's Lambda=0.004, F[30, 234], $p<0.01$) shows that individuals share similar CHCs profiles according to their ability to lay reproductive eggs (figure V.B.4.). Although all groups are

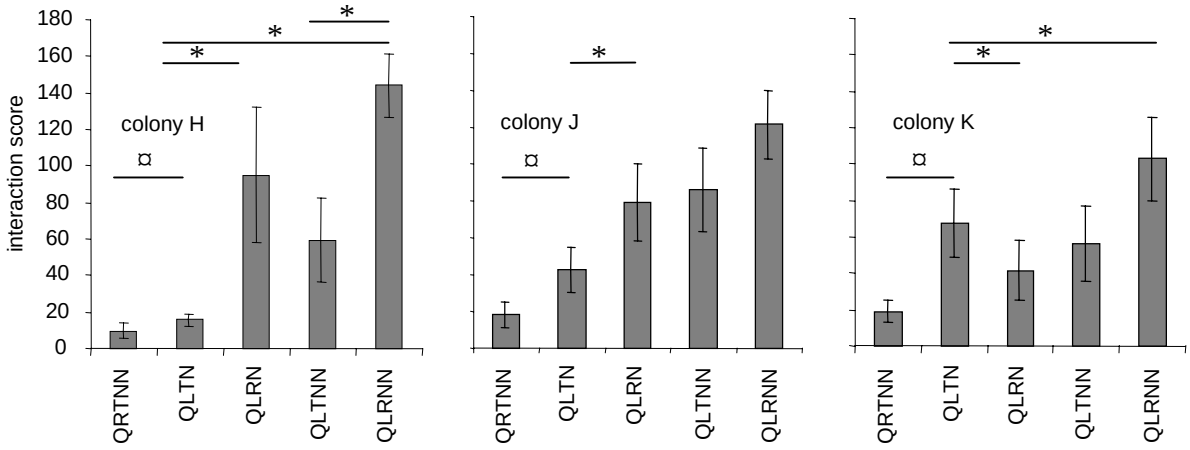


Figure V.B.3.: number of antennation and bites (interaction score) received by non-reproductive and reproductive individuals introduced in their own or in an alien queenright colonies. On the x-axis are the groups of workers introduced in each of the 3 colonies. Abbreviations are as follows. QRTNN: queenright non-nestmates trophic egg-layer; QLTN: queenless nestmate trophic egg-layer; QLRN: queenless nestmate reproductive egg-layer; QLTNN: queenless non-nestmate trophic egg-layer; QLRNN: queenless non-nestmate reproductive egg-layer. ¤ designate significant differences with the Mann-Whitney test ($p<0.05$), * indicate significant differences with the Tukey HSD post hoc test for unequal samples ($p<0.05$).

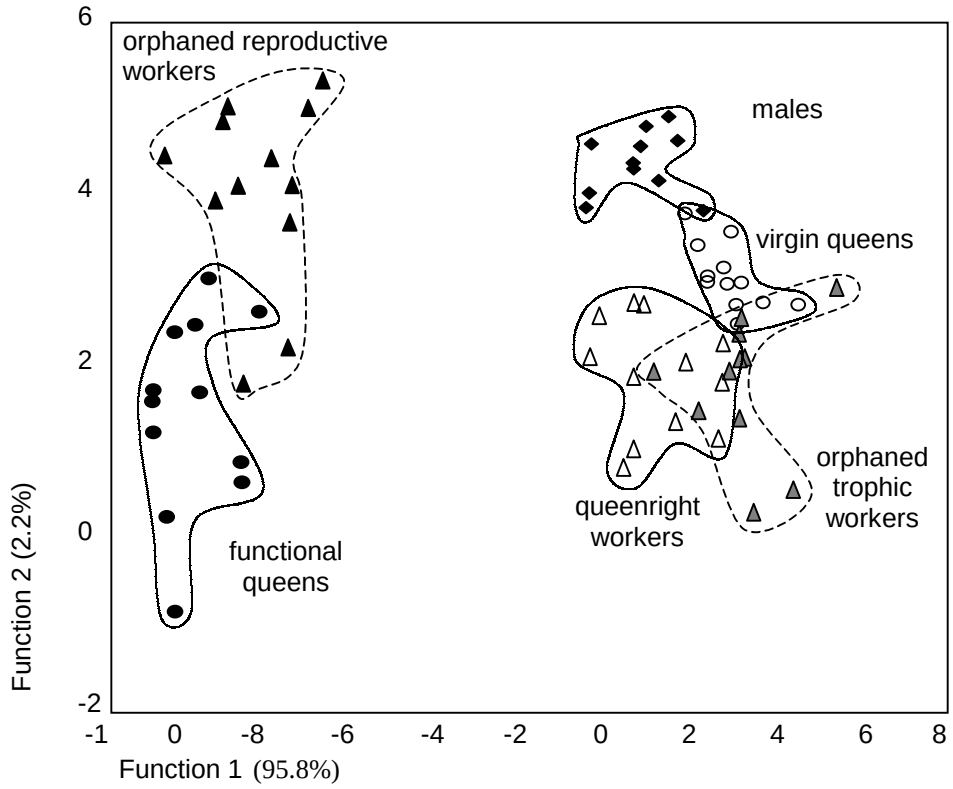


Figure V.B.4.: Discriminant analysis of 6 groups of individuals from 12 colonies. Reproductive and non-reproductive individuals are separated completely on the basis of 16 compound peaks indicated in table IV.B.1. The groups are encircled arbitrarily; those belonging to orphan colonies are encircled by a dotted line, those found in queenright societies by a continuous line.

statistically separated, the males, callow workers, trophic egg-laying workers and virgin queens segregate together, whereas functional queens and reproductive egg-laying workers form a second cluster. These reproductive and non-reproductive poles are separated on the basis of the first discriminant function that explains 95.8% of the total variation. Within these poles, misclassification occurred (table V.B.1.). The model permitted the correct classification of 88.4% of the individuals. The PCA's factor loadings indicated that many compound peaks contributed to the differences in profiles between groups (figure V.B.5.). Comparison of

Table V.B.1.: classification results of the discriminant analysis presented in figure V.B.3.. Reproductive individuals (functional queens and orphaned workers) are totally separated from the non-reproductives.

	percent correctly classified	Q	ORW	VQ	QNRW	ONRW	M	total
functional queen (Q)	83.3	10	2	0	0	0	0	12
orphaned reproductive workers (ORW)	91. 7	1	11	0	0	0	0	12
virgin queens (VQ)	100	0	0	12	0	0	0	12
queenright non-reproductive workers (QNRW)	91.7	0	0	0	11	1	0	12
orphaned non-reproductive workers (ONRW)	70	0	0	2	1	7	0	10
males (M)	90.9	0	0	1	0	0	10	11
total	88.4	11	13	15	12	8	10	

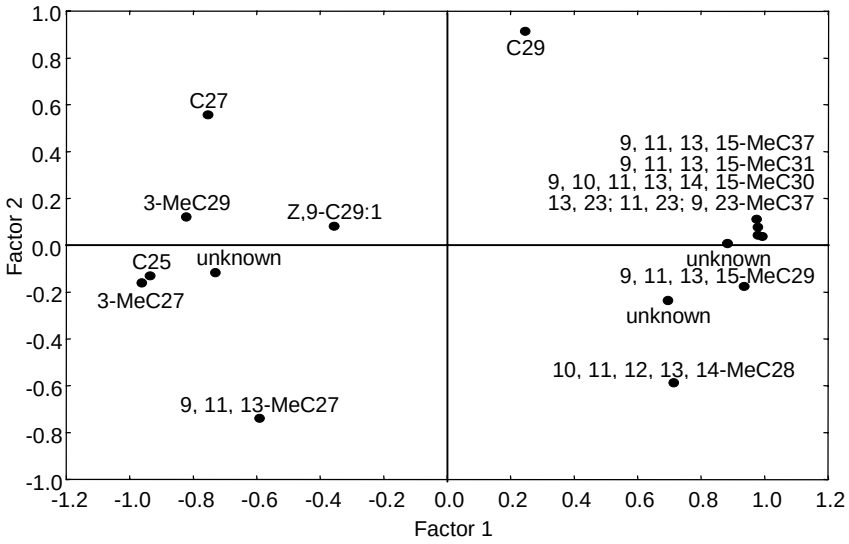


Figure V.B.5.: factor loadings from the PCA performed on the Reyment transformed relative proportions of 16 compounds peaks. Abbreviations for peak identity correspond to those in table IV.B.1.

CHCs profiles showed that most peaks were common to all groups (cf. table IV.B.1.) and that there were few or virtually no qualitative differences in composition of the profiles. The major quantitative differences among the profiles however, were excluded from the DA by the selection rule that was applied to reduce the number of variables used in the analysis. In particular, two peaks present in high proportions were characteristic in reproductive individuals (both for queens and workers), but were absent or found in traces in non-reproductive individuals. Relative areas of these peaks in different groups of individuals are represented in figure V.B.6.

GC/MS analysis of cuticular extracts of reproductive workers (n=7) correspondingly showed minor qualitative differences compared to queens. Reproductive workers possessed the following compounds that were absent in queens: several isomers of methyl- and dimethyltricosane, a linear triterpene, dimethylnonacosane, triacontane, Z-hentriacontene, several isomers of dimethylhentriacontane and of dimethyldotriacontane, methyldotriacontane, methyltritiacontane and several isomers of methylpentatriacontane and methylnonatriacontane. Average relative proportion of these compounds was 0.32%. The other components were shared with either the workers or the queen.

The PPGs (n=7) of reproductive workers similarly contained minor compounds that were not found in queen and in workers: dimethyl- and methylpentacosane, dimethyl- and methylhentriacontane, dimethyl- and methylnonatriacontane, dimethylhentetracontane and dimethyltritetracontane. The average relative area of these compounds was 0.72%.

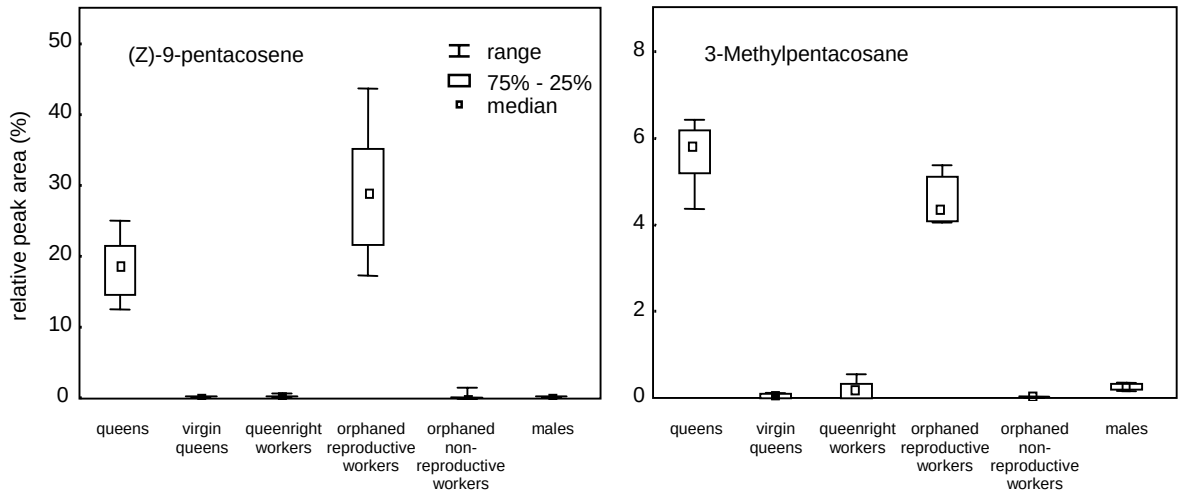


Figure V.B.6.: major quantitative differences in the presence of 9-pentacosene and 3-methylpentacosane between reproductive and non-reproductive individuals. Due to their absence in some individuals, these compounds were excluded by the selection rule of variables to be used in the PCA. Note the difference in scale on the y-axis.

Dufour glands contained tetracosane, a undetermined branched hydrocarbon and 3 terpenes that queens and non-reproductive workers lacked. Their average relative proportions was 0.40%.

Evolution of CHCs profiles in orphaned workers and of their effect on queenright nestmates' behaviour The interaction scores were not strongly correlated with time after orphanage (r=0.37) due to large interindividual variations in the scores (figure V.B.7.). Test workers were classified in 3 groups according to their score. The groups reflected the intensity of interest that individuals elicited in their queenright nestmates (figure V.B.8.).

As in the previous section, the DA (Wilk's Lambda=0.08, F[25, 239]=9.17, p<0.01) showed that control individuals (queen and workers) had distinct CHC profiles according to their reproductive status. When the profiles of the test workers were compared with the latter, it appeared that individuals triggering low or mild attention in nestmates had identical profiles (group A and B respectively, p=0.20), which were closer to non-reproductive queenright workers'. On the other hand, individuals that were attacked (group C) had intermediate to reproductive-like profiles. The group's centroid was closer from the reproductive workers and queens (table V.B.2., figure V.B.9.). Accordingly, some of them possessed reproductive oocytes at the end of the 8 weeks study. In order to show a correlation between CHC profile and attention triggered in nestmates, the interaction scores were regressed against the

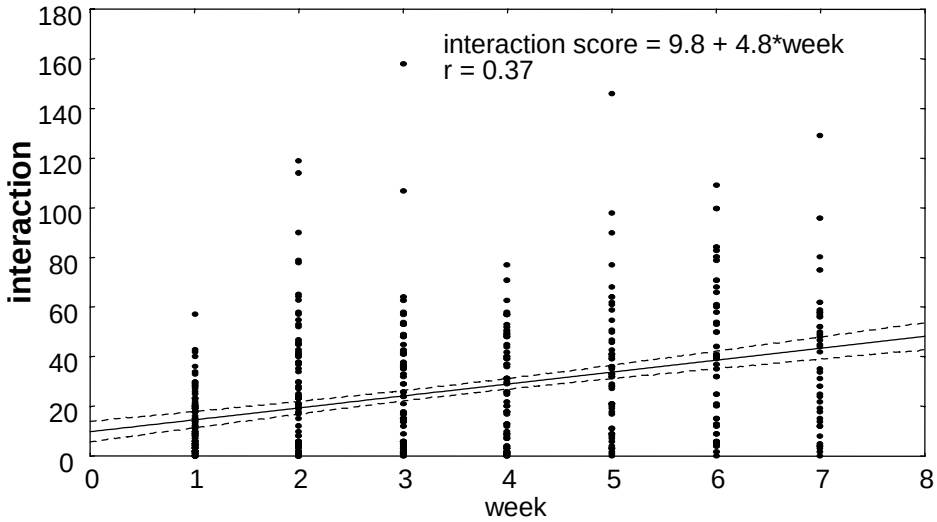


Figure V.B.7.: correlation between the number of antennation and biting (interaction score) received by orphan test workers with weeks since orphanage. Doted lines represent 95% confidence limits.

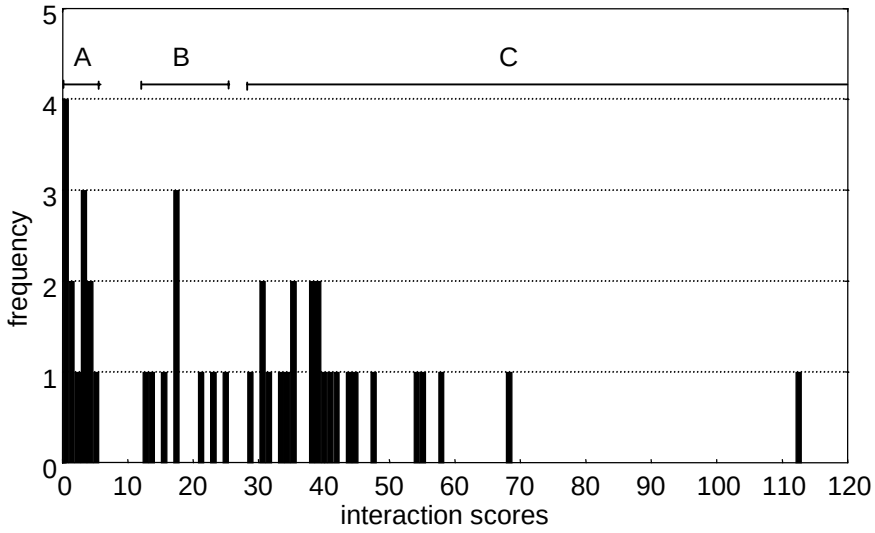


Figure V.B.8.: frequency distribution of interaction scores (IS) of the individuals used in the statistical analysis. Their grouping was determined according to the pattern of observed frequencies: low interest triggered in nestmates 0<IS<5 (group A), mild attention elicited 16<IS<25 (group B), attacks 29<IS<120 (group C).

Table V.B.2.: squared Mahalanobis distances between group centroids of the DA presented in figure V.B.8.

	Q	QRNR	OR	D	E	F
queens (Q)		29.9	1.7	26.5	34.6	20.4
queenright non-reproductive workers	29.9		26.4	5.5	5.7	14.2
orphan reproductive workers (OR)	1.7	26.4		28.3	28.3	8.8
orphan test workers D (D)	26.5	5.5	21.4		1.8	1.8
orphan test workers E (E)	34.6	5.7	28.3	1.8		4.5
orphan test workers F (F)	20.4	8.8	14.2	1.8	4.5	

discriminant value of each individual on the first discriminant function (figure V.B.10.). A weak, negative correlation (r=-0.51) is obtained.

V.B.d. Discussion

In *M. gulosa*, orphan reproductive and trophic workers were discriminated against in all the colonies studied. However, these colonies varied in their ability to discriminate. The intensity of the response toward introduced worker is limited by the number of ants spatially able to gather around a worker to antennate or immobilise it, and should therefore be comparable

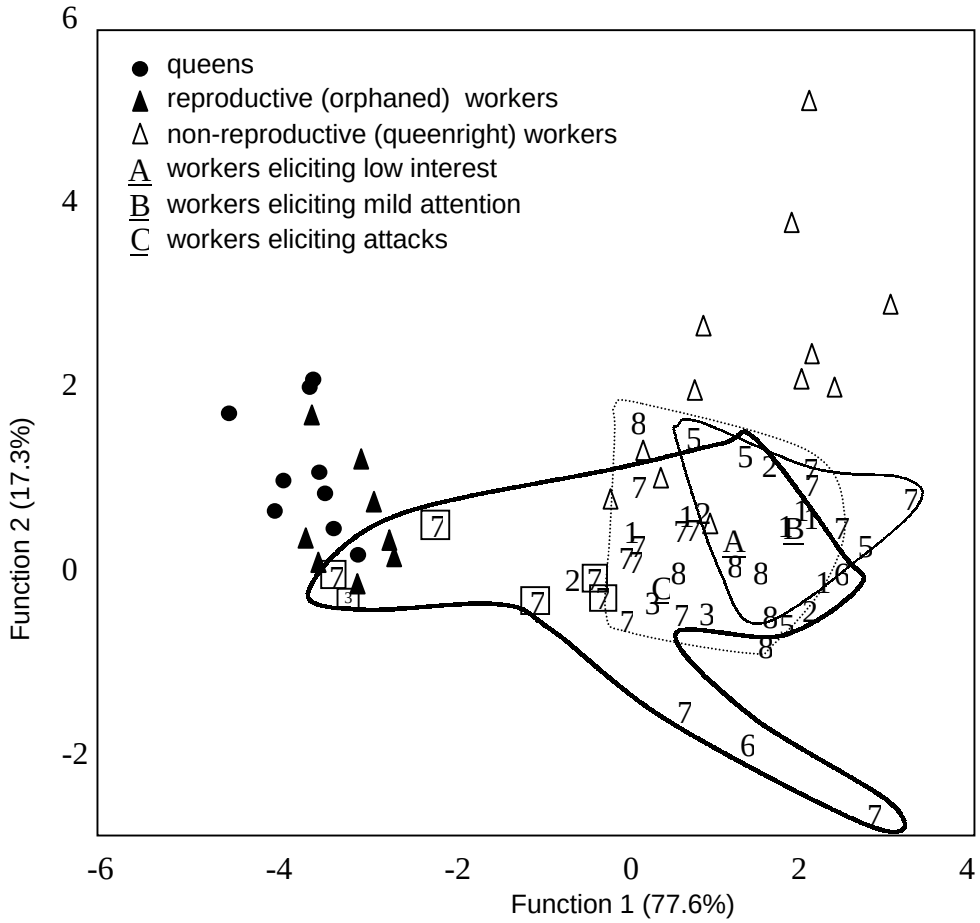


Figure V.B.9.: discriminant analysis of 3 control and 3 test groups. Control groups were queens (n=9), orphaned reproductive individuals (n=8) and queenright non-reproductive workers (n=12). Test groups were constituted of orphaned workers reintroduced after variable durations of orphanage. The numbers represent the weeks (1 to 8) after orphanage, when these workers were extracted. Test individuals were grouped according to the intensity of attention they triggered in queenright nestmates. Group A: low interest (encircled by dotted line), groups B: mild attention (thin line) and group C: attacks (thick line). The groups are encircled arbitrarily. Individuals in a square possessed reproductive oocytes at the end of the study.

among colonies, in which worker density was high enough to allow maximal response. Some colonies showed very good discrimination (colonies C, F and H), whereas others only tended to antennate and bite reproductive individuals more (colony K). Introduced workers did not behave aggressively and some change in their surface chemicals must have triggered the attacks. A possible explanation for intercolonial differences therefore is that workers reacted to differences in cues produced by the orphaned individuals with different thresholds. Workers from the discriminating colonies perceived the difference between reproductive and non-reproductive individuals. These differences may not have been important enough for the workers from the non-discriminating colony K to detect them: reproductive egg-layers were treated as trophic egg-layers.

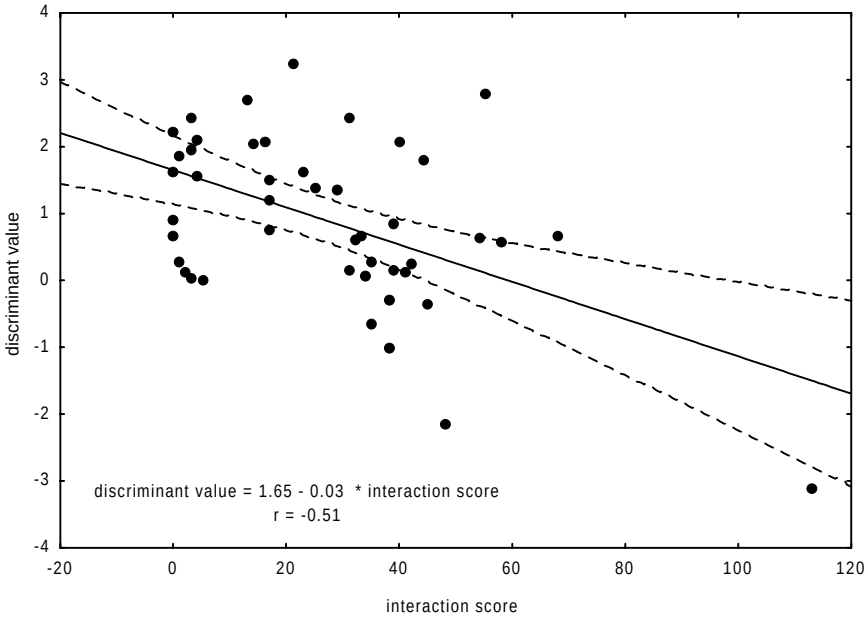


Figure V.B.10.: regression of the discriminant value on the interaction scores of orphaned workers reintroduced in their mother colony. Discriminant values on the first function (explaining 77.6% of the variance) are those obtained from the DA presented in figure V.A.7. Doted curves represent 95% confidence interval.

Overall, in all colonies did workers show more interest in reproductives than in non-reproductives, and this, irrespectively of the colony membership of the introduced ant. Whether the introduced ant was a nestmate or a non-nestmate affected the intensity of the attention elicited for both reproductive and trophic individuals, but not the discrimination. Only in colony K were nestmate orphan reproductive and non-reproductive individuals not discriminated against. The colony however, showed discriminating abilities when exposed to non-nestmate individuals. When the orphan workers from colony K were introduced in foreign colonies, the latter were unable to discriminate between the two kinds of workers, thus proving that the workers lacked the cues enabling discrimination.

In sum, workers of *M. gulosa* are able to recognise reproductive activity in workers, provided that they emit the necessary cue. Based on colony splitting and reunion, such ability to recognise reproductive status of nestmates was already demonstrated in *Vespula atropilosa* (Landolt et al. 1977), in the honeybee (Sakagami 1954, Vander Blom 1991, Visscher and Dukas 1995) and in the ants *Aphaenogaster cockerelli*, *Rhytidoponera confusa*, *Gnamptogenys menadensis* and *Harpegnathos saltator* (Carlin and Hölldobler 1989, Crosland 1990, Gobin et al. 1999, Liebig et al. 1999). The first split and fuse experiments with *Myrmecia* were unwillingly realised by Haskins and Haskins (1950). They kept the queen and

a few workers of *M. tarsata* and *M. nigrocincta* separated from the rest of the colonies for several weeks to months. Upon reunion of the colony parts, workers attacked each other. The authors interpreted these antagonisms in terms of colonial odor, which they assumed diverged in each part after splitting and were not compatible anymore upon reunion. According to Crosland (1990), these fights could be interpreted as the recognition and attack of reproductive workers. The direction of the interactions is however not clear from the scarce description of the attacks in *M. tarsata*, and care should be taken in reinterpreting these observations (cf. Carlin and Hölldobler 1991). In *M. nigrocincta*, the fact that the queen was killed after reunion and that the queenright workers were victims of attacks by their orphaned sisters argue against the idea that the development of workers' ovaries in the queenless fragment induced their attacks.

In *M. gulosa* and in several social Hymenoptera (cf. introduction), CHC profiles of individuals correlate with reproductive status. Reproductive workers and queens (laying male and mostly female eggs respectively), have similar profiles. These are distinct from the profiles of non-reproductive workers, virgin queens or males. All these groups shared most of the compound peaks. The major differences were quantitative, as important variations in the compound peaks' relative proportions occurred (cf. section IV.B.d.ii.). The hypothesis that CHCs could be used as fertility cues or signals that workers could use to recognise the reproductive status in nestmates, is supported. If workers detect changes in CHCs, their behavior should be modulated according to these changes. That orphaned workers at different stages of ovarian development and with changing CHC profiles were treated differently by nestmates was verified.

Although no structured hierarchy seems to limit the number of egg-layers in orphan worker groups (cf. chapter III.), only some of them reproduce. Accordingly, not all the orphan workers suffered intense attacks when reintroduced in their mother nests. Even after 7 to 8 weeks orphanage, some individuals elicited only weak attention, whereas others were already intensely attacked in the third week. Again in this experiment, CHC profiles were correlated with reproductive activity: reproductive eggs were found in the workers with profiles close to those of established reproductives. CHC profiles also correlated with the intensity of interest elicited in nestmates: those of workers belonging to the group that elicited attacks tended to be closer to those of reproductives. Despite important overlap with the other groups, this was confirmed by a correlation between each individual's interaction score with its discriminant value on the first function of the DA. Although the correlation coefficient was relatively low

(-0.51), I suggest that the relationship is meaningful for two reasons. First, the analysis was based on a subjective selection of compounds, of which the ants might use only a fraction, the rest consisting of noise. In the worst case, none of the selected peaks are used, but simply covaried more or less tightly with the relevant cue used by the ants. Second, this first function explained only 77.6% of the variance, whereas the interaction scores reflected 100% of the effect of the cue used by nestmates. A rough estimate of a coefficient accounting for the total variance would be approximately -0.66.

To reintroduce orphaned workers after increasing duration of orphanage provided a way to examine more deeply the relationship between CHCs and recognition of reproductive status. It allowed verifying the constancy of the correlation in the intermediate stages of reproductive development and CHC changes in individuals (see Peeters et al. 1999 for a similar approach). In case the correlation observed was an epiphenomenona, regular reintroduction gave the opportunity to separate the effects of the CHCs and of the cue actually used by workers to detect reproductive status. In other words, if CHC profiles had not yet changed while recognition took place, or alternatively if the profiles had changed before recognition took place, it would have argued against the hypothesis that CHCs play a role in recognition of reproduction in nestmates. Changes in CHCs correlated here with attention triggered by test workers, and therefore support the hypothesis. Two more steps are however necessary to show that CHCs are used as cues involved in regulation of reproduction. Although recognition of reproductive activity in nestmates was demonstrated, it does not show that this recognition is the basis of an inhibition mechanism (Liebig et al. 1999). Furthermore, CHCs have to be extracted, purified and bioassayed to demonstrate that they are perceived and that they mediate information about reproductive status. Whether the recognition of reproductive workers is at the basis of a regulation mechanism.

V.C. Recognition of reproductive status as basis of a regulation mechanism: worker policing

V.C.a. Introduction

As stated by Liebig et al. (1999), recognition of reproductive individuals is the basis of a regulation mechanism only if it leads to suppression of ovarian activity or death. This could not be shown with the two transfer experiments described above, as the test workers were not

left long enough in their mother nests after reintroduction to follow their fate. The transfer method furthermore, possesses another disadvantage that blurred the results: test workers were reared in isolation from their mother colony. The effect of isolation from the stock colony on CHC profiles was thus superimposed on the effect of reproductive activity, so that even non-reproductive workers elicited low to mild attention when reintroduced. A divergence in colonial odour might have been responsible for their ex-nestmates treating them as foreigners. The DA showed that the CHC profiles of these workers indeed changed slightly and became distinct from that of queenright workers. Moreover, such a scenario where workers can become reproductive in isolation and re-enter their nest might be unrealistic and never happen in nature. The behaviour observed following the manipulation might therefore not have been under selection and might represent an artefact. Ideally, to induce reproduction of workers in the presence of the queen is what is needed to show the occurrence of policing behaviours.

The experiment designed by Tsuji et al. (1999) to study the distribution of queen pheromones and already used in section IV.C.b. precisely allows this. As the pheromone regulating worker reproduction is distributed only by direct physical contacts with the queen, those workers prevented from meeting her behaved as orphans and had the opportunity to start reproducing. They were nevertheless continuously in contact with freely moving individuals, which ensured a persistent colony odour and could detect changes in their surface chemistry as they took place. Their reaction could therefore be immediate upon detection (and not dependant on the experimenter) and allows for extraction of CHCs to examine the precise profiles of test individuals. As in this case the test workers were left in the nest, their fate could be monitored. Behaviour of workers exposed to nestmates that start to reproduce was investigated and is described in the present section. The focus is put on worker-worker agonistic interactions and whether they play a role in regulating male production by workers. In other words, whether worker policing occurs in this species was verified.

V.C.b. Methods

Worker reproduction in queenright condition The results presented here were obtained during the experiments described in section IV.C.b. Identity of the individuals engaged in agonistic interactions was noted down during the same observation sessions. Agonistic interactions have already been described in the above-mentioned section. However, a more

precise definition has to be given for a behaviour consisting of several workers biting and pinning an individual down for hours and sometimes days: it was termed “immobilisation”. The dissection of all individuals at the end of the experiment verified their reproductive status.

CHCs and immobilisation In the 4 experiments, CHCs were extracted from 9-20 workers from each of the groups (a, b, c and d). Thanks to the non-destructive SPME extraction method used, it was in some cases possible to carry out observations of ants after they had been extracted. It was moreover possible to measure repeatedly some individuals, at a few days interval. CHC profiles of the different groups were compared by multivariate statistical analysis (cf. sections II.C.b.). Fifteen peaks were selected for a PCA and the 6 principal components were used in a DA.

V.C.c. Results

Worker reproduction in queenright condition Workers in nest parts A (nest part without queen) and C (nest part isolated by a double mesh) were unable to make direct contacts with their queen. Agonistic interactions started in these parts a few days after separation by the double mesh and barrier. Individuals performed antennal boxing and bit each other (cf. section IV.C.b.). Immobilisations (plate 14) appeared after 10.0$\pm$3.2 days (mean$\pm$st.dev., range 7-14) in part A and 11.8$\pm$5.6 days (range 8-20) in part C. A proportion of 11.5 and 9.4% of the workers (a) (having only indirect contacts with the queen) and (c) (having no contacts with the queen, neither direct nor indirect) respectively were the victims of immobilisation. Respectively, 50 (8/16) and 10% (2/20) of these individuals were killed. The behaviour of individuals in nest part B remained unchanged compared to the control. Workers from the isolated group (c) performed in total more agonistic interactions than the other groups (figure V.C.1a.), but among these were fewer immobilisations than in group (a) (figure V.C.1b.). In nest part A, workers of both groups (a) and (d) (worker that have access to both queen and group [a] workers) took part to agonistic interactions. Importantly, workers (d) were aggressive, but were rarely victims of attacks. In part B, workers (b) (workers blocked with the queen) and (d) remained peaceful (figure V.C.1.).

Of particular relevance is the direction of the agonistic interactions, i.e. the identity of individuals initiating the attacks and of their victims. Most of the attacks were directed by

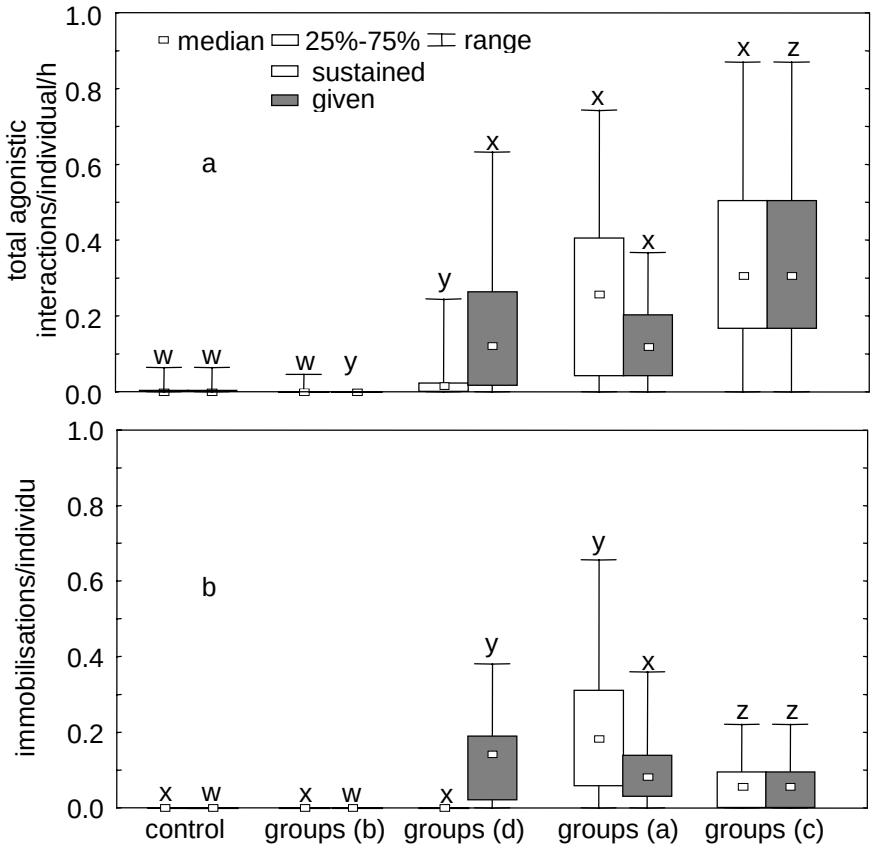


Figure V.C.1.: a) number of agonistic interactions (mean±st. dev.) performed or received by workers in the different groups per hour. Antennal boxing, biting and immobilisation were here considered as different levels of intensity of a single agonistic behaviour. b) Only immobilisations (mean±st.dev.) performed or received by individuals of the different groups per hour were considered. Each worker biting an immobilised nestmate was considered as initiator of an interaction and counted. Numbers of interactions given and received are equal for group (c) as they did not interact with any other group. Data from the 4 replicates were pooled. Different letters indicate significant differences among bars of the same colour ($p<0.01$, Fisher's combined probabilities based on Mann Whitney U-tests).

workers from the group (d) toward workers (a) (51.6%) and by workers (a) toward other workers (a) (38.2%), (figure V.C.2.). In the 4 experiments, the majority of the agonistic interactions performed by workers (d) (74.6±14.8%, range 62.2-94.8%) consisted of immobilisations, which targeted workers (a) exclusively. Although these experiments produced similar patterns of inter-group interactions (figure V.C.2.), colonies differed significantly in the proportion of attacks directed to each group of workers ($Chi^2=55.5$, df=9, $p<0.01$). ON the other hand, the proportion of immobilisation among groups was similar ($Chi^2=4.3$, df=3, $p>0.2$).

The number of workers simultaneously immobilising a nestmate in nest part A varied between 2 and 12 (mean±st.dev.=4.8±1.9). Turnover in the immobilisers maintain some

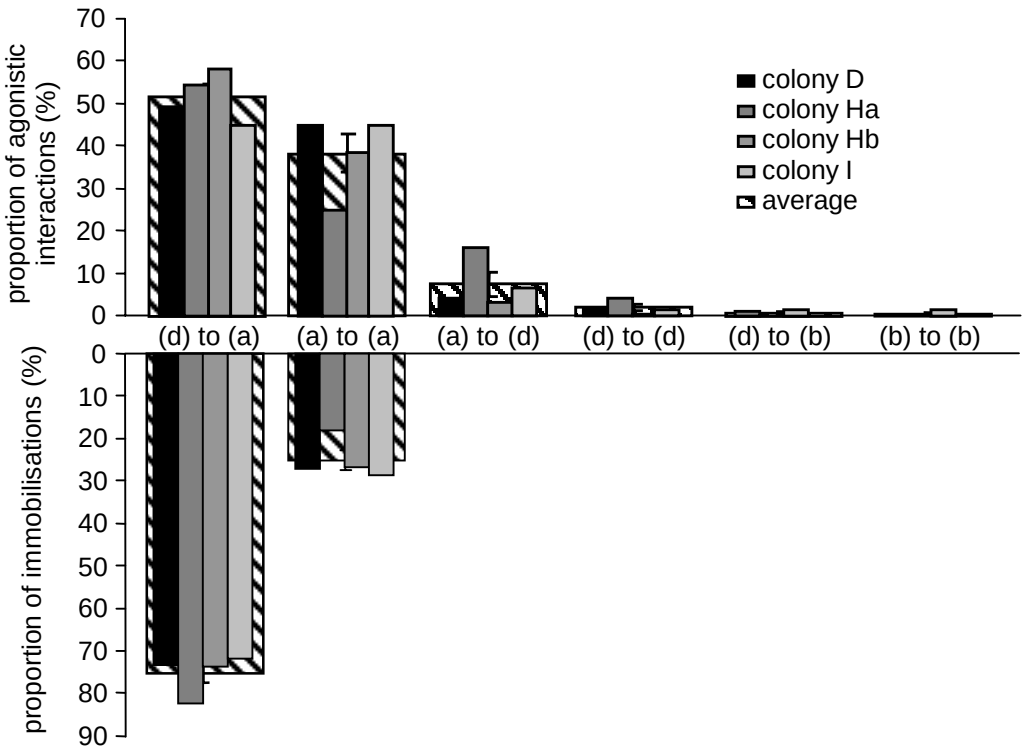


Figure V.C.2.: direction of agonistic interactions (antennal boxing, biting and immobilising) among the worker groups, expressed in proportion of the total number of agonistic acts. Immobilisations alone are represented mirrored. Results are detailed for each colony with the average value across experiments in the background. Error bars represent standard deviation. Workers (d) had the possibility to interact with workers (a) and (b) but workers (a) could not make contacts with workers (b). Workers (c) were isolated and could not interact with the rest of the colony. They are not represented here.

individuals pinned down for several hours or days. There were on average 18.4±11.0 times more individuals in the groups (a) and (d) able to immobilise than individuals actually immobilised at any given moment. In part C, 3.1±1.4 workers on average immobilised a nestmate and there were 14.0±13.4 more workers able to immobilise than workers immobilised. As some workers were killed in both groups or removed for dissection, the actual number of workers immobilised at any given moment was low and ranged from 1 to 2 (figure V.C.3.).

Reproductive worker-laid eggs accumulated in the nests part C from colonies Ha, Hb and I, but not in colony D. The first eggs were laid 18.0±3.6 days (range 15-22) after separation by the double mesh. Whether workers laid reproductive eggs in part A is not known because their eggs could not be distinguished from those laid by the queen. However, dissection showed that some workers (a) had reproductive oocytes in their ovaries in 3 of the replicates. Again, workers in colony D failed to produce reproductive oocytes. In total, 17.0

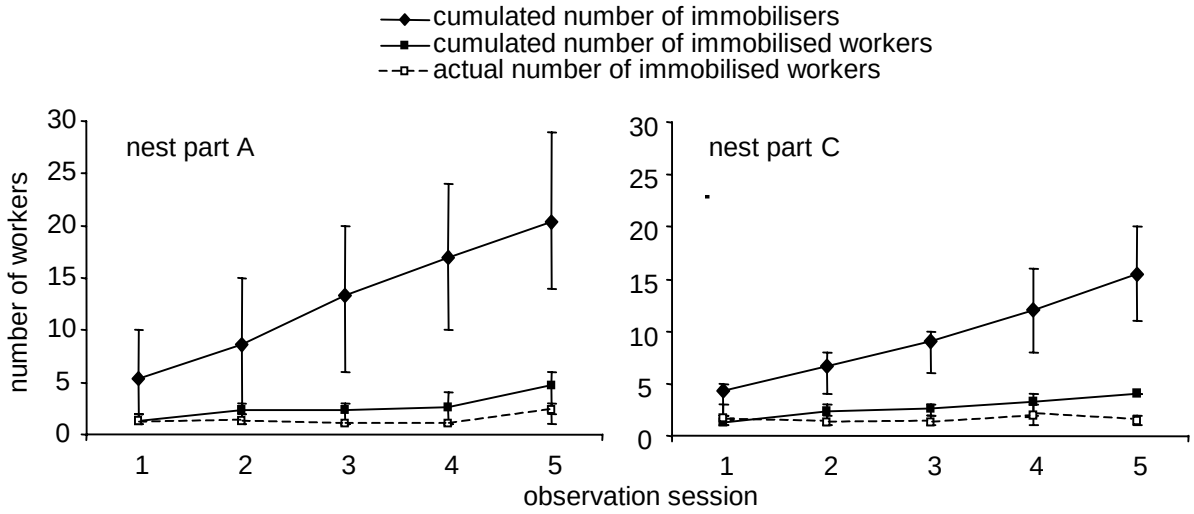


Figure V.C.3.: cumulated number of immobilising and immobilised workers and actual number of immobilised workers in the nest part 1 and 3. Data are shown for the 5 first 1-hour observation sessions. The average for the 4 experiments is given together with range.

and 13.6% of the workers (a) and (c) (27/159 and 29/214 respectively) had reproductive or intermediate oocytes (figure V.C.4.). The proportion of reproductive workers (a) and (c) was different in the 4 replicates (Chi^2=12.4, df=3, $p<0.01$ and Chi^2=17.4, df=3, $p<0.01$).

The workers of (a) dissected soon after the start of their immobilisation (2.2±1.6 days, n=15) or immediately after they were killed by their nestmates had a variable ovarian status. Their ovarioles could be empty, contain either trophic or reproductive oocytes, or intermediate stages between the latter. Most of the workers (a) and (c) which survived immobilisation and which where dissected at the end of the study possessed intermediate or reproductive oocytes (thin and thick hatched areas, figure V.C.4). Some workers possessed reproductive oocytes, but had never been immobilised (66.7 [18/27] and 62.1% [18/29] from the workers [a] and [c] respectively). All the workers from the groups (b) and (d) produced trophic oocytes or had undeveloped ovaries (figure V.C.4.).

CHCs and immobilisation Again in this analysis, non-reproductive and reproductive individuals are clearly separated on the basis of the selected peaks. The control individuals formed the non-reproductive and reproductive poles in the diagram (queenright workers [b] and [d] and the orphaned workers [c] respectively). Profiles from the non-reproductive orphan and impeded workers (c) and (a) were more variable than those of queenright non-reproductive individuals. However, they did not overlap with those of reproductive

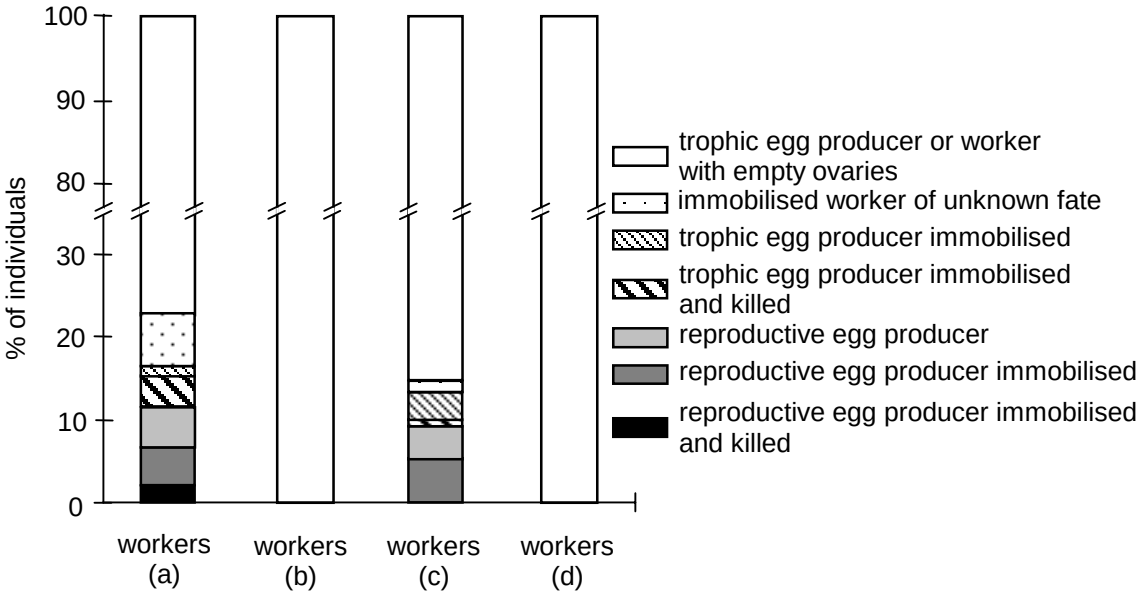


Figure V.C.4.: synthesis of the ovarian development and fate of workers at the end of the experiments. Data of the four experiments have been pooled. Workers (b) and (d) had access to their queen, whereas workers (a) and (c) had no possibility to contact her directly.

individuals. Among the workers prevented from making physical contacts with their queens, some became immobilised by their nestmates. These ants were extracted on the first day they were immobilised and their profile appeared to be intermediate between those of non-reproductive and of reproductive individuals (figure V.C.5.). Immobilised individuals also possessed intermediate proportions of the two peaks (9-pentacosene and 3-methylpentacosane) characteristic of reproductive individuals (data not shown). The DA showed that there was little overlap of their profile with those of non-reproductive individuals, other than with the orphaned workers (c) (table V.C.3.). Some workers were immobilised for several days and could be extracted repeatedly. Most of the individuals whose profile shifted toward the reproductive pole between the two extractions were killed (V, W, X and Y, figure V.C.6.). Only worker Z that reverted to a non-reproductive profile survived (figure V.C.6.).

Four individuals were immobilised in non-manipulated colonies (n=3: C, F and K). Three of them were virgin dealate queens that were forced to stay in the nest and that performed workers tasks. The fourth individual was a worker. They were extracted with the SPME technique and their profile was compared to those of the individuals represented in figure V.C.3., by plotting their position according to the latter DA model (figure V.C.7.). Two virgin queens and possibly the worker, possessed intermediate profile between those of

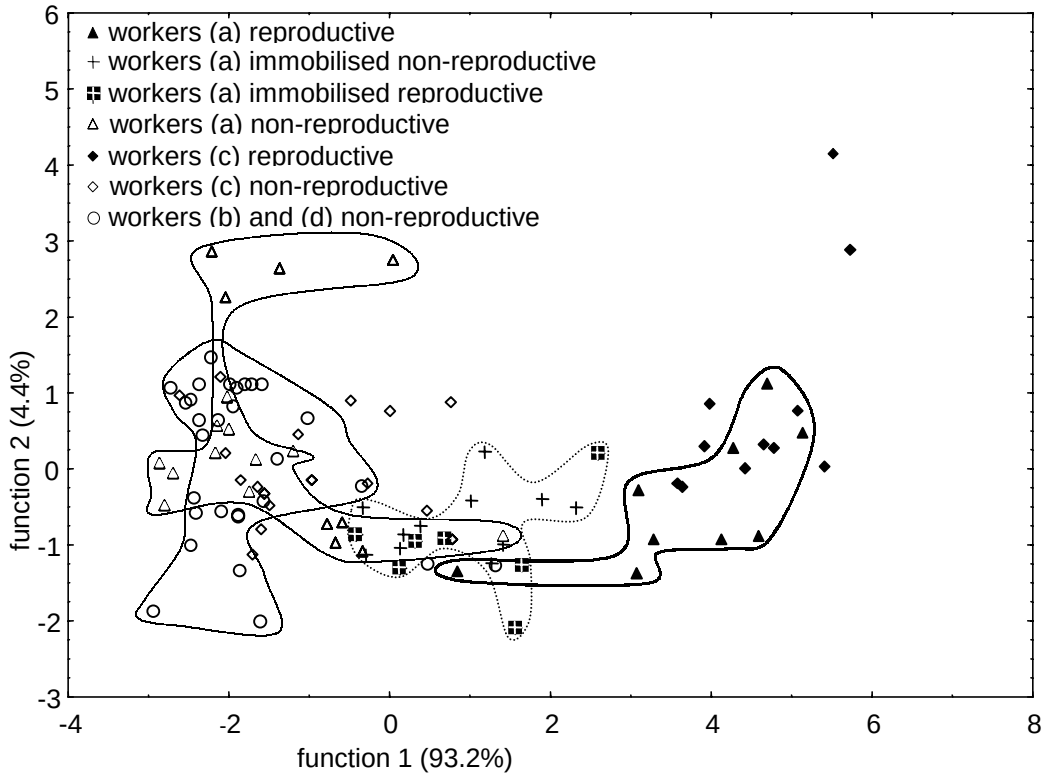


Figure V.C.5.: DA presenting the correlation between CHCs and reproductive status, as well as with immobilisation in the experiment adapted from Tsuji et al. (1999). Workers that had access to the queen (groups b and d) were pooled. Empty symbols represent non-reproductive individuals. Workers that had intermediate or reproductive oocytes at the end of the experiments are represented by plain symbols. Groups were encircled arbitrarily. For clarity, workers from group (c) were not encircled. The dotted line encircles the immobilised workers (a). This group was composed of both reproductive and non-reproductive individuals, whereas the group encircled with the thick line (workers [a] reproductive) was exclusively composed of reproductive workers that had never been immobilised.

Table V.C.3.: classification matrix of the DA presented in figure V.B.5.: groups correspond to reproductive status of workers (a), (b + d), (c) and (d) except for the group AIMM, which is based on a behavioural definition and is composed of all the workers that were immobilised, irrespective of their ovarian development.

	percent correctly classified	CR	AR	BDNR	CNR	ANR	AIMM	total
workers (c) reproductive (CR)	72.7	8	3	0	0	0	0	11
workers (a) reproductive (AR)	55.6	3	5	0	0	0	1	9
workers (b) and (d) non-reproductive (BDNR)	89.7	0	0	26	1	0	2	29
workers (c) non-reproductive (CNR)	15.0	0	0	9	3	4	4	20
workers (a) non-reproductive (ANR)	26.3	0	0	10	3	5	1	19
workers (a) immobilised (AIMM)	83.3	0	1	0	2	0	15	18
total	58.5	11	9	45	9	9	23	

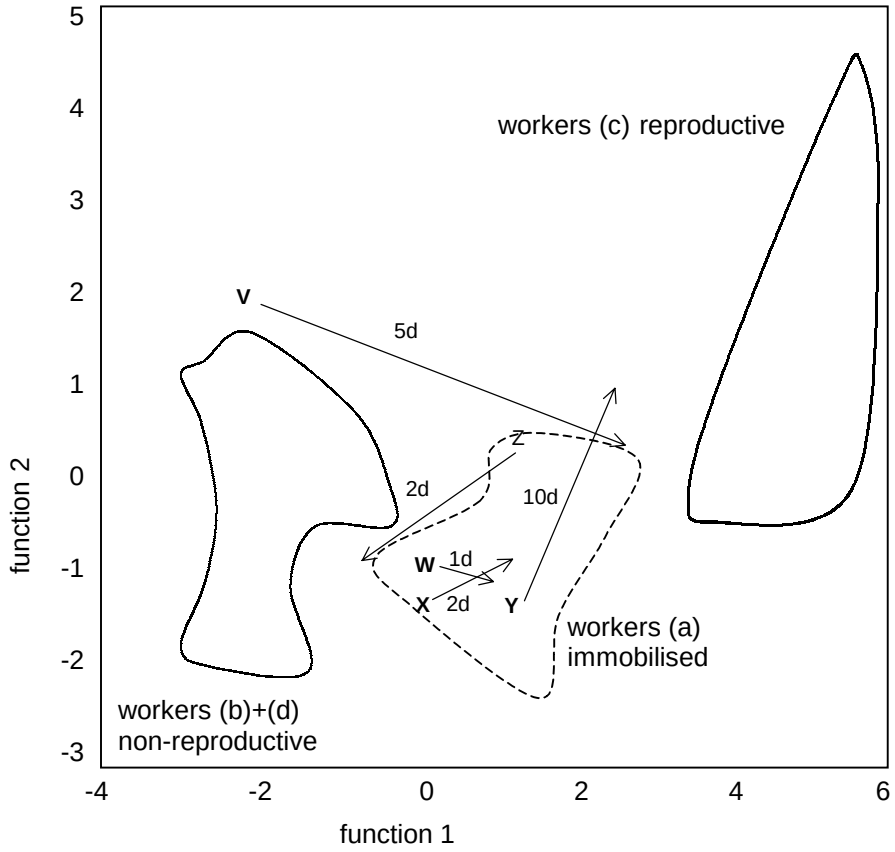


Figure V.C.6.: changes in HC profiles of immobilised individuals. Repeated measures of individuals immobilised for several days were added to the model of the DA presented in figure V.B.5. For clarity, only 3 groups were represented by their encirclements (non-reproductive [b] and [d], immobilised [a] and reproductive [c] workers). The arrows link original (first immobilisation day) and final profiles (death or release day). Number of days between extractions is given on the arrow. Individuals represented with bold letters were finally killed. Individual V was a virgin dealate queen that assumed worker tasks in the nest.

reproductive and non-reproductive individuals. They were therefore similar to those of the workers of which immobilisation was induced experimentally (figure V.C.5.).

V.C.d. Discussion

Workers deprived of direct contact with their queen (group [a] and [c] respectively) behaved as orphaned workers: they became aggressive and some produced reproductive oocytes. The free-moving workers that had access to their queen (groups [d]) also became aggressive. Their attacks, mainly consisting of immobilisations, were exclusively directed toward the workers blocked away from the queen (group [a]). However, workers (d) never produced reproductive

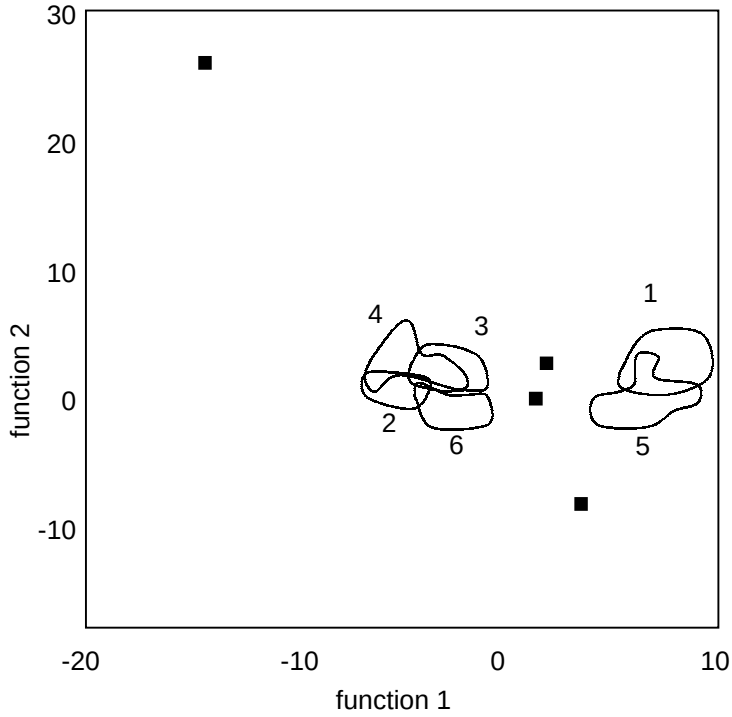


Figure V.C.7.: individuals represented by a ■ were immobilised by their nestmates in non-manipulated colonies (n=3). Their profiles were compared with those of individuals in figure V.B.3. by plotting them according to the latter DA model. The three individuals starting from the top were virgin dealate queens, whereas the fourth, bottommost individual was a worker. Numbers designate the groups of individuals represented by their encirclement: 1=functional queens, 2=virgin queens, 3=queenright workers, 4=orphaned non-reproductive workers, 5=orphaned reproductive workers, 6=males.

oocytes, indicating that their behaviour and physiology were different from those of orphan workers. As all workers shared the same nest, a divergence in colonial odour cannot account for the agonistic interactions. Furthermore, only some of the workers blocked away from the queen were attacked. In the transfer experiment described in the previous section, one or several queenright individuals bit or immobilised reproductive test workers preferentially, indicating that reproductive workers were recognised. Immobilisations observed in the experiment adapted from Tsuji et al. (1999) described here were therefore likely to be due to some workers starting to reproduce and being attacked by their nestmates. However, the link between immobilisation and reproduction in workers was weakened by the fact that some of the immobilised workers lacked reproductive oocytes or oocytes altogether. As the immobilised workers behaved peacefully in each of our experiments, aggressive behaviour was not the cause of their immobilisation. It therefore seems likely that they produced a chemical cue that mediated their recognition at an early stage of the onset of their reproductive activity, in some cases before reproductive oocytes were produced. Supporting

this idea, all the immobilised workers shared intermediate CHC profiles compared to reproductive individuals, independently of their ovarian development status. In contrast, the non-immobilised workers (a) had profiles comparable to non-reproductive workers. This strongly suggests that the immobilised individuals were all shifting to the production of reproductive oocytes. Furthermore, reversion of the profile of an immobilised individual toward that of non-reproductive individuals correlated with its release. Thus CHCs correlate with reproductive status and with behaviour well. This brings strong support to the hypothesis that they mediate the recognition of reproductive status of workers.

Fifty percent of the individuals immobilised in the group (a) were killed as a consequence of the attacks. Among the workers surviving their immobilisation, 25% possessed empty ovarioles or produced trophic eggs at the end of the study. The remaining surviving workers (75%) had reproductive oocytes. Immobilisation in itself therefore did not inhibit reproduction in every worker. However, when immobilisation provoked death of the victim, the phenomenon matched the definition of worker policing given by Ratnieks (1988), as workers "reducing, by whatever means or amount, the fraction of worker-produced males in a colony in favour of sons of the queen". The amount corresponded here to 35% of the workers that started to reproduce in our experimental situation ([8 workers killed + 2 workers surviving immobilisation and producing trophic oocytes]/[8 workers killed + 2 workers surviving immobilisation and producing trophic oocytes + 19 workers which survived immobilisation and had reproductive oocytes]). This proportion may not reflect the natural situation where only rarely would workers try to reproduce. However, the existence of immobilised individuals with intermediate CHC profiles in non-manipulated colonies indicates that the results obtained are no experimental artefact. As discussed by Monnin and Ratnieks (2001), the form of policing observed here matches the definition of punishment: policed workers lose fitness and are prevented from repeating their attempts to reproduce as they are killed by nestmates.

Death of the victims of immobilisation happened less frequently in the control group (c), but was common in larger orphaned groups (n=8). Immobilisation and subsequent deaths persisted in recently orphaned groups kept in the laboratory for some time after the queen's death or removal. This phenomenon was also observed in queenless honeybee colonies and interpreted in terms of worker-worker conflict regarding the timing of oviposition opposed to replacement queen rearing (Miller and Ratnieks 2001). In *M. gulosa*, although orphan workers rear gynes with the diploid brood left by the queen (cf. section III.C.c.), it is not known whether the queen can be replaced. An alternative explanation to the persistence of policing is

that, under queenless circumstances, immobilisation could represent a coarse regulation mechanism of the number of reproductive egg-layers in orphan groups (Gobin et al. 1999, Liebig et al. 1999). Typically, reproductive workers only oviposit and do not take part in other tasks. A too large number of reproductives would therefore decrease work force and affect the number of sexuals produced in the final stage of colony life. Such a mechanism could therefore ensure that, in the absence of a structured hierarchy to regulate the number of reproductives, enough workers contribute to colony maintenance and to food exchanges by producing trophic eggs.

The occurrence of workers with reproductive oocytes and/or intermediate CHC profiles that had never been immobilised, suggests that some individuals escaped policing in queenright colonies. This was clearly not due to a numerical lack of free-moving workers able to immobilise their ”outlaw” nestmates; there were always enough individuals capable of performing immobilisation. Furthermore, workers reacted at any time of the day to the introduction of reproductive individuals (by antennation or biting) and therefore showed a continuous response; their “motivation” to detect reproductive workers seemed constant. No explanation for this phenomenon could be found based on the behaviour of these particular individuals. It could be due to an undetected experimental artefact or to the ability of some individuals to hide their reproductive status by not producing the chemical cue correlated with reproductive status. However, this is unlikely because cuticular hydrocarbons always changed when workers became reproductive, thus showing that the physiological mechanisms linked to reproduction were invariable and presumably unavoidable. Alternatively, these individuals could become accepted by their nestmates even though they produce the cue. A hypothesis is that they produced a cue close enough to that of the queen to become accepted as egg-layers. As workers cannot mate, this implies that the production of this cue is not linked to mating. It seems indeed that cues used to regulate reproduction in *Dinoponera quadriceps* (Monnin et al. 1998), *Harpegnathos saltator* (Liebig et al. 1999, 2000), *Gnamptogenys menadensis* (Gobin et al. 1999) and in *Diacamma* “nilgiri” as well as *D. ceylonense* (pers. obs., Cuvillier-Hot et al. 2001) are only associated with production of reproductive eggs and levels of fertility, but not with insemination status. In *M. gulosa*, reproductive workers acquired queen-like CHC profiles although they were unmated. Several examples support the idea that workers can become queen- or gamergate-like. In *H. saltator*, workers that are allowed to develop their ovaries to the same level as gamergates in isolation are treated like gamergates and are not policed when exposed to nestmates. In contrast, isolated workers that only have partly developed ovaries are policed (Liebig 1999). In the honeybee, workers cannot mate but

some orphan individuals called false queens produce queen-like signals that induce them to be treated like queens by their nestmates. They produce queen-like mandibular pheromones that elicit retinue formation and they prevent ovarian development in nestmates, as queens do (Velthuis et al. 1965, Velthuis 1970, Crewe and Velthuis 1980, Moritz et al. 2000). If such individuals occasionally develop in queenright conditions, they could become accepted and cover their eggs with queen-like substances that protect them against destruction by nestmates (Ratnieks 1995, Katzav-Gozansky et al. 2001). They could thus be the producers of the weak percentage of worker-laid eggs that escape policing (Page and Erickson 1988, Visscher 1989, 1996, Ratnieks 1993). Similarly in wasps, workers were reported to attack reproductive workers (Landolt et al. 1977) and to destroy most, but not all of the worker-laid eggs (Foster et al. 2001). How egg-laying workers or worker-laid eggs become accepted by nestmates in presence of a queen will find an explanation only once the cues regulating policing are identified. Despite productivity and relatedness effects, worker reproduction in queenright colonies seems to be evolutionary stable provided that its frequency remains extremely low (Ratnieks 1993).

The examples of policing of egg-layers in ants show that it the proximate mechanism consists in several workers joining in to immobilise a nestmate (Monnin and Peeters 1999, Gobin et al. 1999, Liebig et al. 1999, this study). In both *Harpegnathos saltator* and *Gnamptogenys menadensis*, when colonies were split up and later reunified, the reproductive workers obtained in the side without gamergates were policed. Immobilisation sometimes provoked death of their victims, but efficiently inhibited the reproduction of the survivors (Gobin et al. 1999, Liebig et al. 1999), in contrast to what could be observed in *M. gulosa*. In *Diacamma* sp. from Japan, colonies were split up to allow the orphaned workers to develop their ovaries. The eggs they laid when the two colony parts were reunified were destroyed (Kikuta et al. 1999), and the workers themselves were immobilised (Tsuji pers. comm.). In the original experiment that was adapted here to *M. gulosa*, *Diacamma* workers that were prevented from establishing physical contacts with their gamergates became aggressive. However, in contrast with the split and fuse experiment described above for the same species, they were not attacked by workers that had contact with the gamergate (Tsuji et al. 1999). The explanation may lie in the fact that *Diacamma* workers usually establish a dominance hierarchy even in the presence of a gamergate. Indeed, a few dominant individuals are able to lay male destined eggs, but these are destroyed by their nestmates (Nakata and Tsuji 1996). When orphan workers that developed their ovaries in isolation are reintroduced in their

mother colony (and therefore did not have the opportunity to establish themselves as dominant egg-layers), they are attacked. Therefore, when establishment of a dominance hierarchy is part of the life history of colonies, workers can impose themselves as new egg-layers. Their reproduction is regulated by destruction of their eggs, allowing efficient control together with rapid gamergate replacement when necessary. In *M. gulosa*, despite the occurrence of agonistic interaction among orphaned workers or workers deprived of contact with their queen, no structured dominance hierarchy is established. Egg laying is not monopolised by a single individual and several workers reproduce (cf. chapter III.). Consistent with this idea, some workers laying reproductive eggs were attacked in both the experiments, when they had the opportunity to become accepted as reproductive egg-layers in their nest and when they did not. As already mentioned, some reproductive workers nevertheless became accepted by their nestmates. These were less aggressive than the average in their group in all but one experiment, indicating that dominance was not involved. Whether these workers reproduced in presence of the queen, i.e. whether they successfully deposited male eggs, is not known. Neither oviposition nor destruction of eggs could be observed. The occurrence of policing on worker-laid eggs had therefore to be tested in a separate experiment.

V.D. Discrimination between queen- and worker-laid eggs

V.D.a. Introduction

Policing on eggs occurs in the honeybee and in wasps (Ratnieks and Visscher 1989, Ratnieks 1993, Halling et al 2001, Foster et al. 2001). Policing on eggs also occurs in the ant *Dinoponera quadriceps* (Monnin and Peeters 1997, 1999) and in *Diacamma sp.* from Japan (Nakata and Tsuji 1996, Tsuji et al. 1998, Kikuta and Tsuji 1999). Mostly the singly mated gamergates destroy reproductive eggs of subordinates, as it is not in their interest to have their sons replaced by grand sons. In contrast, workers should tolerate other workers reproducing as it is in their interest to rear nephews instead of brothers (Monnin and Ratnieks 2001).

The previous section demonstrated the occurrence of policing on the egg-layers in *M. gulosa*. Taking the form of a punishment, policing on egg-layers should be efficient and lead to self-restraint (Ratnieks 1988). Indeed, worker reproduction was never witnessed in queenright colonies (cf. chapter III.). Some individuals experimentally induced to reproduce

in queenright conditions nevertheless escaped policing and had the opportunity to lay male eggs. Whether workers have a second possibility to prevent reproduction of nestmates by destroying their eggs was investigated. Egg eating as a policing mechanism can evolve if workers can discriminate between queen and worker eggs. Whether workers indeed discriminate the two types of eggs was tested. The surface HCs of eggs could represent a basis for discrimination and whether queen and worker eggs HC profiles differ was examined.

V.D.b. Methods

Egg transfer Batches of 4 to 10 eggs were taken from orphaned groups originating in 6 colonies and composed of 20 large workers. They were sucked up with a mouth aspirator regularly cleaned with hexane, and gently dropped in the receiver nests. They were not directly manipulated with forceps in order to avoid injuring them. The first eggs laid by groups of newly orphaned workers were transferred (n=94), as well as eggs laid later on, after larvae already appeared (n=163). As a control, queen eggs were taken and reintroduced into their original colony (n=113 eggs) or in an alien colony (n=73 eggs). Seven colonies were used as donor or recipient. Eggs were always introduced at the same distance from the brood pile (10cm) in queenright colonies. After introduction, handling of the eggs by queenright workers was observed. An egg was considered as accepted if it was not destroyed in the 10 minutes following introduction. Eggs were generally either destroyed or deposited in the pile within 10 minutes. When in the pile, it could not be distinguished from those already present and its fate could not be followed. Usually, no eggs from the pile were destroyed between their deposition and the end of the observation periods. The time at which the eggs were deposited in the pile after introduction was monitored (for 15 worker-laid as well as 15 queen-laid eggs) and compared with a Mann-Whitney test. The age of the eggs transferred was not known. In order to check workers' behaviour facing freshly worker-laid eggs, we removed all eggs present and collected the new eggs (n=16) appearing each hour in 2 orphaned colonies. These eggs were then introduced in their 2 respective mother colonies.

Egg surface HCs SPME technique was used in order to extract exclusively surface chemicals. Eggs were collected from the nests and extracted in a standard manner. Eggs were maintained on a glass cover slide with smooth forceps cleaned with pentane. The SPME fibre was rubbed for 30 seconds on each egg and desorbed in the injector port of the GC as

described in previous sections. Batches of 4 eggs were used to collect enough material. Queen as well as worker eggs of different ages were extracted. All eggs from a nest were removed to obtain newly laid eggs of known maximal age. Statistical analysis was performed as described previously. Fourteen compound peaks were selected for the PCA and the 5 principal components were used as variables for the DA.

V.D.c. Results

Egg transfer Most of the queen-laid eggs reintroduced in their own colony (96%) or introduced in a foreign colony (92%) were accepted. In contrast, more than half the eggs from the first batch produced by newly orphaned workers were destroyed, irrespective of the identity of the receiving colony (mother or foreign colony) (figure V.D.1). Noteworthy, the frequency of hatching of the pool these eggs were collected from was low, indicating a poor viability. When male larvae had already appeared in the orphaned groups, viability of the eggs collected was assumed. When these eggs (n=163) were introduced, most were accepted, again irrespective of the identity of the receiving colony (figure V.D.1.). Viable eggs of less than

Figure V.D.1.: transfer of eggs in *M. gulosa*. Worker-laid reproductive eggs were reintroduced in their mother colonies (i.e. the queenright colony from which the workers originated) or introduced in queenright foreign colonies. Two kinds of eggs were transferred: eggs with low viability, from the 1st batch produced by newly orphaned workers, and viable male eggs. Whether they were destroyed or deposited on the egg pile together with the resident queen's eggs was monitored. As a control queen eggs were collected and reintroduced in the nest or transferred in foreign queenright colony. Sample size is given between parentheses. Small percentages of eggs destroyed prevented statistical comparison.

one hour of age (n=15) were equally well accepted. No colony differed in its level of acceptance. Although viable worker-laid eggs were accepted, workers behaved differently when they inspected them compared to queen-laid eggs. Before being deposited on the pile, the former were repeatedly picked up and dropped at different places of the brood chamber. Workers inspected them for longer before carrying them to the egg pile. Irrespective of their origin (alien or kin), queen eggs were deposited in the pile after 107 seconds (range 10-240), whereas worker eggs were deposited after 335 seconds (range 50-1000, Mann-Whitney test, $U=49.5$, $p<0.01$).

Egg surface HCs The DA (Wilk's Lambda=0.08, F[25, 127], $p<0.01$) showed that the HC surface profiles of each group is distinct from the others, with the exception of the worker-laid eggs less than 24 hours old and less than a week old (Tukey HSD post hoc test for unequal sample, $p=0.19$). Both the queen and the workers' eggs change profile with time. The viable worker eggs (which were all accepted when introduced in queenright colonies) possess the profiles closest to the queen eggs. Profiles of the first batch of eggs laid by orphan workers (of which more than 50% were destroyed by queenright workers) were more variable (figure V.D.2.). GC/MS analysis of the surface HCs was not conducted, but GC results showed that eggs and cuticle shared some but not all compounds.

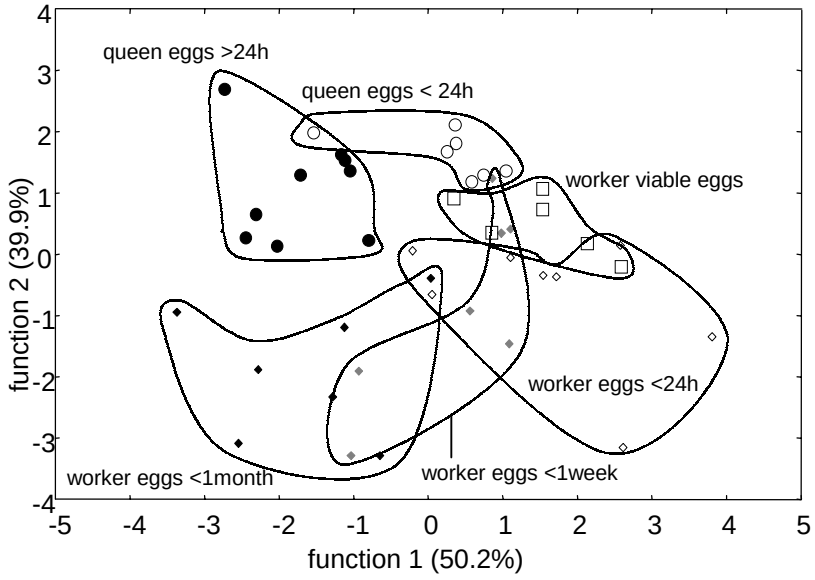


Figure V.D.2.: surface HC profiles of queen and worker reproductive eggs of different ages. Worker eggs of less than 24 hours, 2 week and 1 month belonged to the first batch of eggs laid by recently orphaned individuals. More than half of them are destroyed when introduced in a queenright colony. Worker viable eggs were produced later, as male larvae had already hatched in the nests. Their age was unknown.

V.D.d. Discussion

Workers rarely destroyed queen eggs when they were collected and reintroduced in their nest. Eggs laid by a foreign queen were equally well accepted. Most of the viable eggs laid by orphan workers (97%) were also accepted in all colonies they were introduced in, irrespective of their origin (nestmate vs. non-nestmate). However, more than half the first reproductive eggs that workers produced after orphanage were destroyed upon introduction in queenright colonies. Their destruction by small workers was common in large orphan groups, but the small groups used here as producers of male eggs were composed of large workers that did not destroy them. As only a proportion of these eggs hatched, it is likely that the first eggs worker produce after they became orphan are not viable. They presumably correspond to the oocytes described as being of an intermediate stage between trophic and reproductive oocytes (cf. section IV.C.b.). As they are no true reproductive eggs, their destruction can therefore not be considered to represent policing.

In *Diacamma sp*. from Japan, workers similarly accepted worker-laid eggs when they were transferred in colonies with gamergate (Nakata 1998). Worker policing on eggs was nevertheless shown to occur in this species (Nakata and Tsuji 1996). Indeed, workers or gamergates exclusively destroyed newly laid eggs held in virgin mothers' mandibles, but never the fresh eggs deposited in the pile (Nakata and Tsuji 1996). This suggests that only fresh worker eggs can be recognised as worker-laid because they have a characteristic odour that quickly disappears or becomes scrambled (Monnin and Ratnieks 2001), and/or because they are held by a non gamergate mothers. In *M. gulosa* workers neither adopt the typical egg-laying posture nor hold their reproductive eggs in their mandibles. Fresh eggs simply drop on the floor when ejected from the gaster. This could prevent nestmate from identifying the origin of the egg. Worker reproductive egg laying was never observed in a queenright colony, neither in non-manipulated nor in experimental colonies (cf. section V.C.). Policing on eggs can therefore not be excluded. However, when eggs less than one hour old were transferred in queenright colonies, they were accepted, indicating that even fresh eggs are not destroyed.

Despite apparent lack of policing on eggs, worker-laid viable eggs introduced in queenright colonies were not treated as queen-laid eggs. The former were deposited in the egg pile after a longer handling time compared to queen eggs, suggesting both types of eggs can be discriminated against. The surface HC profiles of worker-laid viable eggs showed that they are distinct, but close to those of queen eggs. Eggs of poor viability (the first produced after workers shift from trophic to reproductive egg-laying) had more different profiles and an

easier discrimination could explain their destruction. However, the ants could detect other criteria such as size or absence of a hard chorion. The longer handling time suggests that workers "hesitated" to accept or reject viable worker-laid eggs. If workers cannot perceive a clear difference in surface chemistry, they should choose to accept the eggs in order to avoid costly mistakes of destroying queen-laid eggs (Nonacs and Carlin 1990, Nonacs 1993, Ratnieks 1993).

Queens of *M. gulosa* neither destroyed worker-laid reproductive eggs nor behaved aggressively toward reproductive workers. Queen policing is therefore absent in the species.

V.E. Source of the CHCs and their role in mediating the recognition of reproductive workers

V.E.a. Oenocytes as source of HCs

Introduction Until recently, the origin of queen pheromones was only sought in structured glands or tissues (e.g. Vander Meer et al. 1980). Their extraction allowed collecting their products in large quantities for bioassaying. The possibility that CHCs could serve as pheromone and therefore that oenocytes constitute its source has only been explicitly envisaged lately (Liebig et al. 2000, but see Coglitore and Cammaerts 1981, Cariou-Etienne et al. 1992 for a similar hypothesis).

Oenocytes are glandular cells spread in the body cavity (Diehl 1975, Wigglesworth 1970, Ismail and Zachari 1984, Lockey 1988, Gu et al. 1995, Colla-Ruvolo and Cruz-Landim 1993, Jensen and Børgesen 2000) and do not constitute a structured gland. Their ability to synthesise HCs was demonstrated in several insects (reviewed in Lockey 1988) and it is likely that they fulfil the same function in ants. Their distribution inside the body of the insects studied varied considerably (Gu et al. 1995, Jensen and Børgesen 2000). Although it must still be influenced by self-grooming, the repartition of the pheromone in the case of a production by diffuse glandular cells could rather be linked to the distribution of the cells inside the body or to internal transport mechanisms (Ismail and Zachary 1984, Schal et al. 1994, Gu et al. 1995). A histological study was conducted in *M. gulosa*, to determine the repartition of oenocytes in the body of workers, males and queens. Extractions of isolated body parts of workers were realised to establish the repartition of the CHCs at their surface. Whether the quantity of CHCs extracted from their thorax and gasters corresponds to the density of

oenocytes inside them was examined. Assuming that oenocytes indeed produce HCs in *M. gulosa*, this helps determine the extent of the latter's translocation from the production site to the cuticle.

Methods Individuals were trisected (head, thorax and gaster were separated), fixed in Bouin and stored in 75% ethanol. Thoraces and gasters were longitudinally cut into two pieces and embedded in soft Araldite (A: 108g, B: 89g, C: 5g, D: 9g). Sections of 15µm thickness were realised using a sliding microtome. Sections were attached to egg albumin-coated slides and stained on a hot plate with Azur II and Methylene Blue 1%.

Oenocytes are clearly recognisable cells of regular shape spread among the fat body cells. The size of 16 oenocytes from the gaster and thorax was measured in a functional queen, a founding queen, a virgin queen, a male, an orphan reproductive and a young and an old queenright non-reproductive worker. Their number was counted under a microscope, every three sections, to avoid counting repeatedly the same cells. Oenocyte number was assessed in the thorax and precisely counted in the gaster of the above-mentioned individuals. Although dissections showed the presence of oenocytes in the head, they were neither counted nor measured. Whether oenocyte density in the gaster is proportional to its size was determined by assessing the volume of the gaster in 2 additional queens and 6 additional workers. The shape of the gaster was approximated with that of a prolate spheroid, and its volume (V) calculated with the corresponding formula: $V=(4/3)Pia^2c$. "c" represents the polar radius, which is greater than the equatorial radius "a". These radii correspond to gaster length and width of the IVth segment respectively. These measures were averaged for the queens and for the workers.

Six queenright workers were trisected and the legs cut off. The sections as well as the mouth were sealed with a melted mixture of colophonium and bee wax in order to avoid contamination from internal HCs. This mixture was preferred to standard glue because of a higher precision in application. The CHCs of head, thorax, legs and gasters were separately extracted in 1ml hexane. The quantity of HC extracted from each part was calculated after removing the compound peaks corresponding to the mixture used to seal the wounds. As compounds of the wax and the ants overlapped, extractions of head, thorax and gaster were repeated with SPME to examine the qualitative differences in their respective HC composition. Peaks representing more than 1% of the total peak area and present in more than 80% of the individuals as well as in the 3 body parts were selected as variables for a principal

component analysis. The 3 principal components constituted the basis of a discriminant analysis (cf. section II.C.b. and IV.B.d.).

To compare the oenocyte density in the gaster with the quantity of HC extracted from it, gaster surface (S) was calculated using the formula for the prolate spheroid $S=2Pia^2+2Pi(ac/c)(\sin e)^{-1}$, with $e=\sqrt{(1-(a^2/c^2))}$. Workers and queens measured are the same as for the estimation of gaster volume.

Results The oenocytes found in thoraces and gasters had comparable sizes in all individuals. However, they are present in much lower numbers in thoraces than in gasters (table V.E.1.). Functional queens have a 2.2 fold ($74.2/33.2mm^3$) larger volume than large workers. This ratio fits that of oenocyte number almost perfectly (2.3, 16642/7406). However, the reproductive worker had an intermediate number of oenocytes compared to non-reproductive worker and the queen. Thus, average quantity of HC extracted from gasters (from 3 queens, 6 non-reproductive queenright workers [cf. section IV.B.d.ii.] and 6 orphaned reproductive workers originating in 3, 6 and 3 colonies respectively) correlated better with oenocyte density (r=0.96) than with gaster surface (r=0.75, figure V.E.1.). Gaster surfaces of queens and workers were 44.1 and 28.6mm2 respectively. Gaster surface of reproductive and non-reproductive workers were assumed to be equal as only large workers were chosen.

Sixty-three to 73% of the total HCs extracted from 6 non-reproductive workers were collected from their head. Nine to 20% originated in their thorax, 6 to 12 from the legs and 5 to 14% from their gaster (figure V.E.2.). CHC profiles from the head, thorax and gaster were

Table V.E.1.: oenocytes diameter and number in thorax and gaster of queens and workers of different ages and reproductive status, as well as of males. The young worker was less than one week old; the old worker was several months old. Individuals originated in several colonies. Only large workers were measured.

	oenocytes diameter (µm)		oenocyte number	
	thorax	gaster	thorax	gaster
functional queen	71.25	63.75	30-40	16642
founding queen	59.50	59.25	30-40	11472
virgin queen	75.19	75.25	30-40	9968
male	53.57	49.31	20-30	-
reproductive orphaned worker	-	-	-	11324
old queenright non-reproductive worker	49.38	66.69	30-40	7406
young queenright non-reproductive worker	48.13	48.56	30-40	-

"-" indicate lack of data

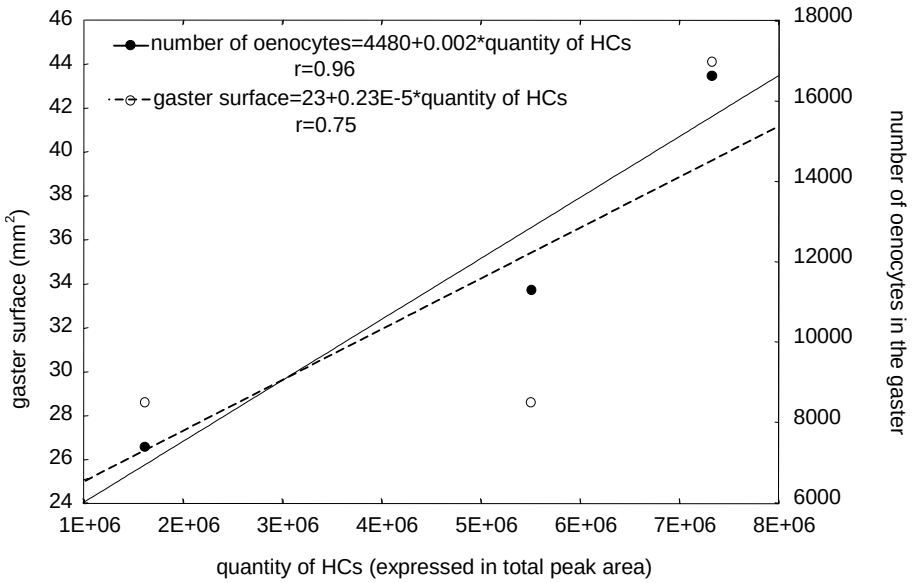


Figure V.E.1.: correlations of average quantity of HCs with gaster surface as well as with oenocytes density in the gaster. Non-reproductive workers are represented by the points on the left, reproductive workers by the middle ones and queens by those on the right. Different pools of queens and large workers were used to obtain each variable.

similar. There were no consistent qualitative differences (figure V.E.3.) and a discriminant analysis based on 15 compound peaks (marked with a star in figure V.E.2.) showed that these substances covered the different body parts homogeneously (Wilk's Lambda=0.80, $F[6,26]=0.52$, $p<0.78$, table V.E.2. and figure V.E.3.). Atypical data points for heads and thoraces could not be explained.

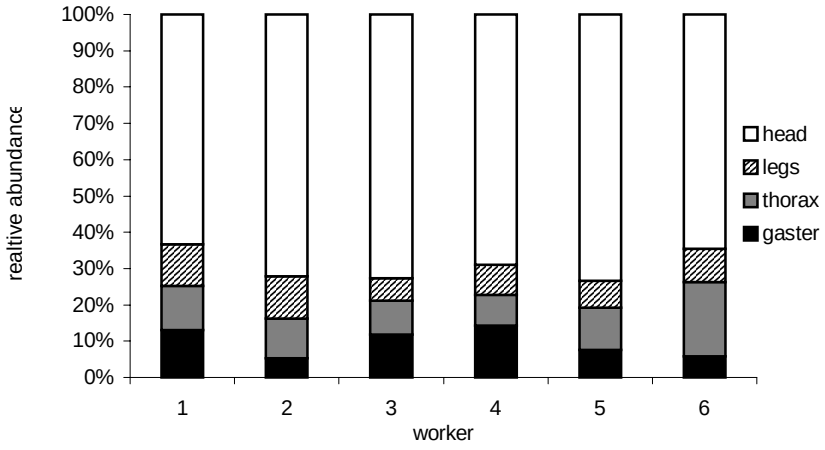


Figure V.E.2.: relative abundance of HC extracted from the head, thorax, legs and gaster of 6 *M. gulosa* queenright workers.

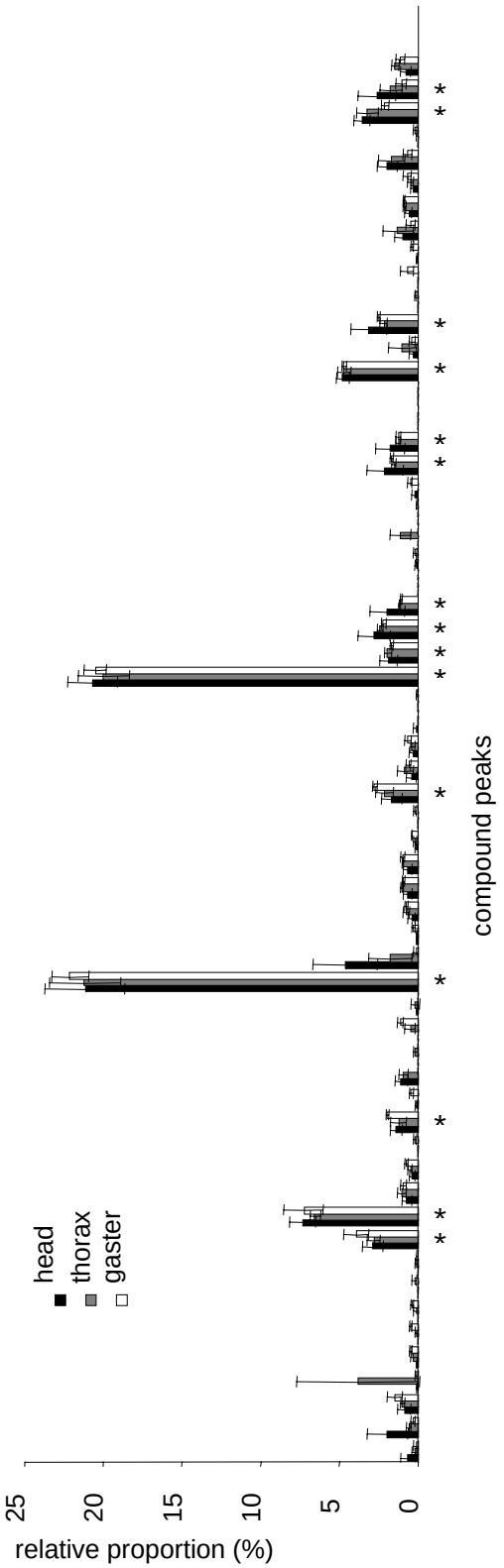


Figure V.E.3.: CHC profiles of heads, thoraces and gasters of queenright workers (n=6) of *M. gulosa*. CHC were extracted with the SPME method. Error bars represent standard deviation. * designate the peaks used in the PCA and DA of figure V.E.3.

Table V.E.2.: p-values obtained from the DA presented in figure V.E.3.

	head	thorax	gaster
head		0.82	0.63
thorax	0.82		0.57
gaster	0.63	0.57	

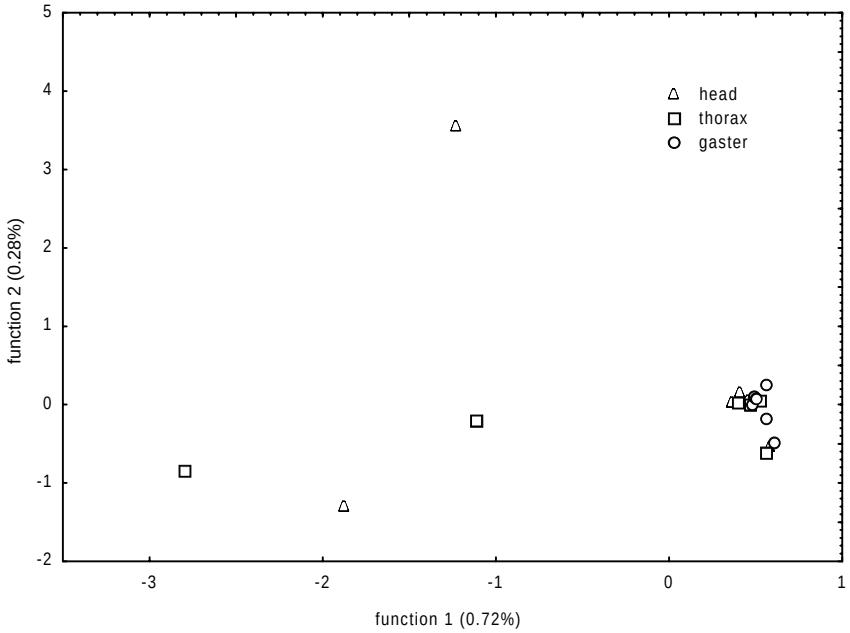


Figure V.E.3.: DA results of CHC profiles of head, thorax and gaster of 6 workers from 3 colonies. CHC were extracted by SPME. Head, thorax and gaster CHC profiles were similar.

Discussion *M. gulosa* queens and workers possessed oenocytes in the head and thorax and gaster. In contrast, oenocytes were only found in the gaster of *Monomorium pharaonis* queens (Jensen and Børgesen 2000). Oenocytes of all *M. gulosa* individuals, irrespective of physical caste and reproductive status, were more abundant in the gaster than in the thorax and presumably the head. Most of the CHCs were nevertheless extracted from heads of queenright workers. Before extraction of heads, precautions were taken to avoid a leak from the PPG that also contain CHCs (cf. section IV.B.d.iii.), indicating that accumulation of HCs on the head is due to external or internal translocation mechanisms. Naumann (1991) demonstrated that self-grooming was partly responsible for the translocation of queen mandibular pheromone in honeybee workers on which labelled synthetic pheromone was applied. In the German cockroach the internal pathway was demonstrated to be more important than the surface translocation of HCs induced by self-grooming (Gu et al. 1995). HCs are transported by

lipophorins in the hemolymph, but nothing is yet known about the mechanisms of extrusion to the cuticle (Katase and Chino, 1982, 1984, Chino and Downer 1982, Gu et al. 1995). Whether functional queens also possess a higher quantity of CHCs on the head is not known. This would indicate that their gaster emit another arrestant pheromone, which would account for the higher interest workers showed in this part of the queens' body (cf. section IV.B.d.i.). The Dufour gland could represent the source of this pheromone (cf. section IV.B.d.iii.). The translocation mechanism produced qualitatively uniform CHC profiles on the whole body of *M. gulosa* workers. In contrast, workers in other species showed variations in the relative proportions of some compounds on the head and thorax (see Howard 1993).

Still preliminary results show a good correlation between oenocyte number and quantity of HC extracted from gasters of different individuals. Differences in size among queens and workers explained the differences in number of oenocytes well. However, with comparable sizes, a functional queen had more oenocytes than a founding and a virgin queen, and a reproductive worker had markedly more oenocytes than a non-reproductive individual. Whether this reflects a real correlation between oenocyte numbers, quantity of HCs produced and reproductive activity needs confirmation. Such difference in oenocyte density between reproductive and non-reproductive individuals of the same physical caste would demonstrate a multiplication of these glandular cells linked to reproductive activity.

Preliminary transmission electronic microscope study of oenocyte ultrastructure could not establish a correlation between cellular and reproductive activity. Oenocytes of a queen were characterised by large dark electron dense inclusions located in vacuoles, whereas reproductive workers lacked these inclusions in such density and size. Presence of these inclusions might nevertheless be correlated with reproductive activity, as the workers examined might not have reached a high level of reproductive activity at the time of fixation. Furthermore, the influence of age on oenocyte activity and the presence of these inclusions could not be controlled, as no old workers were available for comparison.

V.E.b. Hydrocarbons mediate the recognition of reproductive workers

Introduction It was demonstrated in several species of ants that CHCs mediate colony recognition (Lahav et al. 1999, Thomas et al. 1999, Wagner et al. 1999). Whether they also the recognition of reproductive status in individuals has not yet been proven and only correlations were demonstrated to occur (Monnin et al. 1998, Liebig et al. 2000, Cuvillier-Hot et al. 2001). Similarly in *M. gulosa*, very good correlations between changes in CHCs and reproductive status in workers, as well as queens were obtained (cf. sections IV.B.d. and V.B.). The fact that only some orphan workers reproduce whereas most continue producing trophic eggs allows for the separation of the effects of orphanage from that of reproduction in CHC changes. In a first attempt to test the role of CHCs in triggering policing by nestmates, orphan reproductive and trophic egg-layers were extracted in hexane and the extracts bioassayed. As other, more polar substances might be extracted together with the CHCs and be responsible for the effect observed in the bioassay, total lipid extracts were realised. These extracts were then fractioned in HC and non-HC fractions and these fractions were bioassayed in order to determine which is detected and used by workers to recognise reproductive individuals.

Methods

Bioassaying of hexane extracts In two orphaned groups of workers (originating colonies D and G), trophic and reproductive egg-layers were identified as described in the section V.B. A total of 11 reproductive and 12 trophic egg-layers were collected. They were individually extracted by gentle shacking for 2 minutes in 1ml hexane. The extracts were then left under a fume hood to evaporate and were rediluted in 4µl of hexane. Workers from 4 queenright nests (C, D, J and K) were collected and marked with a dot of enamel paint on the head. These workers were immobilised on a metallic rod with fishing string to which weights were tied. The 4µl extracts were applied evenly on their body (legs included) with a micro-syringe. After a 10 minutes allowance for solvent evaporation, the ants were individually reintroduced in their mother colony. The number of nestmates antennating and biting these individuals was monitored at one-minute intervals for 10 minutes. As control for the effect of the solvent, 7 workers were treated with the same volume of pure hexane, reintroduced in their nest and the attention they triggered in nestmates was similarly monitored. The interaction score obtained was tested for normality with the Shapiro-Wilk's test and compared between groups with an ANOVA).

Total lipid extraction, fractioning into HC and non-HC components and bioassaying The fractioning procedure used by Lahav et al. (1999) to demonstrate the role of CHCs in nestmate recognition was adapted to *M. gulosa*. Total lipid extracts of groups of 8-15 ants were realised (Bligh and Dyer 1959). The extracts were loaded onto a silica gel column (Macherey and Nagel, Chromabond 500mg) and eluted with 10ml hexane, 4ml hexane:chloroform (1:1), 5ml chloroform and 5ml methanol. The fractions obtained were left to evaporate under a fume hood. The HC fraction was rediluted in 200µl hexane and all the other fractions were pooled in 200µl chloroform:methanol (1:2) to give the non-HC lipid fraction. The quality and yield of the fractioning process were verified by GC analysis. The quantity of extract to be applied on a test worker was adjusted so as to transfer a quantity of one worker equivalent (Weq) in 4µl of solvent. As for the hexane extracts, queenright workers were chosen, marked with paint, and the 4µl of extracts were spread over their body. After a 10 minutes allowance for solvent evaporation, test workers were reintroduced in their mother colony. They were removed again after 10 minutes observation. Ten minutes later they were tested again in an alien queenright colony. Workers were introduced in pairs: on the first individual, one Weq of reproductive worker extract was applied, whereas on the second, one Weq of trophic egg-layers extract was applied. HC and non-HC fractions were tested in an alternate manner. Antennation and biting elicited by the test individuals was monitored at one-minute intervals for 10 minutes.

Results

Bioassaying of hexane extracts Transfer of hexane extracts of reproductive workers induced them being antennated and bitten. However, most of the effect could be attributed to the solvent (ANOVA, $F=1.88$, $p=0.17$). Individuals treated with the reproductive worker extracts only tended to attract more attention than those treated with the trophic egg-layer extracts (figure V.E.1.). By comparing these scores with those obtained by live reproductive workers introduced in queenright colonies, it appears that the transfer of CHC profiles by extraction/application is poorly efficient in transferring the cues that elicit the strong interest in reproductive individuals or their attack by nestmates.

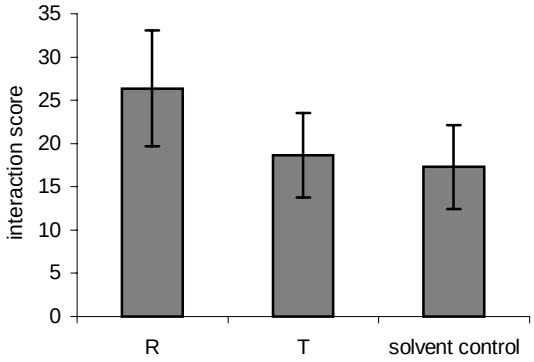


Figure V.E.1.: number of antennation and bites (interaction score) elicited by workers on which extracts of reproductive (R, n=11) and trophic egg-layers (T, n=12) were applied. Control for the effect of solvent consisted in workers on which the corresponding volume of hexane was applied (n=7).

Total lipid extraction, fractioning into HC and non-HC components and bioassaying
Workers carrying the non-HC fractions of trophic or reproductive orphan egg-layers elicited similar attention in nestmates or in non-nestmates (Wilcoxon test, nestmates: n=15, T=26.5, p=0.10; non-nestmates: n=8, T=7.5, p=0.25) (figure V.E.2.). In contrast, workers on which the HC fraction was applied were treated differently whether the extract originated from orphan reproductive egg-layers or orphan trophic egg-layers. Extracts from reproductive individuals elicited stronger attention in their carrier. This was the case when the latter were exposed to nestmates and to non-nestmates (Wilcoxon test, nestmates: n=15, T=18.0, p=0.03; non-nestmates: n=8, T=2.2, p=0.03).

A worker on which the HC fraction of an orphan trophic egg-layer was applied triggered unusual high attention in nestmates (figure V.E.2a.). If this atypical individual is removed from the analysis, the p-value obtained by the comparison of the attention triggered by reproductive and non-reproductive orphan worker extracts becomes 0.006. GC analysis of the HC fractions showed the presence of the typical HC compounds obtained in the hexane extracts (cf. section IV.B.d.ii.). The non-HC fractions of both reproductive and trophic eggs layers were poor in compounds and similar to each other (figure V.E.3.). Their quantity represented 9 to 36% of the total lipid extract.

Discussion As already suggested by the transfer of hexane extracts, fractioning of total lipid extracts into HC and non-HC fractions demonstrated that HCs mediate the recognition of reproductive status in workers and that they most likely are at the basis of the policing

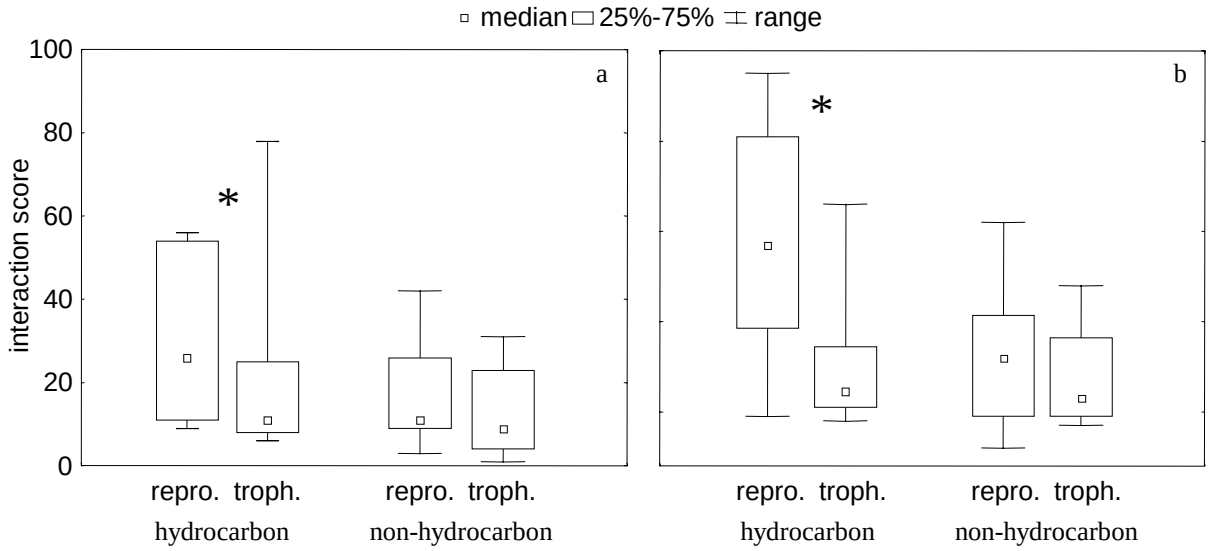


Figure V.E.2.: total lipid extraction of orphan reproductive or trophic egg-layers were realised. The extracts were fractioned in HC and non-HC components. The different fractions were applied on queenright nestmates and the number of antennation and bites (interaction score) elicited by these test workers a) in queenright nestmates, b) in queenright non-nestmates is represented. * designate significant differences (Wilcoxon test, $p<0.05$). "repro." and "troph." designate queenright workers on which HC or non-HC of orphaned reproductive and non-reproductive worker respectively were applied.

mechanism described in section V.B. This is the first time that the role of CHCs in mediating information about reproductive status is shown in a social insect. The attention triggered by the test workers in nestmates or non-nestmates was remarkably lower than when they were exposed to live orphan individuals (cf. section V.B.). This is due to the crude methodology used to transfer the chemical compounds. Workers might either use quantitative variations in one or several compounds to assess reproductive status of individuals, or variations in relative proportions of some components. The application of the extracts realised could have resulted in the dilution of the cues in the cuticular compounds of the dummy workers, thus affecting their detection. For this experiment, live dummy workers were preferred to dead washed ants as it appeared throughout the study that dead individuals are discriminated and elicit different behaviour in nestmates as live workers.

Whether the HCs linked to reproduction and detected by workers represent a fertility signal or simply a cue could not be established. The definition of a signal implies that the emitter benefits from producing it. Advertising their status might be advantageous for the reproductive individuals in orphan worker groups, to receive care or food for example, but no observations were conducted to examine this problem. In a single case was the formation of

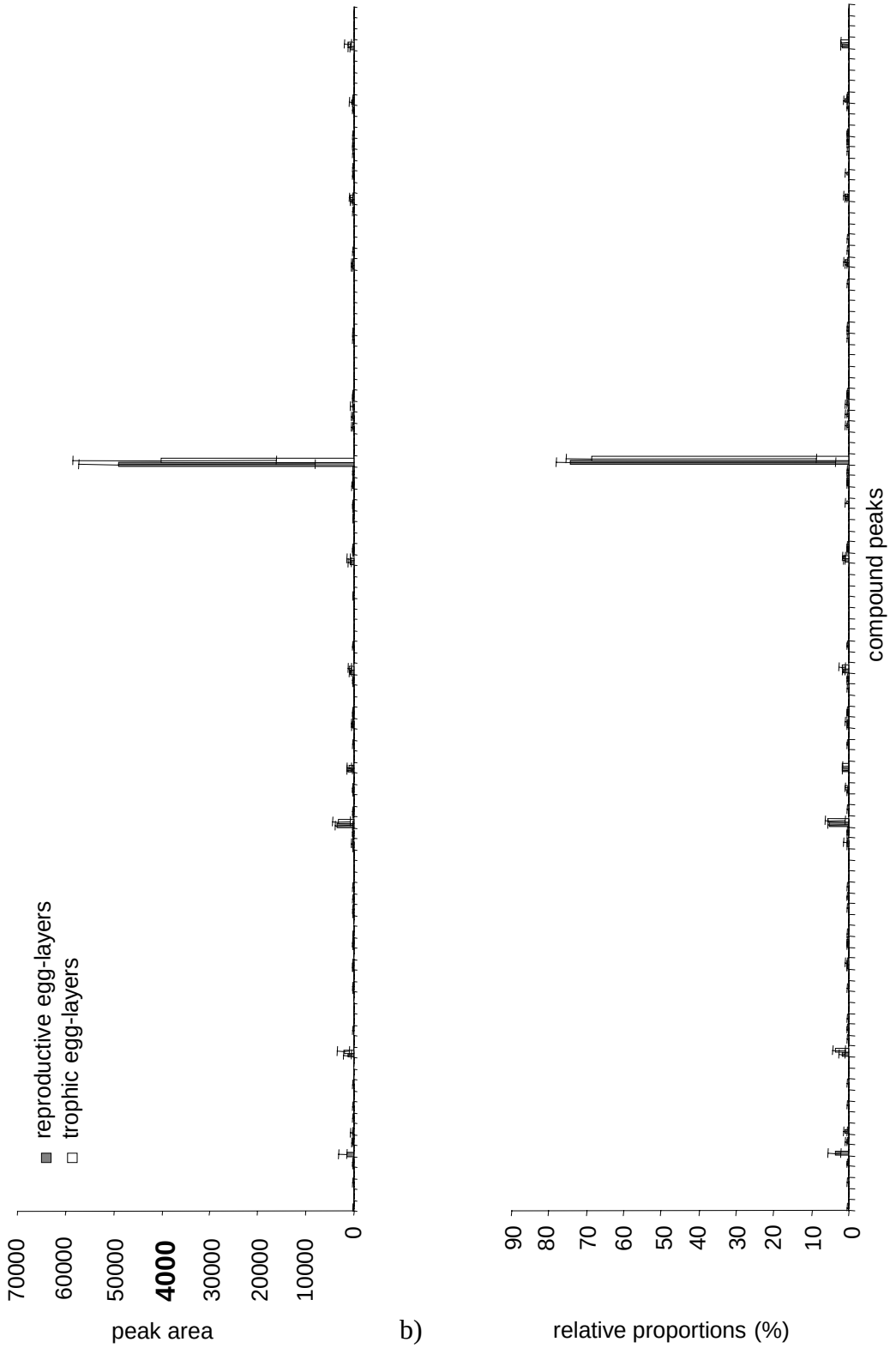


Figure V.E.3.: GC analysis of the non-HC fraction of the total lipid extracts of orphaned trophic (n=3 pools of 8, 12 and 16 individuals) and reproductive egg-layers (n=3 pools of 10, 11 and 12 individuals). a) median of the absolute amount of compound peaks corresponding to a single individual, b) median of the relative amounts of the compound peaks. Error bars represent the range.

retinue observed around a reproductive orphan worker, thus supporting the idea that workers producing a queen-like signal (much like the false queens in honeybees [Velthuis 1970, Crewe and Velthuis 1980]) and could benefit from its emission as the queens do.

V.F. Discussion: regulation of worker reproduction and CHCs

When worker reproduction is experimentally induced in queenright colonies of *M. gulosa*, future reproductives can be policed by immobilisation leading to death. This can occur even before the victims produced reproductive oocytes. Workers recognise the reproductive status of nestmates by detecting changes in the CHC profiles that are linked to reproductive physiology. It is likely that the detection of these changes also trigger the policing of individuals. Further support for this hypothesis comes from the fact that the phenomenon is not irreversible, as an immobilised worker reverting to the profile of non-reproductives was released and survived.

Efficient or costly worker policing leads to the evolution of self-restraint by workers (Ratnieks 1988, Ratnieks and Reeve 1992). In *M. gulosa*, policing is costly because it takes several workers to immobilise a nestmate for long periods. It is also relatively efficient, as it often results in death of the victim. Self-restraint might explain why actual conflict is rarely observed. However, reproductive attempts in the presence of the queen must occur for policing to be maintained over evolutionary time. Consistent with this idea, immobilisation of individuals with intermediate CHC profiles, presumably starting to reproduce, were observed in non-manipulated situation.

The fact that some workers escaped policing under experimental conditions suggests that worker reproduction occurs naturally, as was demonstrated for honeybees (Visscher 1989, 1996, Ratnieks 1993). The absence of specific behaviour or posture during oviposition by reproductive *M. gulosa* workers prevents their visual identification and the low number of dissections realised (compared to honeybees, Ratnieks 1993) do not permit exclusion or confirmation of the possibility.

In the case where some reproductive workers escape policing, nestmates could still prevent their reproduction by destroying their eggs. This is possible provided that workers can distinguish between queen and worker laid eggs in order to avoid costly mistakes of destroying valuable sisters. Although differences were found in the surface HC profiles of worker and queen produced eggs, male worker laid-eggs were accepted when introduced in

queenright colonies. Discrimination by workers is nevertheless suggested by a longer handling time compared to queen-laid eggs. Ratnieks (1993) explains the fact that some honeybee worker-laid eggs escape policing by their closer resemblance to queen eggs. Evidence for this was found in *M. gulosa.* The first eggs laid by workers when they become reproductive are destroyed by nestmates, whereas eggs produced later on are accepted. HC surface profiles of the accepted eggs are closer to those of the queen than are those of the destroyed eggs. However, the low hatching rate of the latter indicates that their viability is low and their destruction might not represent policing.

The origin of ant CHCs has not been fully elucidated yet. Soroker and Hefetz (2000) demonstrated that the fat body is the major source of HCs. Whether oenocytes that are spread among fat body cells or fat body cells themselves produce the HCs is difficult to determine, as both cell types are difficult, if not impossible to isolate from each other. The preliminary results obtained with *M. gulosa* show a correlation between the number of oenocytes in queens and workers, HC quantity on their cuticle and reproductive activity. Increasing sample size will verify the constancy of this link and help determine whether oenocytes produce HCs in ants, as it is the case in various other insects (Lockey 1988).

VI. General conclusion

In the course of evolution of Hymenopteran societies, colony size increased and complex levels of organisation have been reached (Wilson 1971, Hölldobler and Wilson 1990, Bourke 1999). The mechanisms regulating the division of reproductive labour are central to colony organisation. They represent an important constraint on the size colonies can reach. Queen control or policing is assumed to be possible only in species with small colonies, where physical domination of most individuals and/or destruction of their eggs is possible (Ratnieks 1988, Keller and Nonacs 1993). If colony size exceeds the number of individuals that can be controlled by the queen, the latter loses her reproductive monopoly and colony organisation collapses. Pheromones are assumed to represent a more efficient way to reach and regulate the behaviour of numerous workers in larger colonies (Wilson 1971 pp. 299, 302). Colony size affects the reproductive potential of workers (Bourke 1999) and consecutively influences the mechanisms of division of reproductive labour in a feedback process. With colony sizes of a few thousand individuals and a mixture of ancestral (low queen-worker morphological dimorphism and high reproductive potential for workers) and derived features (worker size polymorphism), *M. gulosa* represents an intermediate stage between simple and complex societies (sensu Bourke 1999). To elucidate the mechanisms regulating reproduction of workers in such system can contribute to the understanding of how colony size and mechanisms of division of labour constrained each other in shaping the evolutionary transition between simple and complex social organisations.

Workers of *M. gulosa* are able to recognise queens on the basis of several glandular products. These results support the idea that diverse site of release or biosynthesis represent a general feature of social insect queen pheromones (Vargo and Hulsey 2000, see also Winston and Slessor 1998). The queen pheromonal system of the “primitive” ant *M. gulosa* thus appears as complex as that of “highly” eusocial species. In none of these species is it known whether the various glands produce the same active compounds or different products with the same effect on workers. In addition to elicit worker aggregation, these semiochemicals might possess other functions (Vargo and Hulsey 2000). They may mediate other releaser or primer effects, singly or in association. Their chemical identification and bioassaying is necessary to elucidate their functions and interactions and to understand the complexity of the pheromonal system of Hymenopteran queens.

Queens of *M. gulosa* never interact agonistically with their workers and reproduction of the latter is most likely regulated by a pheromone. This pheromone is not volatile, as revealed by

the double mesh experiments. Workers cannot obtain the pheromone from other workers; they have to establish direct physical contact with their queen to detect it. Whether short-range perception of the queen's presence without physical contact mediates the primer effect could not be established. Queens do not need to actively participate to pheromone distribution by moving in the nest. Neither the increase the number of workers they contact nor the marking of the nest by pheromone was necessary to the maintenance of worker sterility. This indicates that the modest size of the colonies and the resulting interindividual encounter pattern allow for enough direct physical contacts between queens and workers to occur for optimal pheromone detection and subsequent monopolisation of reproduction by the queens.

Based on the results of this study, a model for queen releaser and primer pheromones mode of action in *M. gulosa* is proposed (figure IV.C.1.). The physiology of some workers progressively changes when they are removed from the influence of their queen. Their aggressiveness increases, they start reproducing and rearing new queens. Agonistic interactions appear between 7 and 18 days after the workers are deprived of contact with the queen and reproductive eggs accumulate from the 18^{th} day on. It is hypothesised that perception of the queen's pheromone resets the workers' physiology, so as to prevent these changes. As the endocrine mechanisms triggered by the perception of the pheromone and responsible for this effect is unknown, the existence of an internal queen pheromone level is assumed for simplicity. If workers perceive enough pheromones, their internal level is above a given threshold (T), and their queenright non-reproductive status is maintained. Under these conditions, they only produce trophic eggs, rear exclusively workers and never interact agonistically with nestmates. Workers seem to encounter their queen frequently enough during their normal activities to perceive her pheromones and to remain above the threshold. Their non-reproductive status is thus continuously maintained (1). A lack of perception of pheromone for approximately 24 hours, and the subsequent decrease in pheromonal level below the threshold (T), might induce workers to search for their queen (2). Several situations can then be envisaged. First, the worker finds the queen (3), perceives her presence at a distance and leaves without establishing physical contact (4). Whether this resets their physiology (1) or is without effect is not known. Second, the worker perceives the queen's presence and establishes contact (5); its physiology is then reset (1). In the case the queen is found dead, physiological changes leading to reproduction occur (6). These changes also occur if the worker does not find the queen.

If reproduction of workers is triggered in a queenright situation, the physiological changes in individuals are often detected by nestmates 10-12 days after their deprivation of

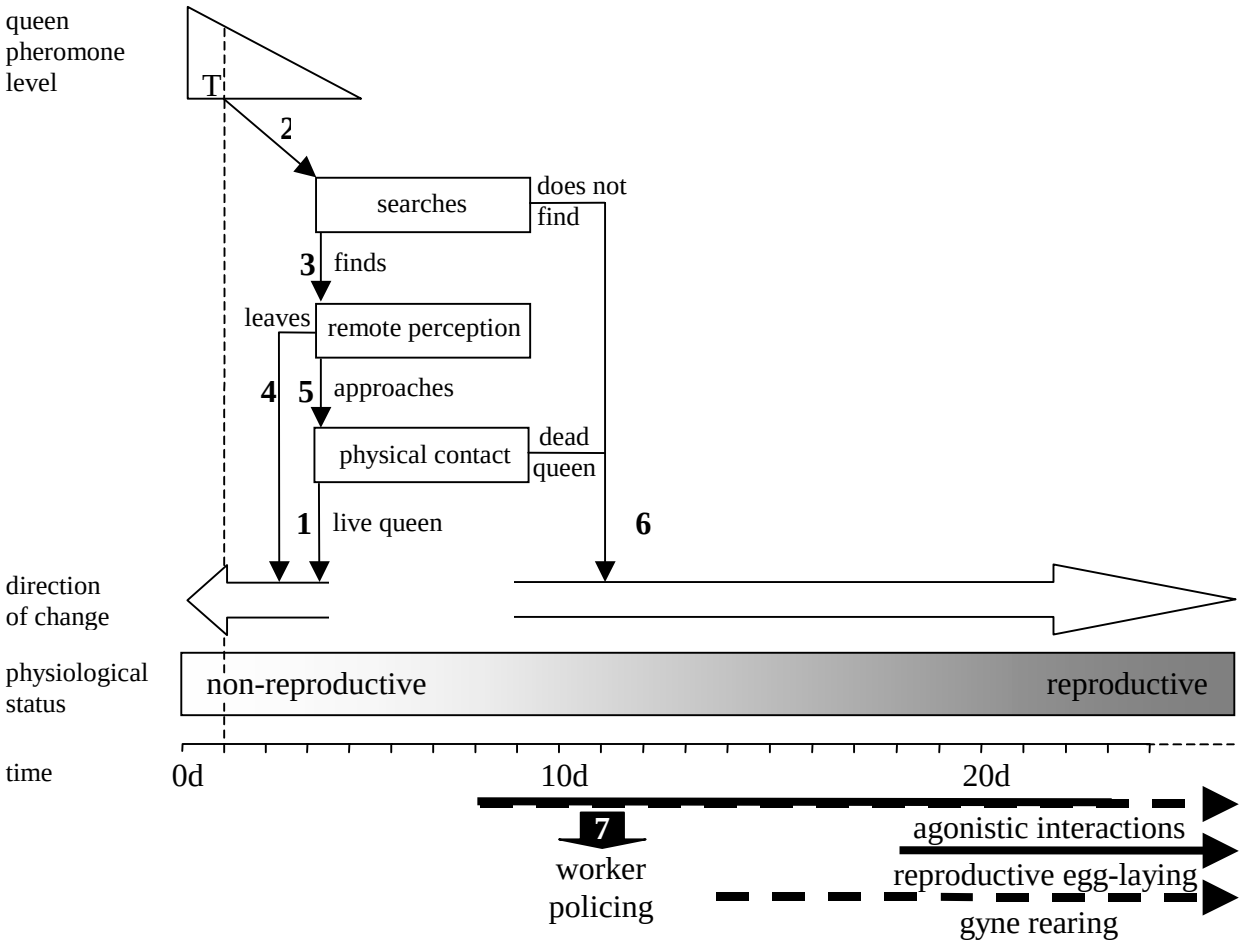


Figure IV.C.1.: proposed model for the mode of action of queen pheromones regulating worker reproduction and gyne rearing in *M. gulosa*. Depending on the frequency of contacts established with the queen, the physiology of workers is reset to a queenright non-reproductive status or progressively changes to a queenless reproductive status. In the first case, workers behave peacefully, produce trophic eggs and rear worker brood, whereas in the second, some workers fight among each other, start reproducing and rear gynes (if diploid brood is available). Within 24 hours of experimental deprivation of physical contact with the queen, workers seem to search for the queen (2). Whether remote perception of queen's presence alone is sufficient to mediate the primer effect and prevent reproduction is not known (4). When gyne production is induced and when frequency of agonistic interactions decline is not known (dotted arrows).

queen pheromones (7). The future reproductives are immobilised and can be killed. In this case, this behaviour represents efficient policing and matches the definition of punishment. If the individuals survive, their reproduction is rarely affected by the immobilisation. Some workers also develop their ovaries without being victim of immobilisations, thus escaping policing. Rare incidence of policing in non-manipulated queenright colonies suggests that reproductive attempts by workers and virgin queens occur naturally. These results are consistent with the evolutionary scenario for worker policing proposed by Ratnieks (1988). An efficient and costly mechanism of policing may lead to the selection of self-restraint by workers. Self-restraint could represent the proximate mechanism responsible for worker sterility and explain the rare occurrence of overt worker policing.

Queen pheromones were commonly envisaged as coercive tools queens used to inhibit workers from reproducing (Wilson 1971 p. 299, Fletcher and Ross 1985, Hölldobler and Bartz 1985, Bourke 1988a, Hölldobler and Wilson 1990 p. 222). The concept of queen control explicitly means that workers are forced to behave in a way contrary to their own interests. A control based on coercive pheromones is assumed to trigger a chemical arm race, with workers trying to develop a resistance or immunity to the substance produced by queens and queens in turn trying to achieve more efficient control. This strategy is likely to be evolutionarily unstable because of low cost-effectiveness. Several authors proposed an alternative hypothesis where queens would signal their presence or fertility. Workers could use such honest signals to behave in a way that maximises their fitness (Seeley 1985 p. 30, Woyciechowski and Lomnicki 1987, Keller and Nonacs 1993). Most of the regulation mechanisms involving queen pheromones can be interpreted on the basis of the signalling hypothesis (Keller and Nonacs 1993). If, as discussed by Bourke and Franks (1995, p. 239), this hypothesis is powerful and correct, to test it and distinguish a signalling from a control mechanism remains difficult. In spite of opposite underlying evolutionary pathways, no experimental proof or disproof has been given so far to establish which of these mechanisms is prevalent. As our understanding of the relationship between queen fecundity and fecundity markers (like CHCs) progresses, there will be a possibility to test some predictions made according to the hypothesis. Identifying queen pheromones and understanding their mode of distribution and action on the endocrine system of workers may also help to disentangle both concepts. The results obtained in the present study clarified the modalities of perception and action of the queen pheromones regulating worker reproduction (see above). However, they can be interpreted equally well under both control and signalling mechanisms.

The different behaviour (attraction and repulsion) shown by workers after they detected the presence of the queen can be interpreted as workers "avoiding" the queen's coercive pheromone or as them "ignoring" her signal. Both these strategies might confer on them a reproductive head start when workers start to compete for egg laying after the loss of the queen (Moritz et al. 2001, 2002, cf. section IV.D.). The availability of several queen pheromones (cf. section IV.B.e.) might allow workers for monitoring more than the queen's presence. Information about her health status or degree of fertility might well be encoded in a blend of semiochemicals. These could represent more than redundant pheromones (Vargo and Hulsey 2000) and constitute a complex and costly honest signal on which workers could rely to adjust their behaviour in a way that maximises their fitness (Keller and Nonacs 1993). On the other hand, a complex blend of queen pheromones can be interpreted as an evidence for a chemical arm race (West-Eberhard 1981).

Queen pheromones have rarely been identified due to the number of potential active compounds and the difficulty of bioassaying (Vander Meer and Alonso 1998, Vargo 1998). The chemistry of *Myrmecia* is relatively well known, as workers have been the subjects of numerous chemical analyses. This interest was motivated by several facts. First, their large size and their easy collection in large number as they defend their colony, allowed for the collection of sufficient material for chemical studies with early methods (Cavill et Williams 1967, Cavill et al. 1970, Brophy and Nelson 1985). Other reasons include the efficient antibiotics they produce (Mackintosh et al. 1998, 1999), as well as the severe allergic reactions their stinging can trigger in humans (Clarke 1986, Taylor 1988, Douglas et al. 1998). Thus, analyses of *Myrmecia* venom are numerous (Cavill et al. 1964, Ewen and Ilse 1970, Wanstall et al. 1974, Clarke 1986, Ford et al. 1991, Matuszek et al. 1992, 1994a, 1994b, Donovan et al. 1993a, 1993b, 1993c, 1995, 1996, Street et al. 1994, 1996, Hodgson 1997, Wu et al. 1998, King et al. 1998, Douglas et al. 1998). However, none of these studies focussed on interindividual differences and their role in communication. In the frame of this study, differences in chemistry of reproductive and non-reproductive individuals were demonstrated to occur in queens as well as in workers. As established in a number of recent studies, variations in CHC profiles correlated with changes in reproductive status in both queens and workers of *M. gulosa*. Correspondingly, differences occurred in the post-pharyngeal gland (which has been shown to sequester CHCs in other species). Differences in composition also occur in the Dufour gland and presumably in the mandibular gland.

CHCs possess several characteristics one attributes to the elusive queen pheromones (e.g. queen specificity, low volatility, spreading over the whole body). The hypothesis of their releaser pheromonal function is supported by the aggregation of workers elicited by hexane extracts of queen cuticle. Furthermore, fractioning of total lipids extracts of workers showed that the HC, but not the non-HC fraction constituted the basis of the discrimination between reproductive and non-reproductive individuals. It is most likely that this discrimination is at the basis of the worker policing mechanism that ensures queen monopoly of reproduction. This supports the idea that the CHC profiles of queens allow their recognition by workers and therefore represent a component of the queen complex "signal".

The identity of the HC compounds mediating recognition of reproductive status in workers has not been elucidated. In solitary insects, CHCs play a role in mate recognition, as sex pheromones and as anti-aphrodisiac (see Howard 1993, Singer 1998 for reviews). The similarity of the pheromonal compounds of some species with the compounds specific for reproductives in *M. gulosa* is noteworthy. For example, Z,9-pentacosene (9P) was identified as being part of the sex pheromone in *Fannia* and *Haematobia* flies. 9P is also thought to release a brooding behaviour in wasps (see Howard and Blomquist 1982, Howard 1993 for reviews). The application of synthetic 9P, or of a mixture of the major "queen CHCs" on *M. gulosa* workers however, did not induce retinue formation or attack behaviour in nestmates, indicating that the compound has no role by itself or in combination with the others major CHCs. Cuticle of *M. gulosa* are covered with approximately 90 different compounds of the three classes of HCs (n-alkanes, methyl-branched alkanes and monoenes). Some of these compounds were demonstrated to possess semiochemical functions in various insects (Howard and Blomquist 1982, Howard 1993, Schiestl et al. 2000). There are thus many candidates for semiochemical activity among the minor CHCs compounds or in different combinations of compounds.

The identification of active compounds represented and still represents a problem in the studies of queen pheromones. As exemplified in this study, the major compounds are not always the active ones. A method of screening the numerous chemicals produced by glands or glandular cells would considerably facilitate the task, by separating the compounds detected by the workers from those without physiological activity. The gas chromatography with electroantennographic detection technique has, for example, been successfully used with bee males to identify the sex pheromones produced by females (Schiestl et al. 2000). The successful adaptation of this technique to study queen pheromones or the development of other screening methods will provide an alternative to random or to tedious systematic bioassaying of queen-specific substances. This would allow to determine the function of the active compounds

obtained and to understand their interactions. Much remains to be discovered on chemical communication in social insects, and especially on the complex queen pheromones that are essential to colony organisation.

VII. Summary

VII.A. Summary

Division of reproductive labour in societies represents a topic of interest in evolutionary biology at least since Darwin. The puzzle of how helpers can be selected for in spite of their reduced fertility has found an explanation in the kin selection theory: workers can overcome the cost of helping and of forgiving direct reproduction by rearing sufficiently related individuals. However, in the Hymenoptera, little is known on the proximate mechanisms that regulate the division of labour in colonies.

Our knowledge is based on several “primitive” ants from the subfamily Ponerinae and two highly eusocial Hymenoptera species. In the former, the dominance hierarchies allowing for the establishment of individuals as reproductives are well understood. In contrast, the pheromonal mechanisms that help maintain their reproductive status are not understood. Similarly in “higher” ants, pheromonal regulation mechanisms of worker reproduction by queens remain largely unknown. The number of putatively biologically active compounds, the interaction of several semiochemicals and the delayed physiological responses of workers complicate bioassaying and explain our poor knowledge.

The aim of this study is to determine the modalities of production, distribution and action, as well as the identity of the queen pheromones affecting worker reproduction in the ant *Myrmecia gulosa*. The species belongs to the poorly studied subfamily Myrmeciinae, which is endemic to the Australian region. The subfamily represents, together with the Ponerinae, the most “primitive” ants: their morphology is close to that of the hypothetical ancestor of ants, and the specialisation of queens is weaker than that of “higher” ants. Simple regulation mechanisms were therefore expected to facilitate the investigation.

The first step in this study was to characterise the morphological specialisation of queens and workers, and to determine the differences in reproductive potential associated with this specialisation. Size of workers varies over a wide range (14-23 mm), exhibiting a bimodal distribution. Growth is monophasic and only slightly allometric. As in most ants, workers do not reproduce in the presence of the queen. However, they possess active ovaries and lay trophic eggs. As they do not perform trophallaxis, trophic egg laying constitutes the main channel of food exchange in the colony. Large workers have more ovarioles than small ones and lay more trophic eggs. The difference in egg output persists when they start to reproduce after the queen’s death or removal. Although small workers are less fecund, they reproduce more readily

following queen removal. Queens have disproportionately more ovarioles than workers and have a 10-fold higher egg-laying rate. Although queen-worker ovarian and morphological dimorphisms are weak compared to those of “higher” ants, they are stronger than in the ponerines studied to date. The study of *M. gulosa* therefore contributes to our understanding of the link between regulation of division of reproductive labour and social complexity. Furthermore, it will help shed light on the reproductive biology in the poorly known subfamily Myrmeciinae.

Queens were recognised by workers on the basis of cuticular as well as gland extracts or products. Each of these releaser pheromones triggers behavioural responses in workers (aggregation). This response is independent from the queen’s behaviour or from the recognition of their shape. It is unlikely that these pheromones mediate a long-range attraction, and they presumably only play a role in queen recognition. In addition to the occurrence of several recognition pheromones, the complexity of the queen pheromonal system in *M. gulosa* was demonstrated by the fact that workers can react differently to the slightly volatile pheromone the queen emits, that they can identify areas of the nest were she stays or from which she is absent and that they can distinguish live from dead queens. What is the exact function of the multiple pheromones identified and how they interact remains to be determined. This could help understand why queen “signal” in a “primitive” ant with weakly specialised queens such as *M. gulosa* appears to be as complex as in highly eusocial species.

Primer pheromones act on workers’ physiology and have long-term effect. Whether workers of *M. gulosa* reproduce or not is determined by the detection of a queen pheromone of this type. Direct physical contact with the queen is necessary for workers to detect this pheromone. Indeed, transmission of the pheromone from contaminated workers to other workers does not occur. The queens’ movements in the nest do not contribute significantly to pheromone distribution: their visits do not considerably increase the number of workers they encounter and the pheromones they deposit on the ground are not necessary to the regulation of worker reproduction. Workers encounter the queen during their usual activities in the nest or may search for her. The resulting frequency of contacts is sufficient for optimal pheromone detection by workers and for the maintenance of their non-reproductive status. Thus, the colony size of *M. gulosa* is compatible with a simple system of pheromone perception by workers based on direct physical contact with the queen.

When prevented from establishing physical contact with their queen, some workers start to reproduce and are policed by nestmates. Some of the individuals thus immobilised do not survive the treatment, whereas others do and continue to produce reproductive eggs. Although some workers escape policing in experimental condition, worker policing could be efficient enough for self-restraint to evolve. Self-restraint could represent the proximate mechanism responsible for the sterility of workers and explain that overt policing is rarely observed in non-manipulated colonies.

Cuticular hydrocarbons (CHCs) represent the major class of lipids present at the surface of insect epicuticle. They are involved in communication in various solitary as well as social insects. Their low volatility, their repartition over the entire cuticle and the existence of queen and worker specific CHC profiles suggest that CHCs constitute a queen pheromone. Supporting this hypothesis, hexane extracts of *M. gulosa* queens, but not of workers, elicited a strong interest in nestmate workers. However, a synthetic blend composed of 15 major queen-specific cuticular compounds was not able to trigger worker aggregation or to inhibit their reproduction. This suggests that semiochemical activity lies in a different combination of HCs or in other classes of compounds. Importance of HC versus non-HC compounds was confirmed by bioassaying purified fraction of both classes of chemicals. Workers discriminated individuals on which HCs from reproductive egg-laying workers were applied. In contrast, they did not show discrimination against workers on which non-HCs fractions of reproductives were applied. Although the role of CHCs in nestmate recognition has been proven in several ant species, only correlations with ovarian activity have been reported to date. This study demonstrates for the first time that they indeed are at the basis of the recognition of reproductive status. This supports the idea that they are also at the basis of the recognition of queens by their workers. As CHCs profiles of workers and queens become similar with acquisition of reproductive status, they represent honest fertility markers. These markers could be used as signals of the presence of reproductives in the colonies, and represent the base of the regulation of division of reproductive labour.

In conclusion, a model for the mode of action of the queen pheromones regulating worker reproduction in this species is proposed. Improving our understanding of queen pheromonal systems will help establish which from the queen control or signal mechanism is at the base of the division of reproductive labour in the social Hymenoptera.

Zusammenfassung

In der Evolutionsbiologie stellt die Arbeitsteilung in Sozietäten spätestens seit Darwin ein Interessensgebiet dar. Die Frage nach der Selektion von Helfern, trotz ihrer reduzierten Fruchtbarkeit, hat eine Erklärung in der Verwandenselektionstheorie gefunden: Arbeiterinnen können die Kosten des Helfens und eingeschränkter direkter Fortpflanzung überwinden, indem sie ausreichend verwandte Individuen aufziehen. Bei den Hymenopteren ist über die proximaten Mechanismen, welche die Arbeitsteilung in den Kolonien regulieren, allerdings nur wenig bekannt.

Unser Wissen basiert auf den Ergebnissen von wenigen Untersuchungen an einigen "primitiven" Ameisen der Unterfamilie Ponerinae und zwei hochsozialen Hymenoptera-Arten. Bei „primitiven" Ameisenarten sind die Dominanzhierachien welche die Bildung von fortpflanzungsfähigen Individuen erlauben, gut untersucht. Im Gegensatz dazu sind die chemischen Signale, welche ihren reproduktiven Status aufrechterhalten, noch nicht aufgeklärt. Ebenso sind die pheromonellen Regulationsmechanismen der Arbeiterinnenreproduktion durch die Königin in "höherentwickelten" Ameisenarten weitgehend unbekannt. Die Zahl der vermutlich biologisch aktiven Komponenten, die Interaktion einiger Semiochemikalien und die verzögerte physiologische Antwort der Arbeiterinnen verkompliziert die Durchführung von Biotests und erklärt unser dürftiges Wissen.

Das Ziel der Studie an *Myrmecia gulosa* war die Bestimmung der Modalitäten von Produktion, Verbreitung und Funktion der Königinpheromone, sowie Aufklärung ihrer stofflichen Zusammensetzung. Die untersuchte Art gehört zu den bisher wenig beachteten Myrmeciinae und kommt endemisch in Australien vor. Zusammen mit den Ponerinae weist diese Subfamilie die "primitivsten" Ameisenarten auf. Die Morphologie der Ameisen ist angelehnt an die der hypothetischen Vorfahren und ihre soziale Organisation ist weniger komplex als die "höherentwickelter" Arten. Es wurden daher einfache Mechanismen erwartet, die helfen sollten, die Regulation der reproduktiven Arbeitsteilung bei "primitiven" Ameisen mit einer morphologisch spezialisierten Königin zu verstehen.

Der erste Teil der Studie sollte die morphologische Spezialisation der Königinnen und der Arbeiterinnen charakterisieren, bzw. den Unterschied im reproduktiven Potential, welcher mit dieser Spezialisierung verbunden ist, bestimmen. Die Größe der Arbeiterinnen variiert stark (14-23mm) und zeigt eine bimodale Verteilung. Das Wachstum erfolgt monophasisch und nur leicht allometrisch. Wie bei den meisten Ameisen reproduzieren die Arbeiterinnen nicht in Anwesenheit der Königin. Allerdings besitzen sie aktive Ovarien und legen trophische Eier. Da

keine Trophallaxis auftritt, findet der Futteraustausch in der Kolonie hauptsächlich über diese Eier statt. Größere Arbeiterinnen besitzen mehr Ovariolen als kleinere Individuen und legen mehr trophische Eier. Der Unterschied in der Eiablage bleibt auch mit Reproduktionsbeginn nach dem Tod oder der Wegnahme der Königin aus der Kolonie erhalten. Obwohl kleinere Individuen weniger fruchtbar sind, reproduzieren sie nach dem Verlust der Königin bereitwilliger. Im Vergleich zur Arbeiterinnen haben Königinnen verhältnismäßig mehr Ovariolen und eine 10mal höhere Eiablagerate. Obwohl ovarieller und morphologischer Königin-Arbeiterin-Dimorphismus im Vergleich zu "höherentwickelten" Ameisen nur schwach ausgeprägt ist, ist dieser doch stärker vertreten als in bis dato bekannten Studien über Ponerinae. Die Untersuchung an *M. gulosa* trägt zum Verständnis der Verknüpfungen zwischen Regulation der reproduktiven Arbeitsteilung und sozialer Komplexität bei. Überdies wird sie helfen, Licht auf die Fortpflanzungsbiologie der wenig bekannten Subfamilie der Myrmeciinae zu werfen.

Königinen werden von den Arbeiterinnen aufgrund ihrer kutikulären sowie ihrer exokrinen Extrakte oder Produkte erkannt. Jedes dieser Releaser-Pheromone löst eine Verhaltensreaktion bei den Arbeiterinnen aus (Aggregation). Diese Reaktion ist unabhängig vom Verhalten der Königin oder der Wahrnehmung ihrer Gestalt. Allerdings ist es unwahrscheinlich, dass die Pheromone eine weitreichende Wirkung besitzen, vermutlich spielen sie nur eine Rolle bei der Erkennung der Königin. Zusätzlich zum Vorkommen einiger Erkennungspheromone wird die Komplexität des Königinpheromonsystems weitgehend durch die Tatsache belegt, dass Arbeiterinnen unterschiedlich auf die leicht flüchtigen Pheromone reagieren, sie können die Teile des Nests erkennen, in welchen sich die Königin aufhält oder wo nicht und sie können zwischen einer toten oder lebenden Königin unterscheiden. Die exakte Funktion der multiplen Pheromone und wie sie interagieren muß noch untersucht werden. Allerdings könnte dies helfen zu verstehen, warum "Königinsignale" bei einer "primitiven" Ameise wie *M. gulosa*, mit einer wenig spezialisierten Königin, anscheinend komplexer sind, als in höheren eusozialen Arten.

Primer-Pheromone wirken sich auf die Physiologie der Arbeiterinnen aus und haben einen Langzeiteffekt. Ob Arbeiterinnen von *M. gulosa* reproduzieren oder nicht, hängt von der Erkennung eines Königinpheromons dieser Art ab. Nur nach direktem physischen Kontakt mit ihrer Königin nehmen die Arbeiterinnen dieses Pheromon wahr. Tatsächlich findet keine Übertragung des Pheromons von Arbeiterin zu Arbeiterin statt. Die Bewegungen der Königin im Nest tragen aber nicht signifikant zur Verbreitung des Pheromons bei. Zudem erhöhen ihre Rundgänge nicht die Anzahl an Begegnungen mit Arbeiterinnen und das Pheromon, welches sie auf dem Boden hinterläßt, ist nicht notwendig um die Fortpflanzung der Arbeiterinnen zu steuern. Die Arbeiterinnen begegnen der Königin während ihrer Aktivitäten im Nest oder suchen

nach ihr. Die daraus resultierende Häufigkeit der Kontakte ist ausreichend für eine optimale Pheromonerkennung durch die Arbeiterinnen und für die Aufrechterhaltung ihres nicht-reproduktiven Status. Daher paßt die Koloniegröße von *M. gulosa* zu dem einfachen System der Pheromonwahrnehmung basierend auf direktem physischen Kontakt zur Königin.

Wenn physischer Kontakt zur Königin unterbunden wird, beginnen einige Arbeiterinnen mit der Reproduktion werden dann aber von Nestgenossen durch “Policing” davon abgehalten. Einige dieser imobilisierten Individuen überleben die Behandlung nicht, wohingegen andere mit der Produktion von Eiern fortfahren. Obwohl das experimentelle Auslösen von Arbeiterinnenreproduktion in Anwesenheit der Königin zeigt, dass einige Arbeiterinnen dem “Policing” entgehen, so scheint es doch effektiv genug auf Selbstbeherrschung (“self-restraint”) zu selektieren. Selbstbeherrschung könnte den “proximaten” Mechanismus darstellen, der für die Sterilität der Arbeiterinnen verantwortlich ist. Dies könnte auch erklären, warum “Policing” in nicht-manipulierten Kolonien offensichtlich nur selten vorkommt.

Kutikuläre Kohlenwasserstoffe (KKW´s) repräsentieren die größte Klasse epikutikulärer Lipide auf der Oberfläche von Insekten. KKW’s beeinflussen die Kommunikation bei verschiedenen solitär lebenden, sowie sozialen Insekten. Ihre geringe Flüchtigkeit, ihre Verteilung über den ganzen Körper und die Existenz von königin- und arbeiterspezifischen KKW-Profilen deuten auf ihre Funktion als Königinpheromon hin. Um die Hypothese zu unterstützen: Hexanextrakte von *M. gulosa* Königinnen, aber nicht von Arbeiterinnen, rufen ein starkes Interesse bei nesteigenen Arbeiterinnen hervor. Allerdings lockt eine synthetische Mischung aus 15 kutikulären Hauptkomponenten der Königin keinerlei Arbeiterinnen an oder inhibierte deren Fortpflanzung. Das zeigt, dass die semiochemische Aktivität auf eine Mischung verschiedener Kohlenwasserstoffe (KW´s) zurückzuführen ist oder in einer anderen Klasse von Chemikalien liegt. Um die Bedeutung der Komponenten zu unterstreichen, wurden KW-Fraktionen gegen Nicht-KW-Fraktionen in Biotests untersucht. Arbeiterinnen sind in der Lage, Individuen mit KW-Extrakten von eierlegenden Arbeiterinnen zu erkennen. Jedoch gelingt ihnen dies nicht bei Nicht-KW-Extrakten. Obwohl bei einigen Ameisenarten ein Einfluß der KW´s bzgl. der Verwandtenerkennung nachgewiesen wurde, konnten bisher nur Korrelationen mit dem reproduktiven Status aufgezeigt werden. Diese Studie demonstriert zum ersten Mal, dass sie tatsächlich die Basis zur Erkennung des reproduktiven Status bilden. Das unterstützt auch die Idee, dass sie als Grundlage für die Erkennung der Königin durch die Arbeiterinnen dienen. Die Kohlenwasserstoffprofile von Arbeiterinnen und Königin gleichen sich mit Erwerb des reproduktiven Status aneinander an. Sie könnten somit ein ehrliches Erkennungsmerkmal für Fruchtbarkeit darstellen. Diese Merkmale könnten als ehrliches Signal der Anwesenheit

reproduktiver Individuen in der Kolonie benutzt werden und die Basis der Regulation der reproduktiven Arbeitsteilung darstellen.

Bei *M. gulosa* konnte ein Modell für die Wirkungsweise der Königinpheromone, welche die Fortpflanzung der Arbeiterinnen regulieren, vorgestellt werden. Die Verbesserung unseres Wissens bzgl. des Pheromonsystems der Königin könnte helfen zu entscheiden, ob Kontrol oder Signal Mechanismen die Basis der reproduktiven Arbeitsteilung bei sozialen Hymenoptera bilden.

VII.C. Résumé

La division du travail reproductif représente un sujet d'intérêt en biologie de l'évolution au moins depuis Darwin. Le problème de la sélection d'individus altruistes malgré une fertilité réduite a trouvé une explication dans la théorie de la sélection de parentèle: les ouvrières peuvent compenser le coût de leur altruisme et de leur stérilité en élevant des individus qui leurs sont suffisamment apparentés. Toutefois, chez les hyménoptères, les mécanismes "proximate" de régulation de la division du travail reproductif dans les colonies sont méconnus.

Nos connaissances sont basées sur plusieurs fourmis "primitives" et deux espèces d'hyménoptères hautement évoluées. Chez les premières, les hiérarchies de dominance qui permettent l'établissement des pondeuses sont bien comprises. Au contraire, les signaux chimiques qui aident au maintient de leur statut reproducteur ne sont pas élucidés. De même, chez les fourmis "évoluées", les mécanismes de régulation de la reproduction des ouvrières basés sur des phéromones restent inconnus. Le nombre de composés potentiellement actifs et le délai de réaction physiologique des ouvrières rendent l'établissement de tests biologiques compliqués et expliquent notre manque de connaissances sur ces phéromones.

Le but de cette étude est de déterminer les modalités de production, de distribution et d'action, ainsi que l'identité des phéromones royales qui affectent la reproduction des ouvrières chez la fourmi *Myrmecia gulosa*. L'espèce appartient à la sous-famille peu étudiée des Myrmeciinae, qui est endémique à l'Australie. La sous-famille représente, avec les Ponerinae, les fourmis les plus "primitives": leur morphologie est proche de celle de l'ancêtre présumé des fourmis, et la spécialisation des reines est plus faible que chez les fourmis "évoluées". On peut donc s'attendre à trouver des mécanismes de régulation simples qui faciliteraient leur étude.

La première étape de cette étude consiste à caractériser la spécialisation morphologique des reines et des ouvrières, et à déterminer les différences de potentiel reproductif qui y sont associées. La taille des ouvrières varie dans une large fourchette (14-23mm), et montre une distribution bimodale. La croissance est monophasique et seulement légèrement allométrique. Comme chez la plupart des fourmis, les ouvrières ne se reproduisent pas en présence de la reine. Toutefois elles possèdent des ovaires actifs et pondent des œufs trophiques. Comme elles ne pratiquent pas la trophallaxie, les œufs trophiques constituent la principale voie d'échanges alimentaires. Les grandes ouvrières possèdent plus d'ovarioles que les petites et pondent plus d'œufs trophiques. La différence de production d'œufs persiste lorsque les ouvrières commencent à se reproduire, après l'enlèvement ou la mort de la reine. Bien que les petites ouvrières soient moins fertiles, une plus grande proportion d'entre elles se reproduit. Les reines possèdent plus d'ovarioles que les ouvrières et ont un taux de ponte 10 fois supérieures. Bien que

les dimorphismes morphologique et ovarien entre reines et ouvrières soient inférieurs comparés à ceux des fourmis évoluées, ils sont plus importants que chez les ponérines étudiées jusqu'à présent. L'étude de *M. gulosa* contribue donc à notre compréhension du lien entre régulation du travail reproductif et complexité sociale. De plus, elle aide à éclaircir la biologie de la reproduction dans la sous-famille méconnue des Myrmeciinae.

Les reines sont reconnues par leurs ouvrières sur la base d'extraits et de produits cuticulaires et glandulaires. Chacune de ces phéromones de type "releaser" déclenche une réponse comportementale chez les ouvrières (regroupement). Cette réponse est indépendante du comportement de la reine ou de la reconnaissance de sa forme. Il est peu probable que ces phéromones exercent une action à longue portée, et elles ne jouent certainement qu'un rôle dans la reconnaissance de la reine. En plus de l'existence de plusieurs phéromones de reconnaissance, la complexité du système de phéromones royal est démontrée par le fait que les ouvrières réagissent différemment à la phéromone légèrement volatile émise par la reine, qu'elles peuvent identifier les zones du nid où la reine séjourne et celles d'où elle est absente et qu'elles peuvent distinguer une reine morte d'une reine vivante. La fonction exacte des multiples phéromones mises en évidence et la façon dont elles interagissent restent à déterminer. Cela pourra aider à élucider la raison pour laquelle le "signal" royal apparaît aussi complexe chez une fourmi "primitive", avec reine peu spécialisée, que chez des espèces "évoluées".

Les phéromones de type «primer» agissent à long terme sur la phisyologie des individus. La régulation de la reproduction des ouvrières est déterminéé par la perception d'une telle phéromone. L'établissement de contacts directs des ouvrières avec la reine est nécessaire à sa détection. En effet, aucune transmission de cette phéromone par l'intermédiaire d'ouvrières contaminées à d'autres membres de la colonie n'a été mise en évidence. Le mouvement des reines dans leur nids ne contribuent pas de façon significative à la distribution de la phéromone: leurs visites n'augmentent pas considérablement le nombre d'ouvrières qu'elles contactent et les phéromones qu'elles déposent sur le sol ne sont pas nécessaires au maintient de la régulation de la reproduction des ouvrières.Les ouvrières rencontrent la reine durant leurs activités usuelles dans le nid ou bien la cherchent. La fréquence de contact qui en résulte suffit à assurer une détection optimale des phéromones par les ouvrières et à maintenir leur statut non-reproducteur. Ainsi, la taille des colonies de *M. gulosa* est compatible avec un système simple de perception des phéromones royales, basé sur l'établisement de contacts directs avec la reine.

Lorsqu'elles sont empêchées d'établir un contact physique avec les reines, certaines ouvrières commencent à se reproduire et subissent un "policing" par les membres de la colonie.

Certaines des ouvrières ainsi immobilisées ne survivent pas au traitement, alors que d'autres y survivent et continue à produire des ovocytes reproductifs. Bien que des ouvrières échappent à ce "policing" dans des conditions expérimentales, il pourrait être assez efficace pour qu'une auto-inhibition ("self-restraint") des ouvrières évolue. Cette auto-inhibition pourrait constituer le mécanisme "proximate" responsable de la stérilité des ouvrières, et expliquer la rareté de l'expression du "policing" dans les colonies non manipulées.

Les hydrocarbures cuticulaires (HCCs) représentent la classe la plus abondante des lipides présents à la surface de l'épicuticule des insectes. Ils sont impliqués dans la communication chez divers insectes solitaires et sociaux. Leur faible volatilité, leur répartition sur l'ensemble du corps et l'existence de profiles HCCs spécifiques aux reines et aux ouvrières suggèrent que ces HCCs constituent une phéromone royale. Le fait que des extraits à l'hexane d'une reine, mais non ceux d'ouvrières, suscitent un fort intérêt chez les ouvrières supporte cette hypothèse. Toutefois, un mélange composé de 15 hydrocarbures (HCs) royaux synthétiques n'a pas déclenché de comportement de regroupement chez les ouvrières, ni inhibé leur reproduction. Ceci suggère que le mélange actif soit constitué d'une autre combinaison d'HCs ou que l'activité biologique réside dans des composés de classe chimique différente. L'importance des HCs face aux composés lipidiques non-HCs a été confirmée par le test biologique de fractions purifiées de chacune de ces classes de composés chimiques. Les ouvrières discriminent les individus sur lesquels des fractions HCs d'ouvrières pondeuses ont été appliquées. Au contraire, elles ne discriminent pas celles sur lesquelles des fractions non-HCs ont été appliquées. Bien que le rôle des HCCs dans la reconnaissance coloniale ait été démontré chez plusieurs espèces, uniquement des corrélations entre HCCs et activité ovarienne ont été montrées jusqu'à présent. Cette étude démontre pour la première fois que les HCCs sont effectivement à la base de la reconnaissance du statut reproducteur. Ceci supporte l'idée que les HCCs sont aussi responsables de la reconnaissance de la reine par les ouvrières. Comme les profiles HCCs des ouvrières et des reines deviennent similaires avec l'acquisition du statut reproducteur, ils représentent des marqueurs honnêtes de fertilité. Ces marqueurs pourraient être utilisés comme signaux de la présence de reproducteurs dans les colonies et constituer la base de la régulation de la division du travail reproductif.

En conclusion, un modèle pour le mode d'action de la phéromone royale de régulation de la reproduction des ouvrières est proposé pour cette espèce. Faire progresser notre compréhension du système de phéromones royal aidera à établir lequel des mécanismes, de signal ou de contrôle royal, détermine la régulation du travail reproductif chez les hyménoptères sociaux.

Plate 1: *Myrmecia gulosa* workers build large nest mounds. Numerous large workers rush out of the nest entrance upon slight disturbance.

Plate 2: the aggressive workers do not hesitate to assault human intruders. The sting of a single worker is enough to discourage further disturbance and avoid the dozens of other guards.
Plate 3: collection site: sandstone area in Waterfall, near Sydney, New South Wales, Australia.

Plate 4: chambers are tightly packed in the nest mound and directly under it. They contained most of the brood. Talc has been blown in the chambers to help in following the tunnels during nest excavation.

Plate 5: deeper underground, chambers are further away from each other and the long tunnels connecting them spread out in several directions. The picture shows the surface occupied by the nest, after excavation. The scale is given by the equipment on the right side.

Plate 6: dissection presenting typical ovaries of a) a queen, b) a large worker and c) a small worker. Queens have 22±3 ovarioles per ovary, large and small workers have 7±2 and 4±1 ovarioles per ovary respectively.

Plate 7: in presence of queens, workers only produce trophic eggs. This egg was initially destined to a larva, but is intercepted by a worker.

Plate 8: thoraces of a) a gamergate and b) a queen of *M. pyriformis*.

Plate 9: ergatandromorph of *M. gulosa*. This individual was male on the left side and female on the right. Notice the mandible, wing bud and color pattern of the gaster that are typical of males. Ergatandromorphs were often attacked by their nestmates (bottom right).
Plate 10: sting apparatus with associated glands and genital apparatus of an ergatandromorph. The individual‘s internal morphology was more female like: Dufour and poison gland were functional and two ovaries, a sting, as well as a spermatheca were present. Only sclerites of the sting apparatus were male.
Plate 11: a male attempting to mate with a worker in an orphan group of *M. gulosa*. Males were often attacked and found dead a few days later. No successful mating was observed.
Plate 12: a worker (left) showing the typical stretching posture at the approach of the queen (right). This behaviour is induced before actual physical contact takes place.

Plate 13: retinue of small workers around a queen of *M. gulosa*Plate 14: workers immobilising a nestmate that was prevented from establishing physical contact with the queen for several days. Workers that develop their ovaries in the queen‘s presence are thus policed.

IX. References

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Dietemann V. and C. Peeters, 2000. Queen influence on the shift from trophic to reproductive eggs laid by workers of *Pachycondyla apicalis.* Ins. Soc. 47:223-228

Dietemann V., C. Peeters and B. Hölldobler (im Druck). Caste specialisation and differentiation in reproductive potential in the phylogenetically primitive ant *Myrmecia gulosa.*

R. Karthik, C. Peeters, S.P. Yuvana, T. Varghese, H.D. Pradeep, **V. Dietemann**, K. Vedham, M. Cobb and R. Gadagkar (submitted). Cues for mutilation reside in the victim, in the ponerine ant *Diacamma.*

Dietemann V. and D. Fresneau (submitted). Reproductive biology in queenright and queenless colonies of the ponerine ant *Pachycondyla apicalis*.

Vorträge:

Dietemann V., C. Peeters and B. Hölldobler, 2001. Worker policing in the ant *Myrmecia gulosa*. Gehalten in Aarhus, Denmark, beim 8. Kongress der European Society for Evolutionary Biology.

Dietemann V., C. Peeters and B. Hölldobler, 1999. Queen signalling in the ant *Myrmecia gulosa.* Gehalten in Losehill Hall, UK, whärend des EU TMR workshop "Social insects as model system".

Dietemann V., 1998/2001. Vorträge gehalten zwei mal im Jahr im Lehrstuhl "Ecologie", Universität Pierre et Marie Curie, Paris, France.

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Dietemann V. and C. Peeters, 1995. Hiérarchie de dominance et régulation de la production de mâles par les ouvrières de la fourmi *Pachycondyla apicalis*. Gehalten in Brussels, Belgium, bei der Tagung der franzosischsprachige Sektion der Union zum Studium sozialler Insekten (IUSSI).

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Dietemann V., C. Peeters and B. Hölldobler, 2001. Changes in cuticular hydrocarbons are linked with immobilisation of orphaned workers in the ant *Myrmecia gulosa.* Vorgestellt in Berlin, bei der Europaische Kongress der IUSSI.**Dietemann V.**, C. Peeters and B. Hölldobler, 2000. Queen pheromone and communication with workers in the phylogenetically primitive ant *Myrmecia gulosa*. Vorgestellt in Florenz, Italien, whärend des EU TMR workhop "Kinship, communication and disease in the evolution of insect societies".

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Erklärung

Hiermit versichere ich ehrenwörtlich, dass ich die vorliegende Dissertation in allen Teilen selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Darüber hinaus erkläre ich, dass die vorliegende Dissertationsschrift weder vollständig noch in Teilen in einem anderen Prüfungsverfahren vorgelegen hat und dass ich weder bereits akademische Grade erworben noch zu erwerben versucht habe.

Würzburg, Mai 2002,

Vincent Dietemann