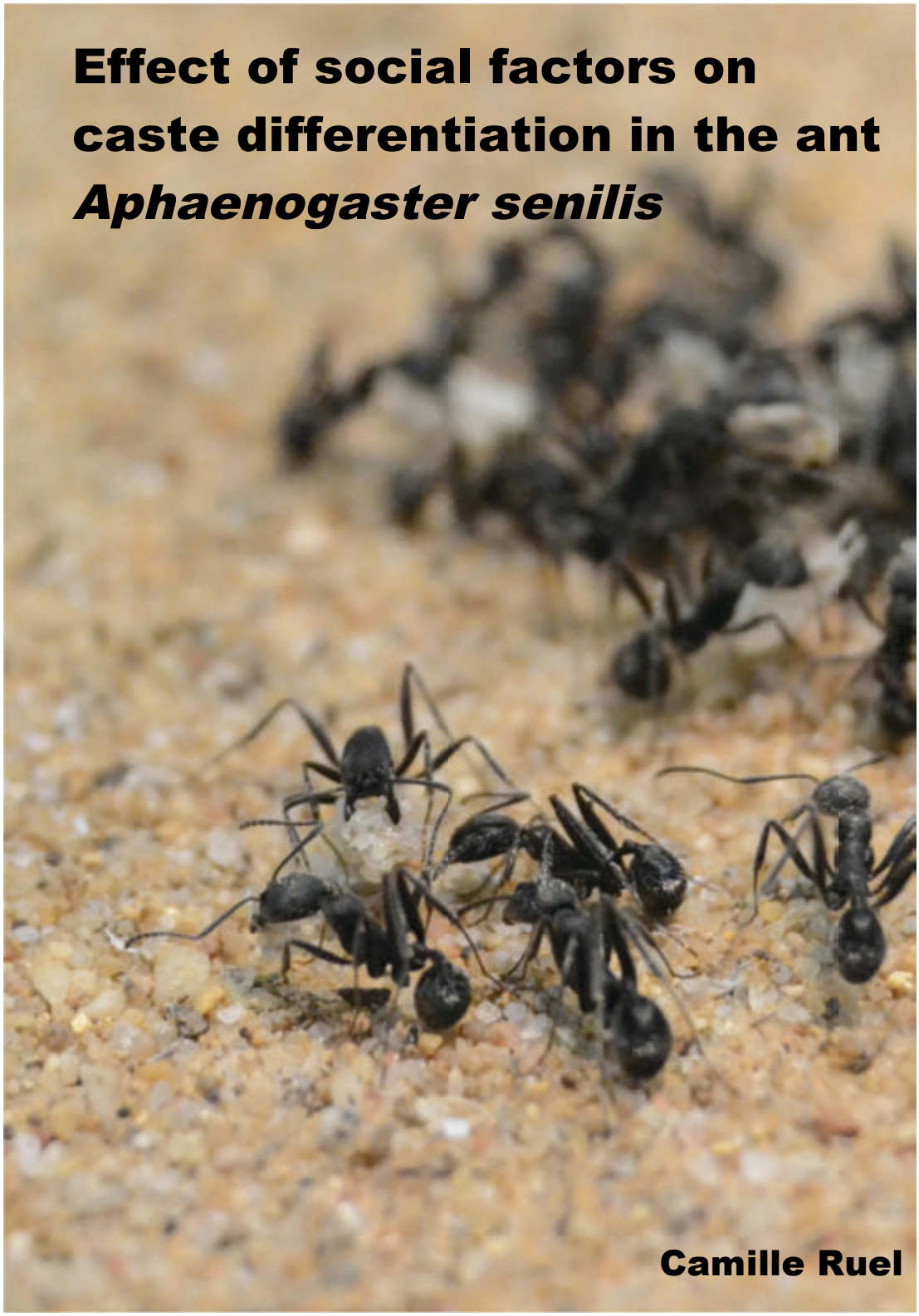
Effect of social factors on caste differentiation in the ant *Aphaenogaster senilis*



Camille Ruel

EFFECT OF SOCIAL FACTORS ON CASTE DIFFERENTIATION IN THE ANT *APHAENOGASTER SENILIS*

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À ma mère, à mon père,

et à notre histoire.

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Évidemment, un grand merci à mes parents, sans qui rien de tout ça n'aurait été possible.

Abstract

In social insects, only one or a few individuals of a colony mate and reproduce. The production of reproductive queens and non-reproductive workers arises from a developmental switch at the larval stage, and is generally regulated by environmental factors. Workers are expected to influence the production of both castes through the control of larval development. Kin selection theory predicts that the current queen, the workers and the larvae have increasing interest in the production of new queens, suggesting potential intracolonial conflicts. These conflicts are likely resolved by signalling the presence of a mated queen. Pheromones are generally involved in conflict resolution, but their chemical nature and effects are largely elusive.

The present thesis aims at characterizing the social factors regulating caste differentiation in ant colonies. We investigated behavioural, physiological and pheromonal mechanisms that influence the production of queens. The monogynous ant *Aphaenogaster senilis* provides a useful model. The queen prevents the production of new queens by means of pheromonal communication. However, if she disappears, a few replacement queens are reared from the totipotent diploid larvae.

Overall, our results confirmed that the workers and the queen constrain colonial reproductive decisions. In chapter two, we showed that the production of queens correlated with the number of workers. Larval fate depended on the realization of food processing tasks by the workers at the group level. The third and fourth chapters confirmed that the mated queen fully inhibits the development of larvae toward queens. She signals her caste and mating status. Chapters three, four and five pointed at cuticular dimethylalkanes, and especially two queen-specific dimethylalkanes, as good candidates for the queen signal. These results contribute to substantial amount of works showing the important role of cuticular hydrocarbons in queen-worker communication. The third chapter asked for the transmission of the queen pheromones. We showed that queen-laid eggs do not transmit queen primer pheromones. Chapter four investigated the role of the queen's behaviour to maintain her reproductive monopoly. We conclude that collective decision-making over the production of sexuals is mainly shaped by the presence and reproductive state of the queen.

Resumen

En los insectos sociales, solo uno o pocos individuos de una colonia se aparean y se reproducen. La producción de las reinas reproductoras y de las obreras no-reproductoras resulta de un cambio del desarrollo en el estadio larval que está, generalmente, regulado por factores ambientales. Sería de esperar que las obreras influyeran sobre la producción de ambas casta mediante el control del desarrollo de las larvas. La teoría de la selección de parentesco predice que la reina actual, las obreras y las larvas tienen un creciente interés en la producción de nuevas reinas, sugiriendo potenciales conflictos intracoloniales. Estos conflictos son resueltos, probablemente, señalizando la presencia de una reina apareada. Las feromonas están, por lo general, implicadas en la resolución del conflicto, pero su naturaleza química y sus efectos todavía permanecen difíciles de conocer.

La presente tesis tiene objetivo de caracterizar los factores sociales que regulan la diferenciación de la casta en colonias de hormigas. Para ello, hemos investigado los mecanismos comportamentales, fisiológicos y feromonales que influyen sobre la producción de reinas. La hormiga monogínica *Aphaenogaster senilis* representa un modelo muy útil. En ella, la reina evita la producción de nuevas reinas mediante el uso de feromonas. Sin embargo, si la reina desaparece, algunas reinas sustitutas son producidas, en muy bajo número, a partir de las larvas diploides totipotentes.

En conjunto, han confirmado que las obreras y la reina limitan las decisiones reproductivas de las colonia. En el capítulo dos, mostramos que la producción de reinas está correlacionada con el número de obreras. El destino de las larvas depende de la realización, a nivel de grupo, de las tareas de procesado del alimento realizado por las obreras. El tercer y cuarto capítulo confirman que la reina fecundada inhibe por completo el desarrollo de las larvas en reinas. La reina señala su casta y su estado de apareamiento. Los capítulos tres, cuatro y cinco apuntan a los dimetilalcanos, y especialmente a dos dimetilalcanos específicos de la reina, como buenos candidatos para la señal de reina. Estos resultados son una aportación a la considerable cantidad de trabajos que demuestran el importante papel de los hidrocarburos cuticulares en la comunicación reina-obrera. El tercer capítulo se pregunta por la transmisión de las feromonas reales. Hemos demostrado que los huevos puestos por la reina no transmiten

las feromonas reales. En el capítulo cuatro investigamos el papel del comportamiento de la reina para mantener su monopolio reproductor. Concluimos que la toma de decisiones colectiva sobre la producción de sexuados es conformada, principalmente, por la presencia y el estado reproductor de la reina.

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Avant-propos

Al científico en ciernes, y al resto del mundo. No busque aplicaciones directas a mi trabajo. No salvo el planeta. No me van a agradecer por todo el bien que habré hecho a la sociedad. Pero creo en mi trabajo. Creo que el conocimiento proporciona sabiduría, libertad y humildad. La biología tiene una pesada tarea que es explicar cómo la diversidad permite la supervivencia del mundo vivo. Esta tesis trata de aclarar cómo la biodiversidad se mantiene con procesos sutiles y eficaces. Se trata de entender cómo la comunicación permite la toma de decisiones y más generalmente el funcionamiento de la vida en grupo. Podéis ver allí paralelismos con nuestra propia sociedad (porque a tantos nos gusta compararnos) o simplemente ver allí un pasito más para la ciencia, pero eso es de cada uno.

Y ahora viene el momento de entrar en el mundo subterráneo y fascinante de estos bichos - que por desgracia a nadie le importa un pepino – le presento a: *Aphaenogaster senilis*. Imagínese ser una larvita que acaba de salir de su huevo, que acaba de nacer. ¡Qué momento tan bonito ! Qué agradable es estar rodeada de otro nuevos nacidos y de olores comunes. ¿Y ahora, cómo va usted a comer ? Crecer ? Desplazarse ? Enfrentarse a su futuro ? No se preocupe, allí están una de sus numerosas hermanas, la obrera y su madre, la de todas : la reina…

Camille Ruel, Sevilla, 2012

Chapter 1

General Introduction

1.1. Communication and decision-making in social organisms

Individual decision-making depends on both intrinsic state and environmental cues. They enable animals to respond adequately to a changing environment. In groups' lifestyles, efficient and up-to-date communication among group members is the key to a well functioning group. Detecting social cues is crucial for regulating social behaviour, and reproductive conflicts are shaped by the interaction between breeders and non-breeders. Individual and group decision-making on "when" and "who" should reproduce should highly depend on the presence and the reproductive state of all the individuals in the colony.

1.2. Reproductive division of labour in social insects

Social insects provide a striking example of polyphenism. There is discrete and temporal or permanent differentiation among individuals in morphology (polymorphism), physiology and behaviour (Anderson and McShea 2001). Apart from notable exceptions, these differences do not arise through differences at the genome level, but are determined by environmental factors (Wheeler 1986; Evans and Wheeler 2001).

One of the most stunning characteristics of social insects colonies is caste polyphenism. Hence, workers often refrain from reproducing to increase their fitness indirectly, helping close relatives –queens- to reproduce (Hamilton 1964; see Box 1.1). This division of reproductive labour between the queen and the worker castes is the result of developmental plasticity at the larval stage (Wheeler 1991; Fig. 1.1). Larvae of similar genotype go through developmental switch, which lead to morphologically, physiologically and behaviourally distinct individuals (Bourke 1999a).

Box 1.1: Kin selection to explain the evolution of eusociality in Hymenoptera

Hamilton's rule (Hamilton 1964; Nowak et al. 2010): Cooperation is favoured by natural selection if relatedness is greater than the cost to benefit ratio.

$r > C/B$

r is the genetic similarity between the recipient and the actor. B represents the offspring produced by the recipient. C is measured as the lost of his own offspring production.

Kin selection in social insects (Bourke 2011a; Foster et al. 2006): In Hymenopteran, relatedness between nestmates can be especially high because of the haplo-diploid sex determination system. Female are diploid while male are haploid. As a consequence, two diploid offspring arising from mating between a diploid queen and a haploid male, share relatedness value of 0.75. Two sisters have therefore more inclusive fitness by helping their mother to reproduce than producing their own offspring (a reproductive individual shares half her genes with her own offspring). Besides, they are expected to bias the production of offspring toward females as they are more related to sisters (0.75) than to brothers (0.25).

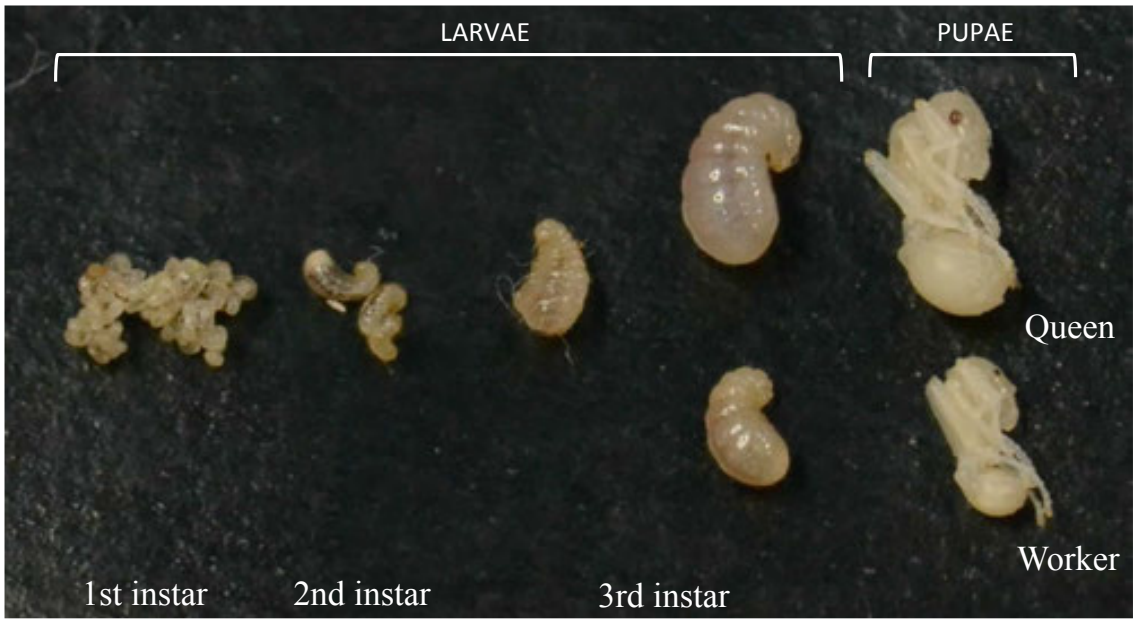


Figure 1.1: Development of *Aphaenogaster senilis* larvae and pupae. In this species, the developmental switch occurs during the second larval instar, but can only be observed at the third instar. (Picture C. Ruel)

1.3. Environmental factors and caste differentiation

Apart from recent studies showing that genetic factors are important in caste determination in some ant species (Volny and Gordon 2002; Julian et al. 2002; Pearcy et al. 2004; Leniaud et al. 2012) and that their role might have been overlooked

(reviewed in Schwander et al. 2009), in most species, environmental factors determine whether an individual become a reproductive queen or a sterile worker. Caste fate depends on abiotic (Wheeler 1986; Cassill and Tschinkel 2000), nutritional (Wheeler 1986) and social factors (Brian 1973; Vargo and Passera 1991). Caste differentiation allows the colony to adjust to external and internal conditions regulating the production of reproductive and non-reproductive individuals.

The role of trophic factors in caste differentiation has been widely demonstrated (Brian 1956; Wheeler 1986; Richards and Packer 1994; Aron et al. 2001; Kaptein et al. 2005; Jarau et al. 2010; Kamakura 2011). The amounts of food (Brian 1956) or specific factors (Kamakura 2011) determine caste differentiation. When nutrition is sufficient, juvenile hormone level rises and coordinates the expression of queen-specific development (Nijhout and Wheeler 1982). Resources are distributed between the brood and skewed resource allocation lead to the production of the reproductive caste in most species. However, social factors are likely the most determinant factors in caste differentiation. Kin selection theory predicts potential intracolonial conflicts over larval fate between all members of the colony, because the queen, the workers, and the larvae have increasing interest in the production of new queens (Box 1.2). Workers control larval development, since they regulate nutritional and abiotic factors to which larvae will be exposed. The queen has a strong impact on the development of larvae, since her presence inhibits the production of new queens in many species (Vargo and Fletcher 1986; Winston et al. 1989; Vargo and Passera 1991; Boulay et al. 2007). The information of the queen presence is thus essential to regulate colony decision to reproduce.

There are few empirical studies on how queen production is controlled. My Ph.D. provides significant results on the factors influencing caste differentiation.

Box 1.2: Kin selection theory predicts intracolonial conflicts over larval fate.

Caste fate conflicts (Bourke and Ratnieks 1999; Wenseleers al. 2003; Ratnieks et al. 2006; Dobata 2012): Kin selection theory predicts that totipotent larvae obtain a greater inclusive fitness by developing as queens rather than as workers, because females are more closely related to their offspring than to those of their sisters. If they control their caste fate, up to 20% of them should develop into queens. However, production of reproductive females should be limited because of a low probability of success for ant queens, a high cost of production (in case of worker-queen dimorphism), and because investing in queens decreases the investment in workers. The switching point at which queens start to be produced should be lower for totipotent larvae than for queens and workers. The queen and workers are expected to limit the investment in queens in order to enhance colony survival.

1.4. Queen pheromones

Although the presence of the queen affects reproductive conflicts, division of labour and social cohesion, queen-workers communication is still not well understood. In most species, the queen informs the colony of her presence through the emission of pheromones with both primer and releaser effects. Releaser pheromones have immediate and short-term effects on the recipient behaviour (Wilson and Bossert 1963; Wilson 1971; Cariou-Etienne et al. 1992; Katzav-Gozansky et al. 2001). Primer pheromones by contrast induce profound physiological modifications, which generally also generate a delayed behavioural response (Le Conte and Hefetz 2008). In my Ph.D., I investigated both primer and releaser effects of queen pheromones in one particular ant species.

In honeybees, the queen pheromones are not a secret anymore. The main component of the honeybee queen mandibular pheromone, 9-oxo-2-decenoic acid, was identified more than 60 years ago (Barbier and Lederer 1960; Butler et al. 1962) and additional components were further discovered progressively (Slessor et al. 1988; Katzav-Gozansky et al. 2001; Wossler 2002; Hoover et al. 2003). The honeybee queen pheromones trigger the workers to form a retinue around her (Katzav-Gozansky et al. 2001), suppress worker ovary development and egg-laying (Hoover et al. 2003), and tend to inhibit the production of sexuals (Winston et al. 1989).

The identity and function of queen pheromones is less well-known in ants than in the honeybee. Yet, most studies point to cuticular hydrocarbons (CHCs; Box 1.3) being the main queen signal (Le Conte and Hefetz 2008). There is a growing evidence that cuticular hydrocarbons correlate with egg laying and serve as fertility signals in ants (Monnin and Peeters 1997; Liebig et al. 2000; Cuvillier-Hot et al. 2001; Hannonen et al. 2002; Heinze et al. 2002; Dietemann et al. 2003; Smith et al. 2008; Holman et al. 2010; Smith et al. 2012b). In the ant *Lasius niger,* queen-specific 3-methyl-hentriacontane was shown to regulate worker sterility (Holman et al. 2010). In *Camponotus floridanus*, the queen's eggs bear a set of hydrocarbons that are specifically found on her cuticle (or are present on workers' cuticle but in much lower proportions). Queen eggs are transported throughout the nest. Workers coming in contact with the queen-laid eggs are informed of the queen's presence and consequently refrain from reproducing (Endler et al. 2004, 2006). Less attention has been drawn on the role of ant

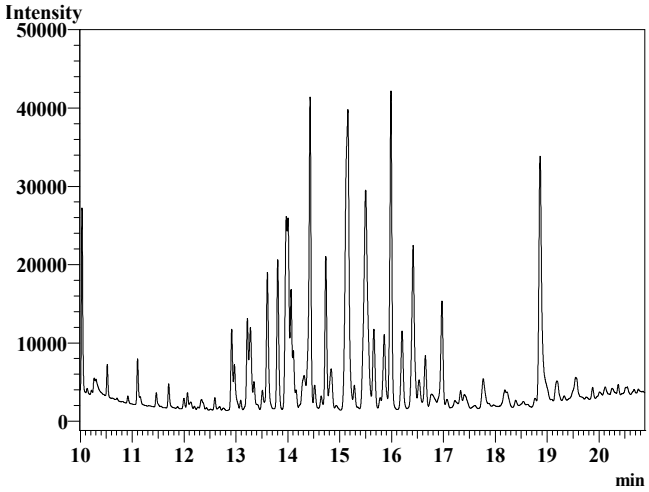
queen glandular secretions, such as the Dufour or the poison gland contents, in queen signalling (Vargo 1997).

Box 1.3: More information about cuticular hydrocarbons in social insects.

What are they? Hydrocarbons are aliphatic chains of carbons and oxygen. Alkanes are the simplest type of hydrocarbons, with single bond between carbons, which are saturated with oxygen. Hydrocarbons can also be methylated, oxygenated or have double bonds between carbons. Usually situated on the epicuticle of the insects, they are perceived after physical contact with the antenna. They are also found in glands, the hemolymph, the stomach, the ovaries, etc.

Properties: Because of a higher melting temperature, linear alkanes are thought to confer less permeability to the cuticle than branched alkanes (Gibbs 2002; Johnson and Gibbs 2004). Linear alkanes should thus be involved in the resistance to desiccation, while branched alkane are likely to be involved in communication functions (Dani et al. 2001; Johnson and Gibbs 2004; Monnin 2006).

Analytical method: Cuticle or glands are rinsed in an organic solvent. The extracts are injected into a gas chromatograph. Each peak from the resulting chromatogram corresponds to different hydrocarbons or mixture of hydrocarbons as well as other compounds (such as ester, alkaloids…) and the area of the peak represents the relative amount of the corresponding compound. Hydrocarbons are identified individually by mass spectrometry analyses.



Chromatogram of a worker's thorax extracted in dichloromethane. To each compound (or mixture of compounds) corresponds a peak with a specific retention time (x axis) and a relative quantity (y axis).

Role of cuticular hydrocabons in social insects: Cuticular hydrocarbons are primarily involved in the resistance to desiccation (Gibbs 2002), but they are preponderant in chemical communication. Composition of hydrocarbons on the cuticle is variable (they vary qualitatively and quantitatively depending on the age, diet, season, caste, colony… Provost et al. 1993; Ichinose et al. 2009c) and the response they trigger is context-depend (Sturgis and Gordon 2012). In many species, cuticular hydrocarbons are involved in colony recognition (D'Ettorre and Lenoir 2010). There are growing evidences that cuticular hydrocarbons advertise of an individual reproductive activity (Monnin 2006).

For the last decades, the studies of queen pheromones were punctuated with strong debates over the manipulative nature -whereby workers are coerced by the queens to act against their inclusive fitness- or honest nature -reliable signals of reproductive capacity- of the queen signal (Keller and Nonacs 1993). Whether queen pheromones are manipulative or reliable signals is hard to test since they lead to a similar outcome. These debates led most authors to relate the emission of queen pheromones to the fertility of the queen. In this Ph.D. thesis, I will separate the signals correlating with caste and mating status and/or egg laying of the queen.

Most studies focused on the effect of queen primer pheromones on worker reproduction (Endler et al. 2004; Dietemann et al. 2005; Cournault 2009; Holman et al. 2010; Brunner et al. 2011), results are lacking concerning the effect of queen pheromones on differentiation of the brood (Vargo and Passera 1991). This is likely due to the lack of adequate model species. I worked on *Aphaenogaster senilis* (Fig. 1.2), a species providing the opportunity to perform simple biological tests on the production of queens. My work contributes to understanding how the presence of the queen affects the development of larvae.

1.5. Model species

Aphaenogaster senilis (Fig. 1.2) is a monogynous and monoandrous ant from the Western Mediterranean Basin. Its density is high in the biological reserve of Doñana National Park, the access of which is restricted to the public. Colonies consist of around 1300 workers and are monodomous (Boulay et al. 2007). Nests are complex structures of underground chambers and tunnels. Although the brood and workers occupy all chambers, the single queen (Fig. 1.2; Fig. 1.3) is usually found in the deepest one. Females have short wings and cannot fly, while males disperse by flying over relatively long distances. New colonies are founded through colony fission (dependent colony foundation). Although in this species, colonies perform many movements during colony life, either through colony fission or emigration (Avargues-Weber and Monnin 2009; Boulay et al. 2010), this does not result in effective dispersion (Galarza et al. 2012). Colony fission concerns many ant species but there are very few in which information is available (Briese 1983; Leal and Oliveira 1995; Fernández Escudero et al. 2001; Amor

et al. 2011; Chéron et al. 2011). Colony fission in *A. senilis* seems to depend on both internal and external factors: at least colony size and intraspecific competition (Boulay et al. 2007; Boulay et al. 2010).

Figure 1.2: Picture of *A. senilis* workers, a pile of first instar larvae, pupae and the mother queen. (Picture C. Ruel)

Both field and laboratory data indicate that in this species, queen pheromones inhibit the development of diploid larvae into new queens (Boulay et al. 2007). Cuticular hydrocarbons, Dufour and poison gland contents have been identified in previous works (Lenoir at al. 2001a; Boulay et al. 2007; Lenoir et al. 2011), but the chemical nature of the queen pheromone remains elusive. If the current queen dies, emergency replacement queens are rapidly reared from the young totipotent diploid larvae, which would otherwise become workers (Ledoux and Dargagnon 1973; Chéron et al. 2009; Fig. 1.1). Brood is totipotent until the second larval instar (Boulay et al. 2009).

Figure 1.3: Picture of three *A. senilis* workers and their mother queen (in the centre). One of her daughter queen (on the right) and her nestmates were added to the colony for aggression tests (see Chapter 4). (Picture C. Ruel)

Because of strict monogyny, the production of only one or a few new queens is sufficient to guarantee colony continuity. Moreover, any excess in the production of new queens inevitably reduces the production of workers necessary for colony maintenance. This is particularly true in fission-performing species like *A. senilis* whereby each new queen leaves her nest with workers protecting her, and only a few queens are needed to guarantee the colony reproductive success. In the field, approximatively 7 out of 10.000 pupae are determined as queens (Boulay et al. 2007). Laboratory experiments have shown that when multiple virgin queens are temporarily present in the nest, they interact aggressively (Cronin and Monnin 2009). The firstborn queen is usually dominant and eventually inherits the colony (Chéron et al. 2009).

1.6. Main objectives

The present thesis aims at characterizing the social factors regulating caste differentiation within ant colonies.

The four following chapters investigate behavioural, physiological and pheromonal mechanisms that influence the investment in female sexuals. I will show that both queen and workers constrain colonial reproductive decision. The presence and reproductive state of the queen are determinant in the development of the brood. The results also suggest candidate molecules involved in queen signalling.

Chapter 2: Behaviour-mediated group size effect constrains reproductive decision in a social insect

This chapter investigates the mechanisms by which workers regulate brood development. The number of workers in a queenless colony influences queen production, since lesser queens are produced in smaller groups. Limitation in the realization of foraging tasks, rather than oophagy of worker-laid eggs explains this result.

Queenless situation is usually rare throughout the colony life cycle. Next chapters (Chapter 3, 4 and 5) analyse communication between the queen, the workers and the brood, how pheromones emission varies with queen state, and what are their effects on reproductive decisions.

Chapter 3: Surface lipids of queen-laid eggs do not regulate queen production in a fission-performing ant

This chapter addresses the question of signal transmission between colony members. We demonstrate that the eggs laid by the queen are biochemically similar to the queen. However, they are not vehicle of the queen primer pheromone. Comparison of workers' and queens' hydrocarbon profiles reveals the existence of two queen-specific dimethylalkanes.

In queenright colonies, chemical divergence between workers and the queen might be explained by the caste or the mating. The next chapter (Chapter 4) aims to test whether queens signal their caste or mating status to the colony.

Chapter 4: Recognition of caste and mating status maintains monogyny in the ant *Aphaenogaster senilis*

The occurrence and resolution of conflicts between queens over the head of the colony are investigated in the chapter. The queen signals her presence to the colony, which maintains her reproductive monopoly. The study is based on the separation of primer and releaser effects of queen pheromones. First, we demonstrate that the colony detects the presence of a mated queen, and consequently prevents the development of larvae into queens. Second, we found that caste is recognized by workers and queens, which eliminate queen challengers. Finally, this study contributes to our understanding of the role of cuticular hydrocarbons in queen signalling. Dimethylalkanes are singled out as possible caste and mating status signals.

The age of the queens was controlled in this experiment because we hypothesised that queen age influences chemical profile, and the increasing age may trigger the emission of queen primer pheromone in unmated queens. The next chapter focuses on the effects of queen and worker age on pheromones emission.

Chapter 5: Differential ontogeny between castes of cuticular and Dufour gland hydrocarbons in the ant *Aphaenogaster senilis*

In this chapter, we investigated whether virgin queens of different age emit the queen primer pheromones. We first confirmed that virgin queens and workers had different amounts of dimethylalkanes. The two castes had distinct ontogeny, with queens having always greater amount and greater and constant proportion of dimethylalkanes. However, the presence of virgin queens did not inhibit queen production.

(Note: Chapters 2 to 5 are presented in scientific paper format entailing redundancy in the introduction and methods' information of the different chapters.)

1.7. List of abbreviations

AIC: Akaike Information Criterion (measures the relative goodness of fit of a statistical model. It is used in model selection)

CHC: Cuticular HydroCarbon

GLMM: Generalized Linear Mixed Models (a generalisation of linear models containing both fixed and random effects and whose response variable follows other than a normal distribution. The model relates to the response variable with a link function)

HC: HydroCarbon

MQ: Mother Queen (reproductive queen which mothered most of the workers and brood of the colony)

NMQ: Newly Mated Queen (reproductive young queen)

QL: Queenless (absence of the queen in the colony or the group)

QR: Queenright (presence of the queen in the colony or the group)

SE: Standard Error

VQ: Virgin Queen

Chapter 2

Behaviour-mediated group size effect constrains reproductive decision in a social insect

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Behaviour-mediated group size effect constrains reproductive decisions in a social insect

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The formation of cooperative entities between lower-level units is characterized by increasing complexity. Apart from the degree of cooperation, social complexity is determined by the number of cooperative individuals. Previous studies have considered the relationships between group size and traits affecting inclusive fitness, such as reproductive efficiency. In social insects, little is known about the conversion of resources into offspring relative to colony size. In the present study, we addressed the importance of worker numbers for the production of queens and workers, and investigated the mechanisms that could affect larval development in the ant *Aphaenogaster senilis*. In this species, if the current queen dies, replacement queens are reared from the totipotent diploid larvae. We found that the number of workers constrained reproductive decisions, since the production of queens was lower in small than in large groups. The number of larvae also limited the success of queen replacement when associated with a small group size. Rearing queens requires an overhead that small worker groups cannot afford. These effects derived from a limitation in the realization of tasks at the group level. The investment in foraging rather than nursing behaviour predicted the production of queens. We tested whether egg-laying workers in queenless nests increased queen production. Although oophagy was likely to occur, the eggs did not affect larval development. Our results suggest that the larval fate does not depend on direct interactions between larvae and workers, but rather relies on collective cooperative performance at the colony level.

Over the last 20 years, a growing field of research has emphasized the importance of cooperation as a pervasive evolutionary force explaining the major transitions of life from genome to animal societies (Maynard Smith & Szathmary 1995; Szathmary & Smith 1995; Queller 2000; Bourke 2011). Principles of social evolution are therefore useful to explain how and why the association of cooperating units leads to the formation of complex entities with enhanced morphological and behavioural specializations. Theoretical models have demonstrated that, apart from the degree of cooperation, the number of cooperative units determines group complexity (Bourke 1999; Kokko et al. 2001; Lehmann & Rousset 2010). Empirical studies have also shown that larger groups have a higher survival rate and/or per capita reproductive success in several cooperatively breeding vertebrates (e.g. Malcolm & Marten 1982; Balshine et al. 2001; Clutton-Brock et al. 2001; Woxvold & Magrath 2005; Gusset & Macdonald 2010). However, it is in insect societies, which function as integrated biological entities, that group size effects are expected to be the most determinant for an individual's inclusive fitness (Wilson 1971; Michener 1974; Bourke 2011). Yet, the mechanisms underlying group size effects in ants, bees, termites and wasps remain elusive.

Insects' colony size varies greatly between species from a couple (e.g. *Thaumatomyrmex*, Jahyny et al. 2002) to several millions of individuals (e.g. *Dorylus* or *Formica* unicolonial species, Hölldobler & Wilson 1990; Dornhaus et al. 2012). Within a species, colony size may also vary by several orders of magnitude depending on colony age, queen number, food availability, etc. Typically, the number of workers increases logistically from colony foundation by the queen to colony maturity, when sexuals (males and virgin queens) are produced (Oster & Wilson 1979; Tschinkel 1988). Unpredicted events, such as predation, immune challenges or extreme weather conditions, are factors that may suddenly reduce the number of workers in the colony. Moreover, in species that disperse by colony fission, during the founding stage the queens are accompanied by workers, which by leaving the mother colony, greatly secure daughter colony foundation. However, mother colonies lose an important fraction of their worker population during each reproductive event (Seeley 2010; Chéron et al. 2011).

One consequence of colony size reduction is a severe constraining of collective decision making and task realization. For

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Abstract

The formation of cooperative entities between lower-level units is characterized by increasing complexity. Apart from the degree of cooperation, social complexity is determined by the number of cooperative individuals. Previous studies have considered the relationships between group size and traits affecting inclusive fitness, such as reproductive efficiency. In social insects, little is known about the conversion of resources into offspring relative to colony size. In the present study, we addressed the importance of worker numbers for the production of queens and workers, and investigated the mechanisms that could affect larval development in the ant *Aphaenogaster senilis*. In this species, if the current queen dies, replacement queens are reared from the totipotent diploid larvae. We found that the number of workers constrained reproductive decisions, since the production of queens was lower in small than in large groups. The number of larvae also limited the success of queen replacement when associated with a small group size. Rearing queens requires an overhead that small worker groups cannot afford. These effects derived from a limitation in the realization of tasks at the group level. The investment in foraging rather than nursing behaviour predicted the production of queens. We tested whether egg-laying workers in queenless nests increased queen production. Although oophagy was likely to occur, the eggs did not affect larval development. Our results suggest that the larval fate does not depend on direct interactions between larvae and workers, but rather relies on collective cooperative performance at the colony level.

Keywords: ant, *Aphaenogaster senilis,* colony size, decision making, oophagy, queen production, task allocation

Introduction

Over the last 20 years, a growing field of research has emphasized the importance of cooperation as a pervasive evolutionary force explaining the major transitions of life from genome to animal societies (Maynard Smith and Szathmary 1995; Szathmary and Smith 1995; Queller 2000; Bourke 2011b). Principles of social evolution are therefore useful to explain how and why the association of cooperating units leads to the formation of complex entities with enhanced morphological and behavioural specializations. Theoretical models have demonstrated that, apart from the degree of cooperation, the number of cooperative units determines group complexity (Bourke 1999a; Kokko et al. 2001; Lehmann and Rousset 2010). Empirical studies have also shown that larger groups have a higher survival rate and/or per capita reproductive success in several cooperatively breeding vertebrates (e.g. Malcolm and Marten 1982; Balshine et al. 2001; Clutton-Brock et al. 2001; Woxvold and Magrath 2005; Gusset and Macdonald 2010). However, it is in insect societies, which function as integrated biological entities, that group size effects are expected to be the most determinant for an individual's inclusive fitness (Wilson 1971; Michener 1974; Bourke 2011b). Yet, the mechanisms underlying group size effects in ants, bees, termites and wasps remain elusive.

Insects' colony size varies greatly between species from a couple (e.g. *Thaumatomyrmex,* Jahyny et al. 2002) to several millions of individuals (e.g. *Dorylus* or *Formica* unicolonial species, Hölldobler and Wilson 1990; Dornhaus et al. 2012). Within a species, colony size may also vary by several orders of magnitude depending on colony age, queen number, food availability, etc. Typically, the number of workers increases logistically from colony foundation by the queen to colony maturity, when sexuals (males and virgin queens) are produced (Oster and Wilson 1979; Tschinkel 1988). Unpredicted events, such as predation, immune challenges or extreme weather conditions, are factors that may suddenly reduce the number of workers in the colony. Moreover, in species that disperse by colony fission, during the founding stage the queens are accompanied by workers, which by leaving the mother colony, greatly secure daughter colony foundation. However, mother colonies lose an important fraction of their worker population during each reproductive event (Seeley 2010; Chéron et al. 2011).

One consequence of colony size reduction is a severe constraining of collective decision making and task realization. For instance, in the ant *Temnothorax albipennis*, the accuracy of new nest searching and colony relocation was shown to depend on the number of workers (Dornhaus and Franks 2006; Franks et al. 2006). The importance of colony size for the formation of self-assemblage (Anderson et al. 2002) is also well exemplified in *Oecophylla smaragdina,* a species in which the construction of chains of workers to bridge gaps requires a critical number of participants (Lioni and Deneubourg 2004). Larger colonies were shown to create more complex collective foraging groups (Beckers et al. 1989), to forage over longer distances, to spend more time foraging, or to allow more foragers to visit each food source than small colonies (Beekman et al. 2004; Thomas and Framenau 2005). Within the nest, colony size enhances division of labour and task specialization in *Rhytidoponera metallica* and *Pogonomyrmex californicus* (Thomas and Elgar 2003; Holbrook et al. 2011) but not in *T. albipennis* or *Cataglyphis cursor* (Retana and Cerdá 1990; Dornhaus et al. 2009). However, how group size translates into offspring production is not well understood. Worker number has been shown to correlate positively with the production of sexuals in some ant species (Elmes and Wardlaw 1982; Savolainen and Deslippe 1996; Cole and Wiernasz 2000; Sorvari and Hakkarainen 2007; Shik 2008), but not in others (MacKay 1981). Similarly, large colony size may increase (Jeanne and Nordheim 1996), decrease (Michener 1964) or have no effect (Bouwma et al. 2006) on per capita productivity.

In the present study, we addressed the importance of worker number for the production of virgin queens in an ant that disperses by colony fission. In many species of ants, young queen-derived diploid larvae are more or less totipotent and can develop into either workers or queens depending on environmental conditions (Wheeler 1986; but see Julian et al. 2002). Workers were hypothesized to exercise great control over caste production through the amount of food they provide to larvae (Brian 1956). In stingless bees, larvae development into queens is triggered by the release of geraniol in their food (Jarau et al. 2010). In the honeybee, *Apis mellifera*, queen prospective larvae receive royalactin, a specific royal jelly protein (Kamakura 2011). In the ant *Pogonomyrmex badius* and in several *Aphaenogaster* species queens have been shown to feed on a more protein-rich diet than workers, although this may not be the determinant for caste fate (Smith and Suarez 2010; S. Caut, M. Jowers, C. Ruel, X. Cerdá and R. Boulay, unpublished).

Workers' decision to rear workers or queens also seems to be related to their perception of pheromones emitted by the current fertile queen. This is particularly striking in our model organism, *Aphaenogaster senilis,* a common gipsy ant in the Western Mediterranean Basin. In this species, both field and laboratory data indicate that queen pheromones inhibit the development of diploid larvae into new queens (Boulay et al. 2007). However, if the current queen dies, emergency replacement queens are rapidly reared from the young totipotent diploid larvae, which would otherwise become workers (Ledoux and Dargagnon 1973; Chéron et al. 2009). In nature, such replacement queens would mate at the entrance of their nest and rapidly start laying eggs to allow the colony to proceed. Since colonies of this species are headed by a single queen (strict monogyny), the production of only one or a few new queens is sufficient to guarantee colony continuity. Moreover, any excess in the production of new queens inevitably reduces the production of workers necessary for colony maintenance. The exact mechanism allowing the colony to adjust caste production precisely to its needs is still unknown.

The number of workers in natural colonies of *A. senilis* recorded in more than 300 colonies ranges between 120 and 3900 individuals (Boulay et al. 2007). Here, we examined the effect of reduced group size on the production of replacement queens versus workers in a queenless (QL) situation. To that end, we tested whether queen rearing covaries with the number of workers, the number of larvae and their ratio in experimentally orphaned groups. If brood care is constrained by a high number of larvae and a low number of workers, we expected that the number of larvae would interact with the number of workers to determine the probability of queen replacement. We tested two nonexclusive hypothetical mechanisms by which worker number could affect larval development: through the care provided by workers and/or through oophagy of worker-laid eggs. First, according to the behavioural mechanism, the larvae would have to receive more care and food in order to develop into queens. Therefore, below a certain threshold number of workers, not enough care or food could be provided to each larva, thus forcing their development into workers. Group size might also constrain the capacity to retrieve food items, which is likely to be determinant for colony food intake and larval development. The faster food is retrieved and processed, the faster it becomes available to larvae. We thus tested whether large groups retrieved large food items more rapidly than smaller groups. Second, there was reason to expect that cannibalism of worker-laid eggs could also affect larval fate, as it would provide

additional nutrition. *Aphaenogaster senilis* workers start laying haploid eggs within days following orphaning (Ichinose and Lenoir 2009a) and before new queens are produced. Preliminary observations indicated that only a small fraction of worker-laid eggs developed into males. It was therefore reasonable to hypothesize that the remaining were cannibalized, possibly by larvae, as observed in other species (Baroni Urbani 1991; Masuko 2003). In a species such as *A. senilis* in which trophallaxis does not exist (Delage and Jaisson 1969; Lenoir et al. 2001b), oophagy could substitute for oral feeding by workers (Crespi 1992). If, as we hypothesized, eating worker-laid eggs triggers larval development into queens, a small worker group may contain fewer laying workers than a larger group and therefore may not produce enough eggs to modify larval development.

Methods

Colony collection and maintenance

Stock colonies of *A. senilis* were collected between July 2008 and July 2011 in the National Park of Doñana (southwestern Spain). In the laboratory, they were housed in 2x20 cm test-tubes, the bottoms of which were filled with water retained by cotton plugs. These tubes were kept in containers measuring 28x18 cm and 11 cm high that served as foraging areas. The inner walls of the containers were lined with Fluon to prevent ants from escaping. Colonies were maintained in total darkness at 28 ± 1 ºC and 50 ± 10% air humidity, and were fed three times a week with mealworms (*Tenebrio molitor*), honey and fruits. All experiments comply with current Spanish legislation.

Experiment 1: Effect of worker and larvae number on queen replacement

A total of 60 QL groups were formed from 13 stock colonies. Groups contained 50, 100 or 200 workers (20 replicates per size category), 50 and 100 being smaller than field colony size. Each group size was tested with every even-numbered increment of larvae from two to 40 (one replicate each: 2, 4, 6… 40). The larvae originated from the respective queenright (QR) stock colony. Of the three identified instar larvae, the first instar has been shown to be totipotent (Boulay et al 2009). Each group was installed in an artificial nest made from a test-tube as described for the stock colonies. Each tube

was connected to a 10x10 cm diameter circular box the inner wall of which was lined with Fluon. All groups received the same nutrition comprising four sliced mealworms, provided every 3 days. Each group was monitored every day until pupation of all surviving brood in order to determine the number of groups producing at least one queen, the total number of queens per group and the first occurrence of worker-laid eggs.

All statistical analyses were performed with the R package software version 2.7.2 (R Development Core Team, Vienna, Austria). Two generalized linear mixed models (GLMM; lme4 package, Bolker et al. 2009) were fitted to test the effect of worker numbers (three-level factor), larvae numbers (continuous variable), their interaction and the ratio between worker and larvae numbers (continuous variable) on the production of at least one queen (binomial distribution), on the number of queens produced (Poisson distribution) and the occurrence of worker-laid eggs (binomial distribution). The stock colony was included as a random factor. For each model, the significance of explanatory variables was tested by analysis of variance based on the Akaike information criterion (AIC). The significance of each factor level was assessed by contrast analysis. Finally, linear and logarithmic regression models were fitted to test the relation between the number of larvae and the proportion developing into queen.

Experiment 2: Task allocation and group size

Three colonies were each divided into one group of 50 and one group of 200 workers. The workers from the foraging area and the nest were mixed together and collected randomly. Each group was provided with 10 first- and 10 last-instar larvae. In each group 20 focal individuals were marked with a dot of colour oil-based paint (Mitsubishi Pencil UniPaint) on the head, thorax and gaster. They were housed in transparent plastic CD boxes (12x12 cm and 0.2 cm high) allowing behavioural observations. The CD boxes were kept in larger containers (28x18 cm and 11 cm high) that served as foraging areas. Water was provided by connecting the CD boxes to a small test-tube filled with water retained by a cotton plug.

A total of 5913 behavioural observations were then carried out over 6 days. Focal individuals' behaviour was scanned by the instantaneous sampling method approximately 10 times per day on days 2, 3, 4, 7 (before the worker oviposition period), 14 and 15 (oviposition period). A principal component analysis whose first three components accounted for 90% of the variation in ant activities allowed all acts to

be regrouped into four categories: (1) foraging (exploration outside the nest, prey retrieval), (2) brood care (making contact with a larva, grooming, antennation with brood), (3) inactivity and (4) other (social interactions, nest maintenance and guarding the nest entrance). Three GLMMs were fitted to test the effect of group size and period of observation on the proportion of foraging, brood care and inactivity per individual (all binomial distributions). Stock colony was included as a random factor. To extrapolate the amount of nursing and foraging activities performed not only by marked workers but also by the whole group, we multiplied the total number of acts observed in each group by 2.5 (because 20 marked workers were observed in a 50-worker group) or 10 (because 20 marked workers were observed in a 200-worker group) for 50- and 200-worker groups, respectively.

Since this experiment showed that 200-worker groups had a higher absolute number of foragers (see Results), we also tested whether this translated into faster prey retrieval at the group level. One mealworm was introduced every day for 1 week into the foraging area. After providing the mealworm, we observed each group for 30 min to determine the time taken to retrieve the prey. The effect of the number of workers on the time taken to retrieve the prey was tested by survival analysis, including the colony as a random (frailty) factor.

Experiment 3: Worker ovarian development

Twelve colonies were each divided into two groups of 300 workers. One group (nurses) was composed of workers collected inside the nest and performing internal activities (brood care, inactivity or other). The other group (foragers) was composed of workers collected in the foraging area. Six nurse and six forager groups contained the mother queen while the remaining groups were QL. Approximately 10 workers were collected from each group every 72 h for 21 days. They were dissected and their ovarian development assessed on a binary scale. The ovaries were considered developed when they contained growing oocytes (stage E1, E2, E3, V, R1v, R1 and R2 of Fénéron and Billen 1996) and undeveloped when no developing oocyte was observed (stage J and D).

A GLMM was fitted to test the effect of ant activity, day of collection (continuous variable) and queen presence/absence on the proportion of workers with developed ovaries (binomial distribution). Stock colony was considered a random factor.

Experiment 4: Effect of worker-laid eggs

Worker eggs were laid in 27 QL groups (hereafter Source) of 200 workers from 27 stock colonies. When at least 10 eggs were observed in a group, 35 sets of three QL groups were immediately prepared from the respective stock colonies. Seventeen sets were composed of three groups as follows: one group contained 200 workers ($200_{control}$); the second group contained 50 workers ($50_{control}$); the third group (50_{egg}) also contained 50 workers and received the respective Source group's egg production every day for 21 days. The remaining 18 sets of three QL groups were composed as follows: one group contained 200 workers ($200_{control}$); the second group contained 100 workers ($100_{control}$); the third group (100_{egg}) also contained 100 workers and received the eggs produced by the respective Source group every day for 21 days. All the QL groups within the 35 sets, except the Source group, contained 20 first-instar larvae from the respective QR stock colonies. Eight of 27 stock colonies were large enough to create two sets. All brood reaching the pupa instar were counted and collected three times a week for 2 months. Two GLMMs were fitted to test the effect of group size and egg supplementation on the total number of diploid pupae (Poisson distribution) and on the proportion of queens among them (binomial distribution). The stock colony was included as a random factor.

Results

Experiment 1: Effect of worker and larvae number on queen replacement

One group containing 50 workers and 36 larvae was removed from the analysis because of high worker mortality during the course of the experiment. Group size strongly affected larval caste fate. The probability of producing at least one replacement queen in a QL situation depended on the interaction between the number of workers and the number of larvae provided (Table 2.1, Model 1). Hence, with 50 workers the probability of producing at least one queen increased significantly with the number of larvae (Fig. 2.1). With 100 and 200 workers it was significantly higher and did not depend on the number of larvae. However, there was no effect of the workers-to-larvae ratio on the probability of producing at least one queen (Table 2.1, Model 1).

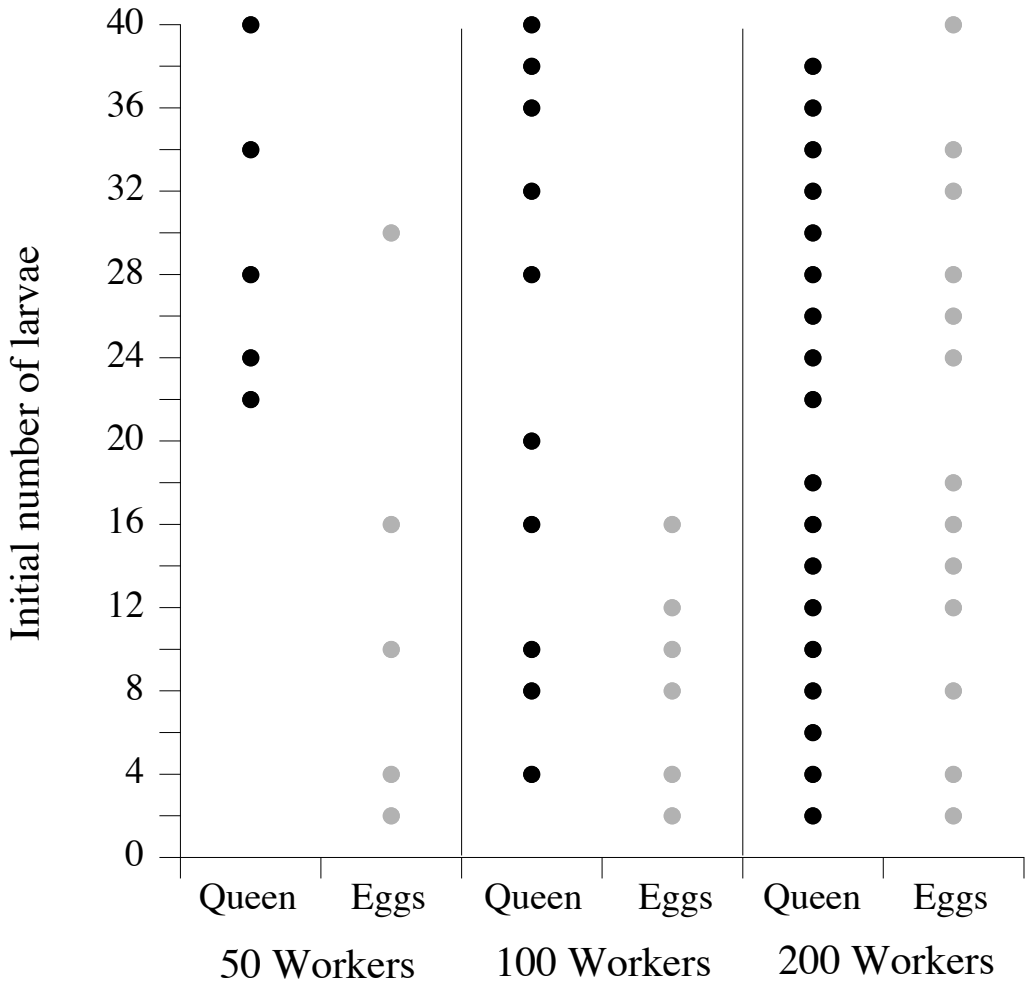

Figure 2.1: Effect of the number of larvae on the production of at least one queen (black circles) and on the occurrence of worker-laid eggs (grey circles) in groups of 50, 100 and 200 workers. One replicate of each even number of larvae from two to 40 was tested for each group size. Circles represent groups in which at least one queen was produced. The 50-worker and 36-larvae group was not included in the analysis because of high mortality.

The number of queens produced per group ranged between zero and six and increased significantly with both the number of larvae and the number of workers but did not depend on their interaction (Table 2.1, Model 2). Groups of 100 workers produced significantly more queens than those composed of 50 workers (mean ± SE: 0.32 ± 0.13 versus 0.95 ± 0.29; $Z = 2.374$, $P = 0.017$) and fewer than those of 200 workers (2.35 ± 0.36; $Z = -3.212$, $P = 0.001$). There was no effect of the workers-to-larvae ratio on the number of queens produced (Table 2.1, Model 2).

In both 50- and 100-worker groups the number of larvae only explained a small amount of variation in the proportion of larvae becoming queens (Fig. 2.2; linear fit: $R^2 = 0.24$, $P = 0.03$, $R^2 = 0.07$, $P = 0.26$, respectively; log fit: $R^2 = 0.19$, $P = 0.06$, $R^2 = 0.07$, $P = 0.263$). However, in the 200-worker groups the proportion of larvae

developing into queens decreased logarithmically with the number of larvae initially provided (log fit: $y = 0.528–0.301 \log(x)$, $R^2 = 0.63$, $P < 0.001$; linear fit: $R^2 = 0.47$, $P < 0.001$).

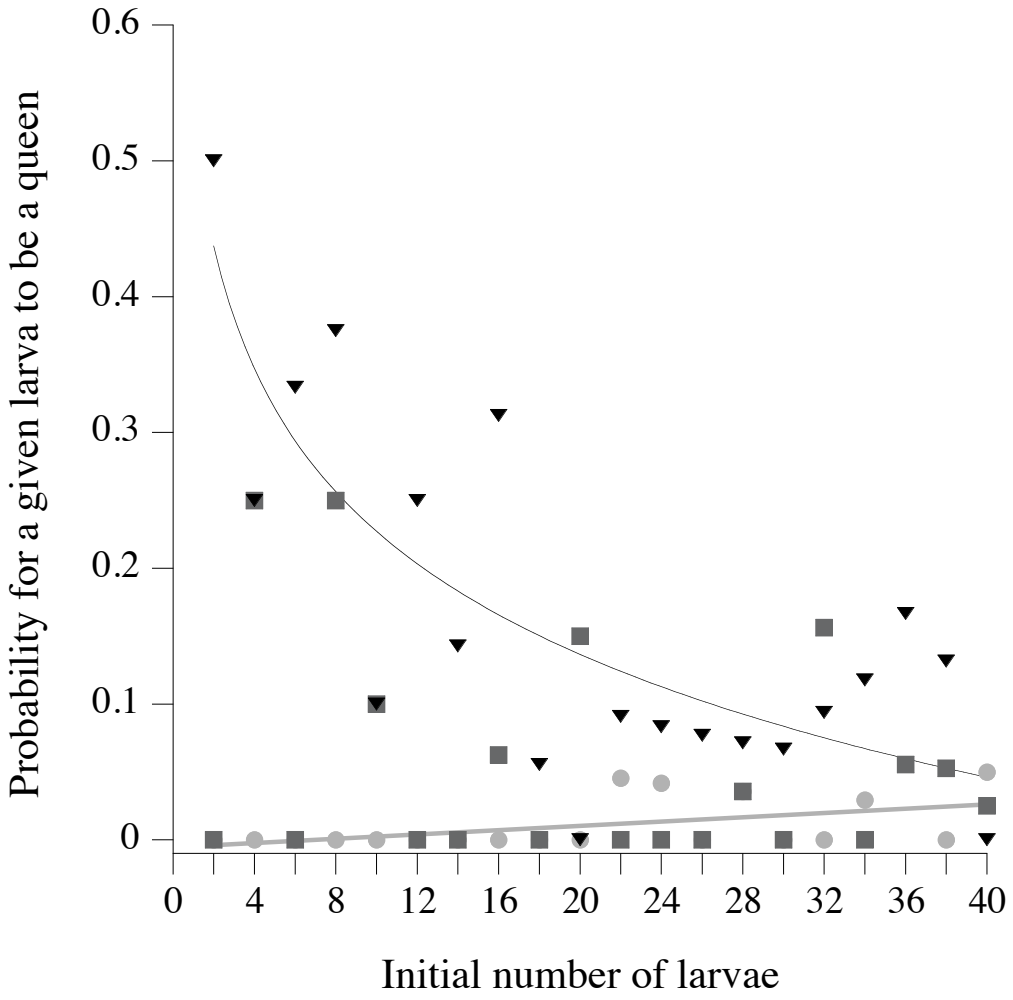

Figure 2.2: Probability to develop as queen as a function of the number of larvae in groups of 50 (grey dot), 100 (grey square) and 200 (black triangle) workers. The best fit for 200 workers (black line) was logarithmic (y = 0.528 - 0.301 log(x), $R^2 = 0.63$, $P < 0.001$). For 50 workers linear functions explained a very small proportion of variance (linear fit: grey line $R^2 = 0.24$, $P = 0.03$; log fit: $R^2 = 0.19$, $P = 0.06$). For 100 workers, the probability to develop as queen does not depend on the number of larvae (linear fit: $R^2 = 0.07$, $P = 0.26$; log fit : $R^2 = 0.07$, $P = 0.263$).

Twenty-four of 59 groups produced eggs. The first piles of worker-laid eggs appeared on average 13.5 ± 0.4 days after orphaning. The presence of eggs depended on the interaction between worker and larval numbers (Table 2.1, Model 3). While 65% of 200-worker groups laid eggs irrespective of the number of larvae, worker-laid eggs were found in only 26% and 30% of the groups of 50 and 100 workers, respectively. In addition, in 50- and 100-worker groups egg piles were more likely to occur when the number of larvae was small (Fig. 2.1). As for queen production, the workers-to-larvae ratio did not affect the presence of worker-laid eggs (Table 2.1, Model 3).

Table 2.1: Results of the model selection (GLMM) testing (1) the effect of the number of workers, the initial number of larvae and their interaction on production of at least one queen (Model 1: binomial distribution), number of queens produced (Model 2: Poisson distribution), and presence of worker-laid eggs (Model 3: binomial distribution); (2) the effect of the number of workers and the period of observation on nursing activity (Model 4: binomial distribution), inactivity (Model 5: binomial distribution) and foraging activity (Model 6: binomial distribution); (3) the effect of the task each individual performed when the experiment began, the day they were collected and the presence/absence of the queen on proportion of workers with developed ovaries (Model 7: binomial distribution); (4) the effect of the number of workers and presence of eggs on number of diploid pupae produced (Model 8: Poisson distribution) and proportion of each caste (Model 9: binomial distribution).

	Models	*df*	AIC	χ^2	$\chi^2 df$	*P*
Model 1	**Production of at least one queen**					
	Workers*Larvae + ratio	8	68.719	0.17	1	0.68
	Workers*Larvae	7	66.89	7.02	2	**0.03**
	Workers + Larvae	5	69.909			
Model 2	**Number of queens produced**					
	Workers*Larvae + ratio	8	76.868	0.58	1	0.446
	Workers*Larvae	7	75.448	3.419	2	0.18
	Workers + Larvae	5	74.867	6.741	1	**0.009**
	Workers	4	79.609			
Model 3	**Presence of worker-laid eggs**					
	Workers*Larvae + ratio	8	70.509	0.639	1	0.424
	Workers*Larvae	7	69.148	6.261	2	**0.044**
	Workers + Larvae	5	71.409			
Model 4	**Nursing**					
	Workers*Time	5	1311.2	1.182	1	0.277
	Workers + Time	4	1310.4	286.17	1	**<0.001**
	Workers	3	1594.6			
Model 5	**Inactivity**					
	Workers*Time	5	536.55	17.629	1	**<0.001**
	Workers + Time	4	552.18			
Model 6	**Foraging**					
	Workers*Time	5	1188.7	40.932	1	**<0.001**
	Workers + Time	4	1227.7			
Model 7	**Workers with developed ovaries**					
	Task+Time+Queen	5	1986.8	0.591	1	0.442
	Task+Time	4	1985.4	2.541	1	0.111
	Task	3	1985.9	118.67	1	**<0.001**
	Intercept	2	2102.6			
Model 8	**Number of diploids**					
	Eggs+Workers	5	299.24	3.184	2	0.204
	Eggs	3	298.42	0.135	1	0.714
	Intercept	2	296.42			
Model 9	**Caste proportion**					
	Workers+Eggs	5	134.71	0.078	1	0.780
	Workers	4	132.79	40.344	2	**<0.001**
	Intercept	2	169.13			

Significant results are shown in bold.

Experiment 2: Task allocation and group size

The number of workers significantly affected individual time allocated to inactivity compared to other tasks. The number of workers outside the nest was similar for the two

group sizes (50 versus 200 workers: 15.3 ± 1.9% versus 14.6 ± 2.2%). In the groups of 50 workers, the observed individuals performed 4.6 times more brood care (20.4 ± 2.8% versus 4.4 ± 1%) and were 1.5 times less inactive (35.3 ± 2.1% versus 53.4 ± 2.3% of the observations) than in the groups of 200 workers. As a result, the activity rate of each focal worker was higher in 50-worker groups.

The likelihood of a focal individual performing brood care activities was significantly higher before oviposition than in the oviposition period, in both 50- and 200-worker groups (Fig. 2.3; Table 2.1, Model 4). In the 50-worker groups, a decrease in brood care was associated with an increase in inactivity in the oviposition period (Table 2.1, Model 5). However, inactivity in the 50-worker groups remained lower compared to the 200-worker groups during the two periods. By contrast, in 200-worker groups, brood care was replaced by increasing foraging activity (Table 2.1, Model 6). Foraging remained stable over the two periods in 50-worker groups. Therefore, 200-worker groups had more foraging activities than 50-worker groups during the oviposition period, while 50-worker groups had more before oviposition.

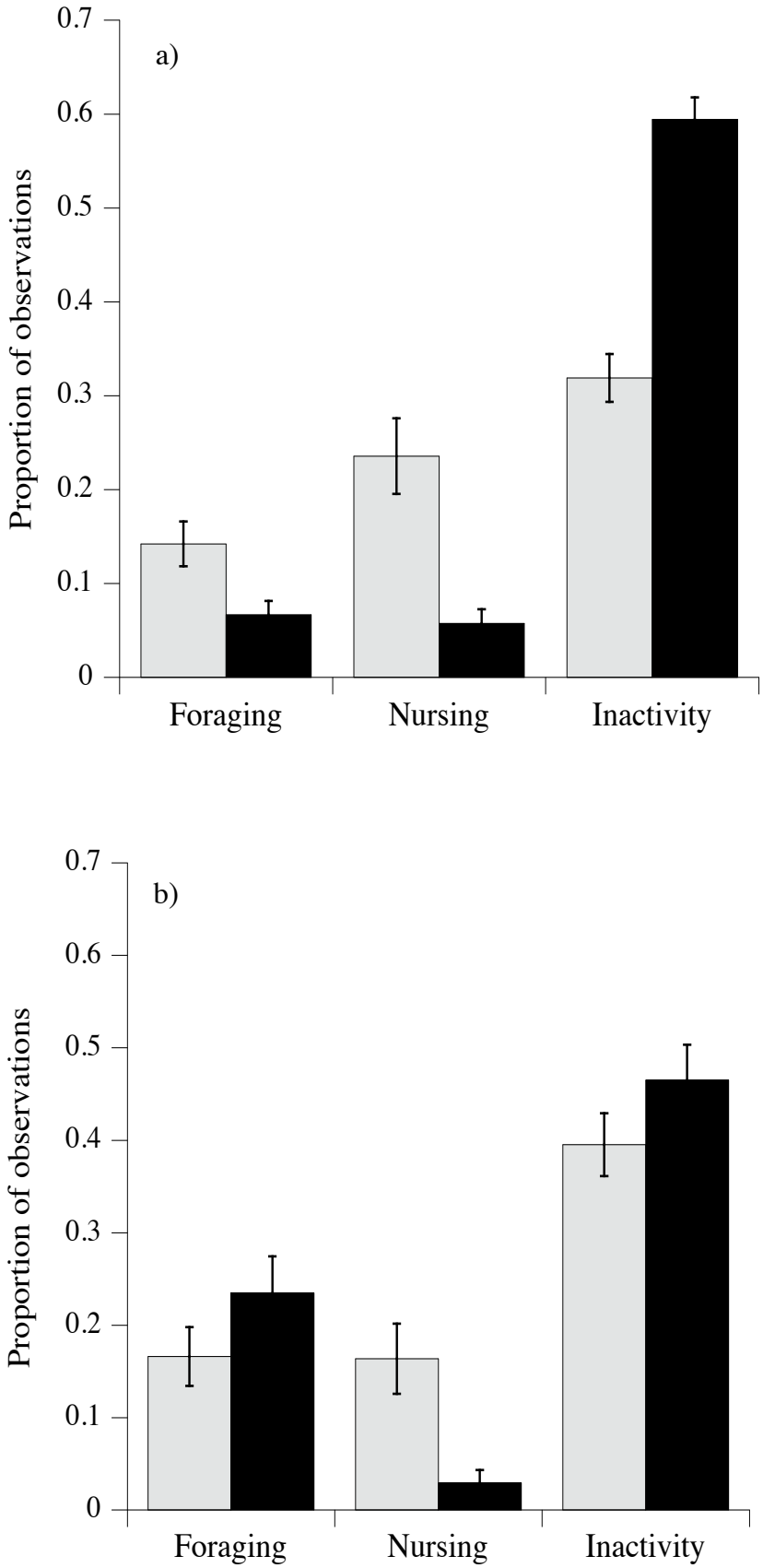

Figure 2.3: Proportion (mean ± SE) of foraging, nursing and resting activities by 20 focal workers in groups of 50 (grey bars) and 200 workers (black bars) during the 2 periods of observation: a) period 1: days 2, 3, 4 and 7 (before worker oviposition); b) period 2: days 14 and 15 (after oviposition).

Only 40% and 10% of the individuals in the 50- and 200-worker groups were observed. To assess differences in brood care and foraging activities at the group level, the behavioural profiles of focal individuals were multiplied by 10 and 2.5 for 200- and 50-worker groups, respectively. The results indicated that throughout the period of observation the absolute number of brood care activities performed by all 50 workers would have ranged between 348 and 746 in the 50-worker groups. In the 200-worker groups, the 20 larvae would have received between 171 and 787 brood care activities. The absolute number of foraging activities would have ranged between 225 and 474 and between 707 and 1716 foraging acts, in the 50- and 200-worker groups, respectively. In other words, although each individual spent less time being inactive in a 50-worker group, this was not sufficient to compensate for the reduction of foraging activities. As the absolute number of foraging acts was higher in the three 200-worker groups than in the 50-worker groups, prey retrieval was significantly faster in the former than in the latter (survival analysis: $\chi^2_5 = 18.8$, $P = 0.002$).

Experiment 3: Worker ovarian development

Dissections revealed that a high proportion of workers had developed ovaries, irrespective of the presence of the queen (Fig. 2.4; 44.7% versus 42.7% QR and QL situations, respectively; Table 2.1, Model 7). The proportion of workers with developed ovaries did not vary significantly during the 3 weeks of the experiment (Table 2.1, Model 7). However, nurses' ovaries were significantly more developed than those of foragers (57.3% versus 30.3%; Table 2.1, Model 7).

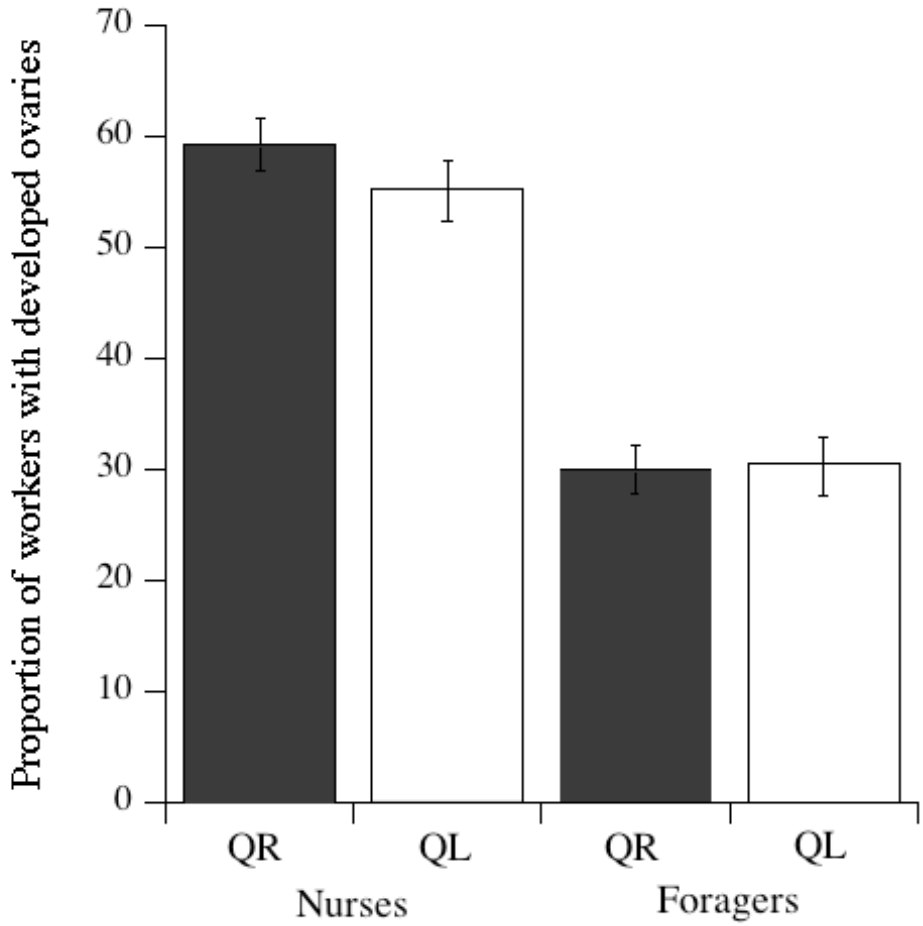

Figure 2.4: Development of workers ovaries (% mean ± SE) in queenright (QR, black bars) and queenless (QL, grey bars) conditions and according to their task when the experiment started.

Experiment 4: Effect of worker-laid eggs

Of 20 larvae that were initially provided to each group, 10.2 ± 0.5 reached the pupal stage. Overall, the number of diploid pupae (irrespective of the caste) did not differ significantly according to the combination of group size and the addition of worker-laid eggs (Fig. 2.5; Table 2.1, Model 8).

Each Source group produced on average 4.4 ± 0.4 eggs per day. The amount of eggs added to the 50_{eggs} groups did not differ from that added to the 100_{eggs} groups (Mann–Whitney test: $U = 171$, $N_1 = 17$, $N_2 = 18$, $P = 0.563$). The presence of worker-laid eggs did not have any effect on the proportion of each caste produced (Table 2.1, Model 9).

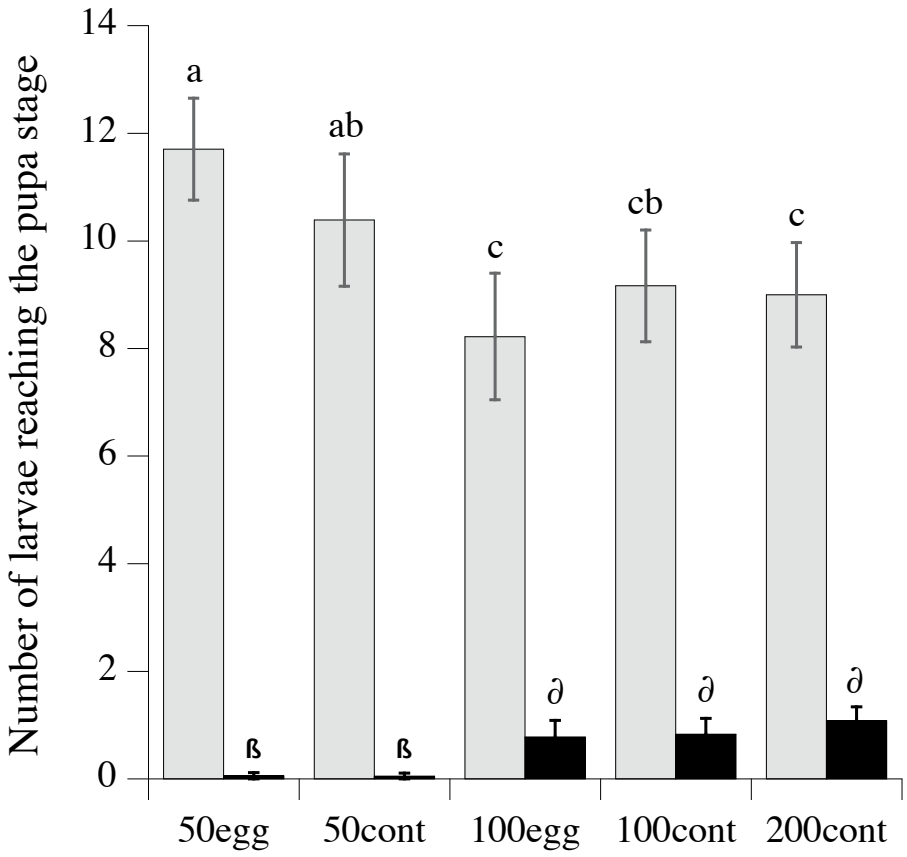

Figure 2.5: Number (mean ± SE) of worker (grey bars) and queen (black bars) pupae produced as a function of the number of workers and the addition of eggs from the source colonies. The letters a, b and c denote significant differences between groups in worker production (50cont vs 100eggs and 50cont vs 200cont: $P \leq 0.05$; 50eggs vs 100cont: $P < 0.01$; 50eggs vs 100eggs and 50eggs vs 200cont: $P < 0.001$). The letters ß and ∂ denote significant differences between groups in queen production (50eggs vs 100eggs, 50eggs vs 100cont, 50cont vs 100eggs and 50cont vs 100cont: $P < 0.01$; 50eggs vs 200cont and 50cont vs 200cont : $P < 0.001$).

The proportion of larvae that developed into workers was significantly higher in the $50_{control}$ than in $100_{control}$ or $200_{control}$ groups (Fig. 2.5). Therefore, as group size increased, the proportion of workers decreased while that of queens increased. However, when all the $200_{control}$ groups were compared separately, there was no significant correlation between the number of queens and workers produced (Pearson correlation: $r = -0.11$, $t_{34} = -0.648$, $P = 0.522$).

On average only 0.53 ± 0.14 males were produced during the 60 days of the experiment in the control groups. Significantly more males were produced in the 50_{egg} and 100_{egg} groups (2.14 ± 0.57; Mann–Whitney test: $U = 879$, $N_1 = 72$, $N_2 = 35$, $P = 0.003$). This difference was due to an increase in male production in both groups from day 47. The few males produced before this date derived from some of the 20 larvae initially provided to each group (haploid larvae occurred in small numbers in laboratory colonies and are not distinguishable from diploid larvae). The excess of males produced

after day 47 in the 50_{egg} and 100_{egg} groups derived in all likelihood from the added eggs. Yet the average number of eggs that eventually reached the pupal stage was extremely low compared to the number of eggs that were added during the first 3 weeks.

Discussion

Elucidating how group size effects operate at the different levels of social organization is important for understanding the evolution and maintenance of cooperation. So far, however, the mechanisms underlying the effect of group size on reproductive decisions have not been investigated in depth. The ant *A. senilis* is a good model system for such studies because the production of queens is highly predictable even in the laboratory (Boulay et al. 2007). Hence, in the QR condition, all diploid brood develops into sterile workers while queen removal immediately triggers the production of a new queen from the totipotent larvae. Our results show that the workers-to-larvae ratio did not affect the production of queens and that there were a critical number of workers below which queen replacement was less likely. The numbers of workers and larvae had a nonadditive effect on the success of queen replacement (e.g. the probability that at least one larva would develop into a queen; experiment 1). Hence, the number of larvae limited queen replacement in 50- but not in 200-worker groups. From 20 initially provided larvae, the 50-worker groups were able to rear the same number to the pupal stage as groups of 200 workers. But the 200-worker groups had a higher reproductive potential since the proportion of larvae developing into queens was higher (experiment 4). However, behavioural observations also indicated that a group of 50 workers was capable of enough plasticity to maintain a similar amount of brood care per larvae as a group of 200 workers (experiment 2). Workers laying eggs could explain the high level of inactivity observed in the 200-worker groups. Although our results support the hypothesis that many worker-laid eggs are cannibalized, lower egg provisioning in 50-worker groups did not limit queen production (experiments 3 and 4).

Ant larvae are completely dependent on adults for feeding. The latter must forage, process the food and distribute it to the larvae in addition to other types of care (e.g. cleaning). The entire process from foraging to larval feeding is probably a determinant for larval development and is constrained by the number of workers. Our

results (experiment 2) indicate that task allocation among colony members was plastic and varied with group size and time. The percentage of inactive individuals decreased from 55% in 200-worker groups to 35% in 50-worker groups. In small groups, inactive workers were therefore reallocated to nursing. During the oviposition period, 200-worker groups became more similar to 50-worker groups. Multiple factors could explain these changes in colonial behavioural profile. Such behavioural plasticity has been observed in several other social insect species (Gordon 1996). The degree of worker specialization in various tasks has also been shown to increase with worker number (Thomas and Elgar 2003; Holbrook et al. 2011; but see Dornhaus et al. 2009). The high proportion of apparently inactive individuals may constitute a reserve allowing the colony to cope with unpredictable worker loss (Anderson and McShea 2001; Cassill 2002; Jeanson et al. 2007). It has also been suggested that large colonies could afford a high number of inactive individuals because higher inactivity of some individuals might be compensated in large colonies by a higher degree of specialization of other individuals, enhancing overall group efficiency (Jeanson et al. 2007).

The result of experiment 2 showed that individual behavioural plasticity allowed a group of 50 individuals to maintain a colonial level of brood care similar to that of a group of 200 workers. In this experiment, the number of larvae was fixed to 20 in all the groups, which corresponds to the lower limit at which 50 workers were able to produce a new queen. The change from inactivity to other activities may be stimulated by the brood. Larvae are known to beg for food in several ant species (Creemers et al. 2003; Kaptein et al. 2005). Moreover, in the honeybee, the number of workers engaged in nursing behaviour was shown to increase with the number of larvae in the hive requiring food (Schmickl and Crailsheim 2002). It can therefore be hypothesized that, in *A. senilis*, inactive individuals must be stimulated by a small workers-to-larvae ratio or a critical number of larvae to shift to brood care activities. This contrasts with the behavioural hypothesis prediction that more queens would be produced in groups with fewer larvae because high brood care per larvae would be provided. Moreover, this could explain why queen replacement failed in groups of 50 workers containing fewer than 20 larvae. In addition, behavioural plasticity did not allow the group to maintain the same level of foraging and food retrieval in 50-worker as in 200-worker groups. As a consequence, workers required significantly more time to retrieve prey to the nest, which may have affected the entire food-processing chain up to the larvae, also reducing brood care quality.

In groups of 200 workers, the probability of becoming a queen decreased logarithmically with the number of larvae (experiment 1). This suggests that in the QL condition, a negative feedback operates at the colony level to limit the production of queens to a few individuals. The mechanism underlying this feedback is unknown. The first larvae developing into queens might inhibit the development of other larvae into new queens either directly, by chemical signals, competition for food or aggressive interactions, or through worker behaviour. Hence, the lack of a significant negative correlation between the number of workers and queens produced across group sizes (experiment 4) suggests that several larvae that did not develop into workers did not develop into queens either. Workers may be able to detect and kill larvae developing into extra queens, as occurs in *Linepithema humile* and *Solenopsis invicta* (Passera et al. 1995; Klobuchar and Deslippe 2002). Independently of the mechanism, the limitation of queen production in *A. senilis* is important because this species is strictly monogynous and only one queen is necessary for the colony to proceed. Moreover, during fission, the new queen leaves her nest with workers protecting her, which favours high queen survival. As a consequence, colonies are expected to limit their investment in sexuals. Extra individuals may serve as 'life insurance' in case the first new queen should accidentally die (Chéron et al. 2009). However, a massive investment in new queens that would compete among themselves would be useless and would probably jeopardize colony growth and maintenance.

Although the number of queens increased with group size, the total number of diploid pupae (workers + queens) produced from the 20 larvae provided in 50-, 100- and 200-worker groups did not differ significantly (experiment 4). This suggests that the capacity to rear the brood is maintained irrespective of group size, but rearing queens requires an overhead that small worker groups may not afford. Hence, our results show that in small groups, workers did not sacrifice worker-destined larvae in order to produce more costly queens. The specific cost of producing a queen instead of a worker is difficult to estimate because we still lack information on the physiological mechanisms triggering larval development into one or the other caste. In species with worker–queen dimorphism such as *A. senilis,* queens need to receive an excess of food compared to workers. Individual dry weight measurements of 196 workers and 60 queen pupae revealed the latter were only 1.83 times heavier than the former (R. Boulay, unpublished data). This difference seems relatively small but may limit the production of queens. Variation in food quality in the larval diet may also trigger larval

development into one or the other caste, especially in the field where the colonies have access to diverse resources (Smith and Suarez 2010). In our experiment, only one food source was provided to the groups. This suggests that either variation in food quality was not a determinant of larval development or the larvae had access to another resource such as worker-laid eggs.

The first pile of worker-laid eggs appeared after 10 days under QL conditions (Ichinose and Lenoir 2009a). Several lines of evidence suggest that many worker-laid eggs were cannibalized. First, very few males were produced, even when four eggs were added daily to 100- and 50-worker groups. Second, worker ovarian development did not depend on the presence of a queen but was related to their activity, which most probably also reflected their age (experiment 3). Whether workers lay eggs in a QR situation remains unknown. The delay between queen removal and the appearance of a pile of worker-laid eggs may reflect the time for switching from trophic to reproductive eggs in a QL situation. The presence of a chorion suggested that the eggs laid by QL workers in experiment 4 were all viable, but the presence of trophic eggs in a QR situation has not been investigated in this species. Although workers may keep developed ovaries in anticipation of the mother queen's death (Bourke 1988), this hypothesis is unlikely given the relatively long queen life span (> 5 years, personal observation). Therefore, workers developing ovaries in both QR and QL situations would be selected for by larval nutritional needs rather than for potential worker reproduction. Oophagy has been shown in other species such as *Messor semirufus* (Baroni Urbani 1991). In the ponerine ant *Amblyopone silvestrii* (Masuko 2003) small larvae mostly feed on queens' eggs, while large larvae are transported on the prey. Similarly, the large larvae of *A. senilis* feed directly on the prey (Agbogba 1986) while the diet of the small larvae is unknown. It is therefore reasonable to expect that, as in *A. silvestrii,* young *A. senilis* cannibalized eggs too. This hypothesis is supported by the finding that worker-laid eggs appeared when few larvae were present (experiment 1). However, consumption of worker-laid eggs, if confirmed, did not affect larval caste fate.

In insect societies, colony fitness can be constrained by group size. Larger groups differ from small groups in their task distribution (Thomas and Elgar 2003; Holbrook et al 2011). They outcompete small groups in foraging (Beckers et al. 1989; Beekman et al. 2004; Thomas and Framenau 2005) and invest their resources in reproductive individuals (Jeanne and Nordheim 1996; Cassill 2002). Colony fitness

could also be limited by very large group sizes. The individual and group efficiencies may peak and then decline because of group dynamics constraints (Bruce and Burd 2012). The larval fate does not solely depend on direct interactions between larvae and workers, but rather relies on collective cooperative performance at the colony level. The evolution of sociality leads to a dependency not only on other members in the group, but also on the global performance of the group as a whole.

Chapter 3

Surface lipids of queen-laid eggs do not regulate queen production in a fission-performing ant

Camille RUEL, Alain LENOIR, Xim CERDÁ, Raphaël BOULAY

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ORIGINAL PAPER

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Camille Ruel · Alain Lenoir · Xim Cerdá · Raphaël Boulay

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Abstract In animal societies, most collective and individual decision making depends on the presence of reproductive individuals. The efficient transmission of information among reproductive and non-reproductive individuals is therefore a determinant of colony organization. In social insects, the presence of a queen modulates multiple colonial activities. In many species, it negatively affects worker reproduction and the development of diploid larvae into future queens. The queen mostly signals her presence through pheromone emission, but the means by which these chemicals are distributed in the colony are still unclear. In several ant species, queen-laid eggs are the vehicle of the queen signal. The aim of this study was to investigate whether queen-laid eggs of the ant *Aphaenogaster senilis* possess queen-specific cuticular hydrocarbons and/or Dufour or poison gland compounds, and whether the presence of eggs inhibited larval development into queens. Our results show that the queen- and worker-laid eggs shared cuticular and Dufour hydrocarbons with the adults; however, their poison gland compounds were not similar. Queen-laid eggs had more dimethylalkanes and possessed a queen-specific mixture of cuticular hydrocarbons composed of 3,11+3,9+3,7-dimethylnonacosane, in higher proportions than did worker-laid eggs. Even though the queen-laid eggs were biochemically similar to the queen, their addition to experimentally queenless groups did not prevent the development of new queens. More studies are needed on the means by which queen ant pheromones are transmitted in the colony, and how these mechanisms correlates with life history traits.

Keywords Social insect · *Aphaenogaster senilis* · Egg marking · Cuticular hydrocarbons · Queen pheromones

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Introduction

In animal societies, collective behaviors and individual decision making rely on an efficient transmission of information among colony members. Identifying reproductive potentials of other individuals is essential in mediating conflicts over reproduction and maintaining social cohesion. In social hymenopterans, queens have a central role in colony-level reproductive decisions. Their presence inhibits worker ovarian development and the production of numerous haploid male eggs (Hoover et al. 2003; Bhadra et al. 2010; Holman et al. 2010). Moreover, in several species, the queen prevents diploid larvae from developing into future queens (Vargo and Fletcher 1986; Vargo and Passera 1991), either directly by interacting with larvae or by affecting worker nursing behavior (Vargo and Fletcher 1986; Jarau et al. 2010; Penick and Liebig 2012). This phenomenon is manifest in species that reproduce by colony fission like the honeybee *Apis mellifera* and the gypsy ant *Aphaenogaster senilis* in which almost the entire diploid brood is reared into

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The means by which the queen signals her presence is still unclear for many species, but the size of the colony seems critical. In a small group, the queen may control workers and brood through physical suppression (Brian 1970; Bartels 1988; Peeters 1989; Retana and Cerdá 1990; Vargo and Passera 1991). However, as colony size increases, direct behavioural interactions rapidly become inefficient, and chemical communication prevails (Keller and Nonacs 1993). In the honeybee, the queen pheromones that inhibit worker ovarian development and the development of the larvae into queens are secreted mostly by the mandibular glands and are composed of 9-oxo-2-decenoic acid (Barbier and Lederer 1960) and other minor components (Slessor et al. 1988; Katzav-Gozansky et al. 2001; Wossler 2002; Hoover et al. 2003). These secretions have a low volatility and are disseminated by physical contact. Messenger workers acquire the queen signal through extensive contact with the queen and redistribute it throughout the hive, thus informing the colony of the queen presence (Seeley 1979; Naumann et al. 1991).

The identity and function of queen pheromones is less well known in ants than in the honeybees. Yet, most studies point to cuticular hydrocarbons (CHCs) being the main queen signal (Le Conte and Hefetz 2008). In the ant *Camponotus floridanus,* whose colonies contain several thousands of workers, the queen's eggs bear HCs that are specifically found on her cuticle. Workers presumably transport queen-laid eggs to the multiple nest chambers and, by so doing, inform their nestmates of the queen's presence. Hence, workers coming in contact with the queen-laid eggs refrain from reproducing (Endler et al. 2004, 2006). A queen-specific HC, 3-methylhentriacontane, was also found on queen-laid eggs in *Lasius niger* (Holman et al. 2010). This unique compound limits ovarian development in workers. In addition to informing the colony of the queen's presence, egg marking with queen-specific HCs allows workers to discriminate between queen-laid and worker-laid eggs, and thus conditionally destroy the latter (Ratnieks 1995; Monnin and Peeters 1997; D'Ettorre et al. 2004a; Shimoji et al. 2012). Egg marking by the queen is an important process in the social regulation of worker reproduction.

Few studies have tested whether the presence of queen-laid eggs also inhibits the production of new reproductive females. In *Solenopsis invicta* and *Linepithema humile* the queen pheromones prevent diploid brood from developing into queens. These pheromones also trigger the workers to attack larvae oriented toward queens. However, the daily addition of eggs to queenless groups of workers and larvae did not limit the production of queens in either species (Vargo and Fletcher 1986; Vargo and Passera 1991).

We used the gypsy ant *A. senilis* as a model system to test whether the queen-laid eggs may be used as vehicle to distribute the queen signal within colonies and prevent larvae from developing into future queens. First, we analyzed queen-laid and worker-laid egg surface chemicals and compared them to adult cuticular compounds and secretions from two abdominal glands (the Dufour and poison glands). We hypothesized that some compounds are queen-specific and are transmitted to the surface of queen-laid eggs. Second, we tested whether the frequent addition of fresh queen-laid eggs inhibited the production of queens from diploid larvae. If the queen-laid eggs prevent the production of queens, then we may expect the queen pheromone to be present on the egg surface.

Methods

Colonies were collected and housed in the laboratory in ø10x10 cm circular boxes connected to ø2x20 cm test tubes.

Pictures of queen- (n = 233) and worker-laid eggs (n = 78) were taken with a stereomicroscope (Zeiss Stemi 2000) equipped with a digital camera (Axiocam lcc1 Zeiss). Egg volume was obtained using the formula: $V = 1/2 \times \pi \times a \times b \times c$ where a, b, and c are the length of three perpendicular egg axis measured using the program AXIOVISION 2010 v.4.8.2.

Chemical analyses

The queens of seven colonies were housed with 100 nestmate workers for five days. Eggs were collected after the 1st and the 5th day. Egg samples were prepared by pooling 10 eggs from the same queen. However, two queens apparently stopped laying eggs after 24h. As a result, we were able to prepare seven samples of 10 one-day-old eggs but only five samples of four-day-old eggs. The seven queens were then dissected to collect their thoraces, Dufour glands and poison glands. The workers started laying eggs 8 days after queen removal. When 10 worker-laid eggs appeared in a queenless group, they were collected.

The chemical compounds contained on the ant thoraces, in their glands and on the surface of the eggs were extracted in 50µL of dichloromethane and the extracts stored at 4ºC until chemical analyses. First, the HC profiles of the queens thoracic cuticles were compared to those of two whole QR workers per colony and the pools of 10 queen- or worker-laid eggs. Samples were injected into a gas chromatograph (GC 2010 Shimadzu) equipped with a Flame Ionization Detector. Oven temperature was programmed to run from 130ºC to 240ºC at 15ºCmin^{-1}, and then from 240ºC to 300ºC at 3ºC min^{-1}. Second, the profiles of eggs (n = 10 for queen-laid eggs and n = 4 for workers-laid eggs) were compared with those of adults for Dufour and poison gland secretions (n = 6 for queens and n = 7 foreign workers). The gas chromatograph column temperature was programmed to run from 60ºC to 210ºC at 5ºC min^{-1}, and then from 210ºC to 300ºC at 15ºC min^{-1}. Two hundred nanograms of eicosane were added to each sample as an internal standard. Compound identification was achieved by performing mass-spectrometry (GC-MS Perkin-Elmer) on 50 eggs, 1 queen, and 10 workers from 2

colonies under the same chromatographic conditions, and compared to previously published identifications (Lenoir et al. 2001a; Boulay et al. 2007; Lenoir et al. 2011).

Compound proportions were standardized by subtracting the mean proportion of a compound across samples to each individual proportion and dividing by the result by standard deviation of the mean (see Boulay et al. 2007). By so doing, all the variables have a zero mean and unit standard deviation. The differences in chemical profiles were analyzed by means of multivariate analyses. The 18 and 12 major compounds present in cuticular and Dufour gland extracts, respectively, were selected. The proportions of each of these compounds, in at least half of the ants in one group, were more than 2 and 3% of the total quantity of compounds. A principal component analysis was performed in order to reduce the number of variables. The factor scores of the first components explaining most of the variance in our data (45.9%, 22.9% and 8.9% for CHCs; and 32%, 21.1%, 13.7%, and 10.1% for Dufour gland compounds) were used in a discriminant analysis to test whether the predefined groups (queen's eggs, workers' eggs, queen and workers) could be distinguished according to their chemical profiles. Mann-Whitney tests were performed to compare the total amounts of compounds found on queens versus eggs, and the proportion of the single HCs found on adults versus eggs, between the adults, and between queen- and worker-laid eggs. The sequential Holm-Bonferroni correction was used to control for family-wise error across the multiple comparisons.

Effects of queen-laid eggs on larval fate

Thirty-three colonies were each divided into three groups of 200 workers collected inside the nest and 15 first-instar larvae. Of the three identified larval instars, only the first was shown to be totipotent (Boulay et al 2009). Each group was subjected to a different treatment. One group contained the mother queen (QR, hereafter) while the other two groups were queenless (QL and QL-egg). All the eggs produced in the QR group were carefully transferred with smooth forceps every second day to the corresponding QL-egg group. After 20 days, the queen and the eggs were removed from the QR and the QL-egg groups, respectively. Given that egg incubation time in this species is approximately 30 days (C. Ruel, pers. Obs.), the eggs added to the QL-egg groups did not have time to hatch. Larvae were monitored every day observing throughout the test-tube to record their gender, caste, survival rate, and development time until all surviving larvae had pupated. We also measured the egg-laying rate of 18

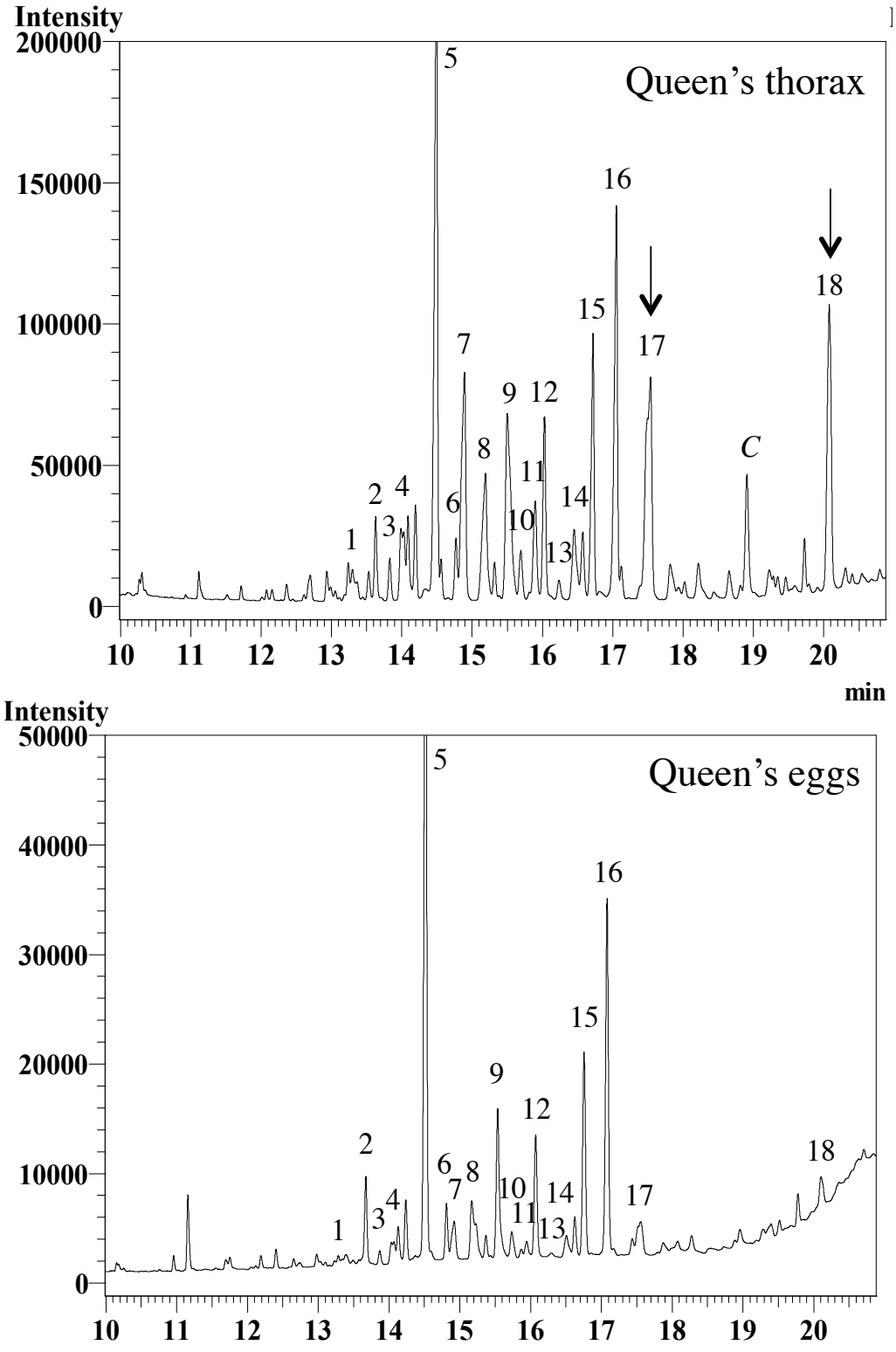
queens by counting the eggs transferred from the QR to the QL groups every second day during the first 16 days of experiment.

Data were analyzed with the R-package software v. 2.7.2 (R Core Team, Vienna, Austria). Two generalized linear mixed models (GLMM; lme4 package) were fitted to compare the probability of producing at least one queen during the 40 days of experiment and the proportion of surviving larvae among the QR-, QL-, and QL-egg treatments. Both models were fitted using the binomial error distribution and logit link function. Colony was included as a random factor. For groups that produced at least one queen, a mixed model (nlme package) was fitted to compare the time to production of the first queen in each group among treatments. The response variable was square-root transformed to fit model assumptions.

Results

Adult long-chain cuticular hydrocarbons found on the egg surface

Forty-three long-chain saturated HCs (from 25 to 32 carbons) or mixtures of HCs were found on queens' and workers' cuticles and on fresh eggs, albeit in different amounts and proportions (Fig. 3.1). Discriminant analysis highlighted major differences between the profiles of both castes and their eggs (Fig. 3.2a). All samples types were well classified by the model. The first axis, which explained 86% of the variance, distinguished the workers from the queens and their eggs. The second axis, which explained 13% of the variance, separated the queens from their eggs.


Intensity
200000
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100000
50000
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Queen's thorax
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Intensity
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Queen's eggs
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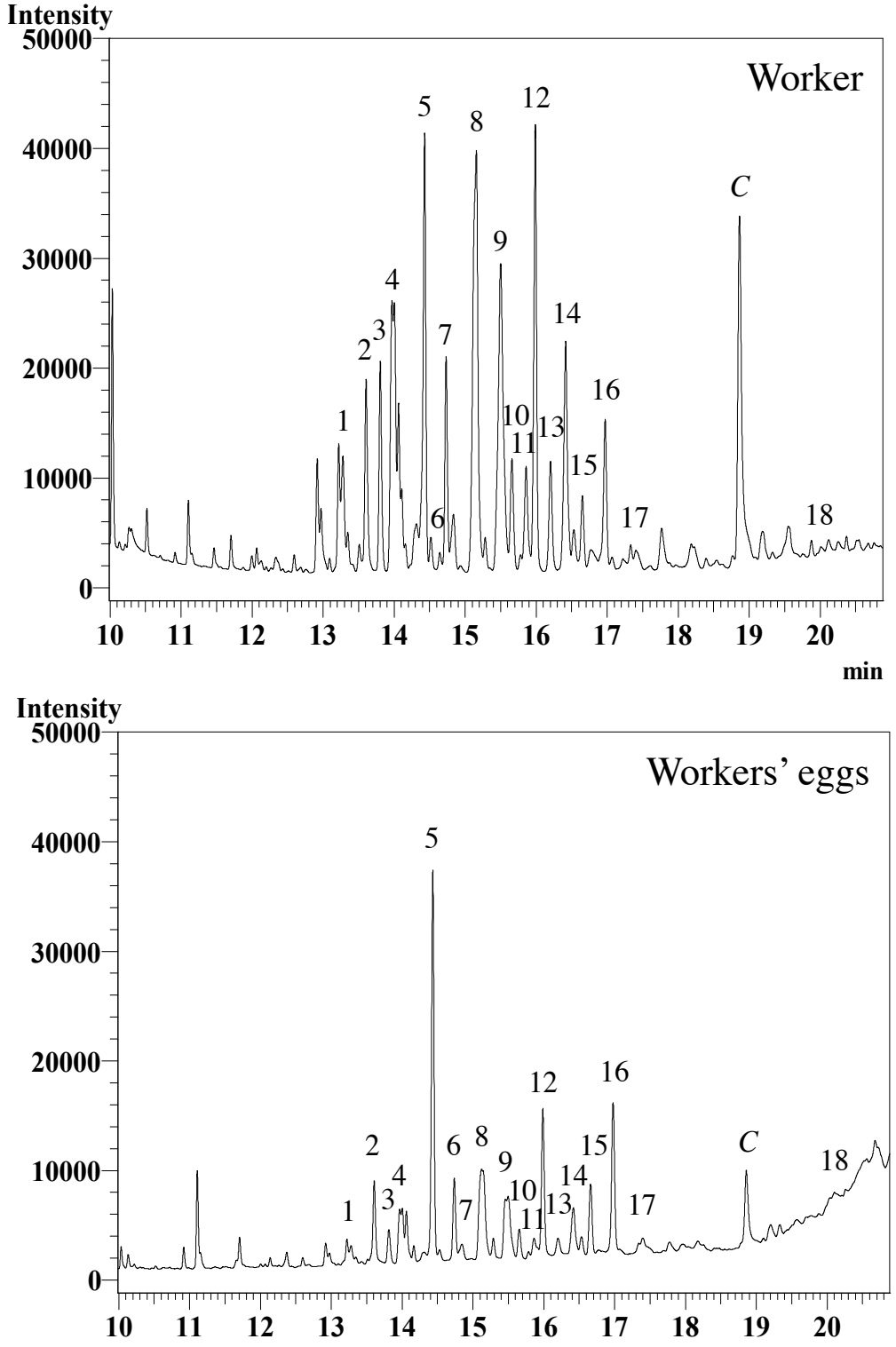


Figure 3.1: Four examples of cuticular HC profiles of a queen's thorax, a nestmate worker and 10 of their respective eggs. Numbers are given to the major compounds: 1 = 8,12+6,10DiMeC26, 2 = C27, 3 = 4,8,12TriMeC26, 4 = 7+9+11+13MeC27, 5 = 3MeC27, 6 = C28, 7 = 3,7+3,9DiMeC27, 8 = x,yDiMeC28+10MeC28, 9 = 4MeC28, 10 = 6,10DiMeC28, 11 = 4,8DiMeC28, 12 = C29, 13 = 4,8,12TriMeC28, 14 = 11MeC29, 15 = 5MeC29, 16 = 3MeC29, 17 = 3,11+3,9+3,7DiMeC29, 18 = 3,9+3,11DiMeC31. (*C* = cholesterol). A complete list of cuticular HCs is given in Lenoir et al. (2001a).

Queen and worker profiles differed mainly in their percentage of eight compounds (Table 3.1). In particular, two mixtures of long dimethylalkanes, 3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane (peaks 17 and 18 marked with an arrow in Fig. 3.1), represented 13.1 ± 1.3% (means ± SE) and 10.1 ± 2.3% of the queens' profiles, respectively, and were almost absent in workers (0.6 ± 0.1% and 0.5 ± 0.1% respectively). More generally, a queens' cuticle contained relatively more dimethylalkanes and fewer monomethylalkanes than a worker's cuticle.

Table 3.1: Percentage (Mean±SE) of the major cuticular HCs, classified by chemical nature, for queens, workers, and their respective eggs (respectively n = 7, 14, 12 and 7). Cuticular HCs of queens versus workers and queen- versus worker-laid eggs are compared (Mann-Whitney test with Holm-Bonferroni correction). Absent or undetected compounds were given the minimum threshold detected by the gas chromatograph. The dimethylalkane x,yDiMeC28 was not identified. The corresponding peak number in Figure 1 is given for each compound.

Peak number in Fig. 3.1	Compounds	Queen *vs* Workers	Queen's eggs *vs* Workers' eggs
2	C27	3.6 ± 2.3 *vs* 2.3 ± 0.2 ns	**3 ± 0.2 *vs* 7.5 ± 3.4** **< 0.006**
6	C28	0.9 ± 0.1 *vs* 2.4 ± 0.4 ns	**1.8 ± 0.1 *vs* 3.9 ± 0.4** **< 0.004**
12	C29	2.4 ± 0.4 *vs* 4.8 ± 0.6 ns	**4.5 ± 0.3 *vs* 7 ± 0.4** **< 0.004**
	Total linear alkanes	*9.3 ± 3.7 vs 11.1 ± 1.3* *ns*	***10.3 ± 0.5 vs 19.9 ± 2.9*** **< 0.025**
4	7+9+11+13MeC27	**3.7 ± 0.7 *vs* 11.9 ± 1** **< 0.005**	**2.5 ± 0.2 *vs* 5.2 ± 0.6** **< 0.005**
5	3MeC27	10.1 ± 0.1 *vs* 8.2 ± 0.6 ns	25.4 ± 0.9 *vs* 21.6 ± 2.2 ns
9	4MeC28	5.6 ± 0.9 *vs* 10.8 ± 0.9 ns	6.2 ± 0.1 *vs* 5.3 ± 0.4 ns
14	11MeC29	2.5 ± 0.3 *vs* 3.8 ± 0.7 ns	**1.5 ± 0.1 *vs* 3.5 ± 0.3** **< 0.003**
15	5MeC29	3.8 ± 0.6 *vs* 2.2 ± 0.4 ns	**7.5 ± 0.4 *vs* 4.5 ± 0.3** **< 0.003**
16	3MeC29	5 ± 0.9 *vs* 3.3 ± 0.4 ns	9.8 ± 1.8 *vs* 9.7 ± 0.6 ns
	Total methylalkanes	***44.3 ± 4 vs 64.5 ± 0.9*** **< 0.017**	*65.6 ± 2.3 vs 66.3 ± 3.7* *ns*
1	8,12+6,10DiMeC26	**1.1 ± 0.2 *vs* 2.4 ± 0.2** **< 0.004**	**2.7 ± 0.8 *vs* 7.1 ± 1.1** **< 0.006**
7	3,7+3,9DiMeC27	**7.6 ± 1 *vs* 1.5 ± 0.1** **< 0.004**	**2.9 ± 0.2 *vs* 1.3 ± 0.1** **< 0.003**
8	x,yDiMeC28+10MeC28	**5.8 ± 0.6 *vs* 14.1 ± 0.6** **< 0.003**	**3 ± 0.3 *vs* 7.8 ± 0.5** **< 0.004**
10	6,10DiMeC28	**1.3 ± 0.1 *vs* 2.3 ± 0.1** **< 0.003**	1.2 ± 0.1 *vs* 1.5 ± 0.1 ns
11	4,8DiMeC28	3.3 ± 0.5 *vs* 2.8 ± 0.1 ns	1.4 ± 0.2 *vs* 1.6 ± 0.1 ns
17	3,11+3,9+3,7DiMeC29	**13.1 ± 1.3 *vs* 0.6 ± 0.1** **< 0.003**	**4.7 ±0.4 *vs* 1 ± 0.2** **< 0.003**
18	3,9+3,11DiMeC31	**10.1 ± 2.3 *vs* 0.5 ± 0.1** **< 0.004**	5.1 ± 0.8 *vs* 1.6 ± 0.9 ns
	Total dimethylalkanes	***53.7 ± 3.1 vs 26.2 ± 0.5*** **< 0.025**	***48.5 ± 1.4 vs 32.7 ± 1.3*** **< 0.017**
3	4,8,12TriMeC26	**1.2 ± 0.2 *vs* 3.9 ± 0.2** **< 0.003**	0.6 ± 0.1 *vs* 1.2 ± 0.1 ns
13	4,8,12TriMeC28	1.6 ± 0.5 *vs* 2.5 ± 0.1 ns	**0.4 ± 0.1 *vs* 1.4 ± 0.2** **< 0.005**

The HC profiles of 1- and the 4-day-old queen-laid eggs were similar and were consequently pooled together. The profiles of queens' and workers' eggs were

dominated by 3-methylheptacosane (24 ± 1.1%; peak 5 in Fig. 3.1). Overall, queens' eggs contained significantly more long-chain dimethylalkanes and fewer linear alkanes than workers' eggs. The mixtures of 3,11+3,9+3,7-dimethylnonacosane (peak 17) and 3,9+3,11-dimethylhentriacontane (peak 18) were present in relatively low proportions on eggs, but the proportion of the former was significantly higher on queens' than workers' eggs. The total amount of HCs did not differ significantly between queens' and workers' eggs (87 ± 9 ng vs 73 ± 13 ng for volumes of 31.7 ± 0.3 μm^3 vs 25.9 ± 0.4 μm^3; Mann-Whitney test: $U = 51$, $N_1 = 12$, $N_2 = 7$, $P = 0.482$).

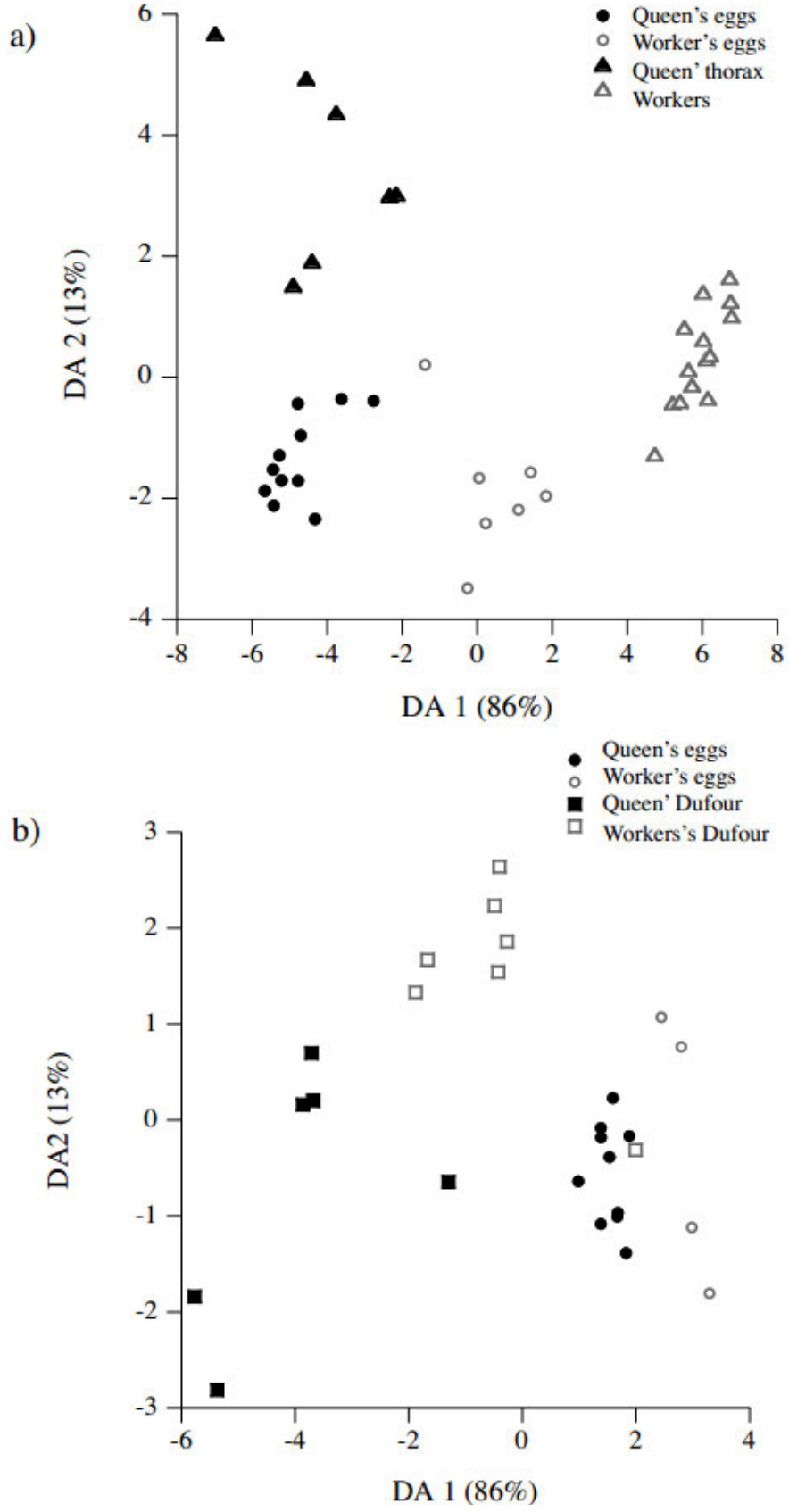
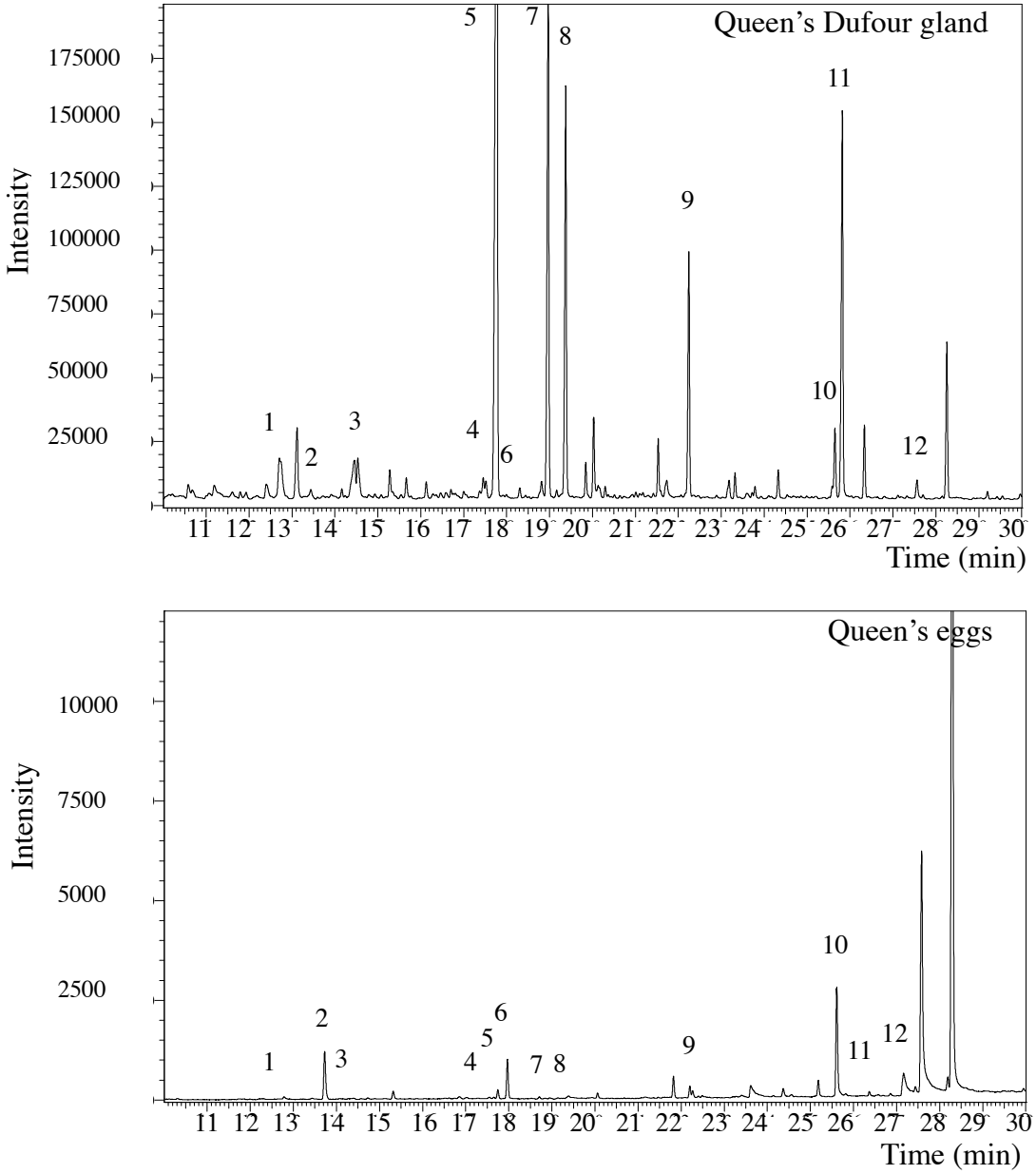


Figure 3.2: Discriminant analysis of a) cuticular HC profiles of queens' thoraces (filled orange triangle), entire workers (empty blue triangle), queen-laid eggs (filled red circle), and worker-laid eggs (empty blue circle); b) Dufour HC and alkaloids profiles of queens' glands (filled orange triangle), workers' glands (empty blue triangle), queen-laid eggs (filled red circle) and worker-laid eggs (empty blue circle). The percentage of variance explained by the first two discriminants is given for each axis.

Adult abdominal gland compounds found on the egg surface

The queens' and workers' Dufour glands contained thirty-three saturated and unsaturated HCs (from 13 to 19 carbons) plus small amounts of an aldehyde, hexadecanal (Fig. 3.3; Table 3.2). Twenty HCs were specific to the Dufour gland, as they were never present in the poison gland.



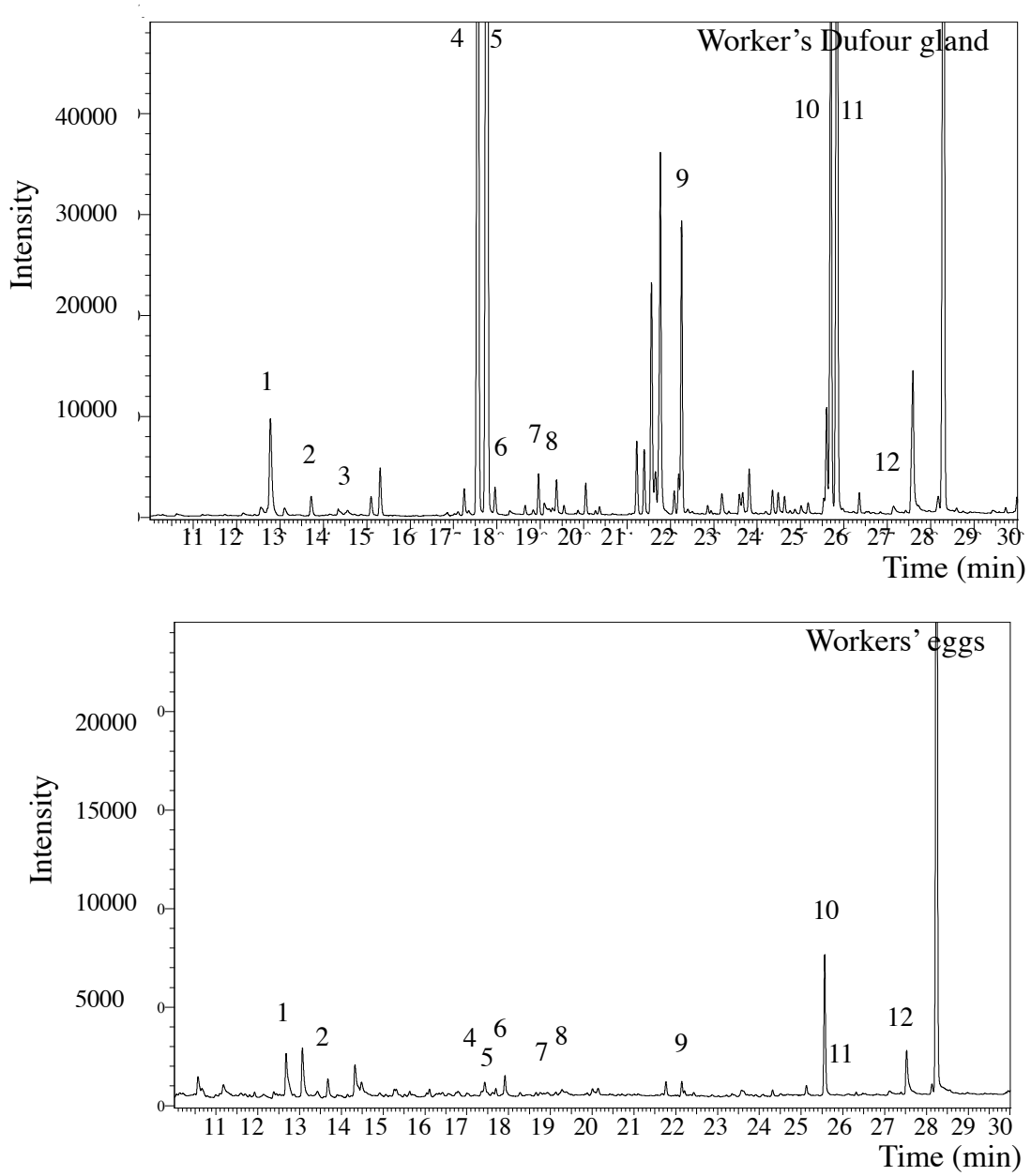


Figure 3.3: Four examples of Dufour gland profiles and compounds deriving from the Dufour gland of a queen, a worker and 10 of their respective eggs. Numbers are given to the major compounds: 1 = C13, 2 = 5MeC13, 3 = Unidentified HC1, 4 = C15:1, 5 = C15, 6 = Terpene, 7 = 5MeC15, 8 = 3MeC15, 9 = C17, 10 = C19:2, 11 = C19:1, 12 = 9MeC19. (The compound with a retention time of 28.3 minutes is the internal standard eicosane).

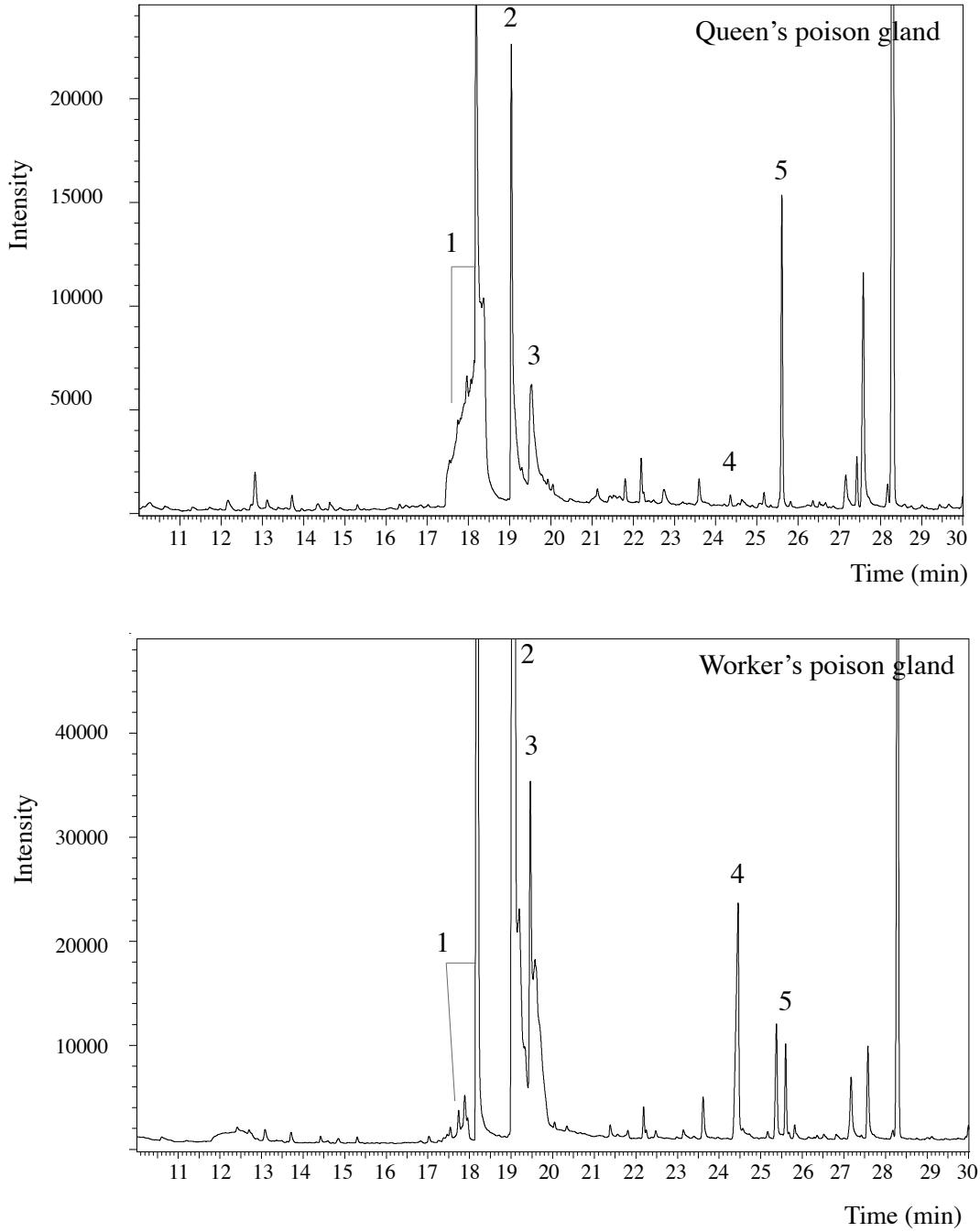


Figure 3.4: Two examples of a queen's and a worker's poison gland profiles. Numbers are given to the five major compounds: 1 = Anabasine, 2 = Anabaseine, 3 = Unidentified alkaloid, 4 = C18, 5 = C19:2. (The compound with a retention time of 28.3 minutes is the internal standard eicosane).

Thirteen of these Dufour-specific HCs were detected on the egg surface. The discriminant function analysis correctly classified 88.9% of the samples and revealed extensive overlaps among the 4 groups (Fig. 3.2b). The first axis mainly separated the eggs, irrespective of their origin, from the adults. Hence, queen- and worker-laid eggs did not differ significantly with respect to major Dufour gland compounds. The total amount of Dufour HCs did not differ significantly between queens' and workers' eggs

(20 ± 3 ng vs 10 ± 2 ng; Mann-Whitney test: $U = 28$, $N_1 = 10$, $N_2 = 4$, $P = 0.304$). The egg surface diverged from the adult glands by seven HCs. The eggs had higher proportions of tridecane, 5-methyltridecane, 9-methylnonadecane, an unidentified HC, and an unidentified terpene than the adults (Table 3.2). Adults' Dufour glands, on the other hand, contained more pentadecane and nonacosene. The second axis of the discriminant function analysis mostly differentiated workers and queens. This difference was due to a higher proportion of 5- and 3-methylpentadecane in the former (in bold in Table 3.2).

Finally, 17 compounds, including HCs and three alkaloids (anabasine, anabaseine, and an unidentified alkaloid), were found in queens' and workers' poison glands (Fig. 3.4; Table 3.2). Specific poison gland compounds were absent from the surface of queen- and worker-laid eggs. Alkaloids were overrepresented in workers' and queens' poison glands, reaching 66 ± 12.7% and 66.6 ± 8.4% of the total content, respectively. Queens and workers did not differ in any of the poison gland compounds.

Table 3.2: Percentage (mean ± SE) of Dufour and poison glands compounds in queens (n = 6), workers (n = 7) and on the surface of 10 queen- and worker-laid eggs (n = 10 and n = 4, respectively). The blanks indicate that a compound is absent or undetected. These compounds were given the minimum threshold detected, explaining why the total does not add up to 100%. Three hydrocarbons, one terpene, and one alkaloid could not be identified. Gas chromatographs profiles of the Dufour and poison glands are published in Boulay et al. (2007) and Lenoir et al. (2011) respectively.

Retention time	Compound names	Queen-laid eggs	Worker-laid eggs	Queen's Dufour gland	Worker's Dufour gland	Queen's poison gland	Worker's poison gland
12.5	C13:1		1.7 ± 0.4	0.3 ± 0.2	1.3 ± 1.1		
12.8	C13	8.9 ± 3.9	8.4 ± 4.3	1 ± 0.2	2.9 ± 1	4.9 ± 2.9	5 ± 2
13.7	5MeC13	5.7 ± 1	7.2 ± 2.3	0.8 ± 0.4	0.8 ± 0.3	0.9 ± 0.2	1.3 ± 0.6
14.3	Unidentified HC 1		6 ± 2.1	0.3 ± 0.2	3.6 ± 3.3	5.1 ± 3.2	4.6 ± 4
14.5	3MeC13	4.7 ± 1.6	1.6 ± 0.3	0.7 ± 0.3	2.4 ± 1.3		
14.7	C14:1					1.1 ± 0.5	0.7 ± 0.3
15.3	C14	2.4 ± 0.3	1.5 ± 0.1	0.6 ± 0.1	1.6 ± 0.5	1 ± 0.4	2.1 ± 1.1
16.8	3MeC14	2.3 ± 0.3	1.2 ± 0.1	0.4 ± 0.1	0.6 ± 0.2		
17	C15:2	2.6 ± 0.2		0.4 ± 0.1	0.8 ± 0.3		
17.5	C15:1	4 ± 1.1	3 ± 0.8	2.2 ± 1.6	14 ± 3.3		
17.7	C15		1.8 ± 0.2	22.3 ± 4.5	14.9 ± 4.9		
17.9	Unidentified terpene	4.5 ± 0.8	7.6 ± 2.8	0.8 ± 0.4	0.6 ± 0.1		
18.15	Anabasine					23.8 ± 4.4	19.9 ± 4.3
18.8	7MeC15		1.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.2		
18.9	5MeC15	2.3 ± 0.3		**9.2 ± 2.6**	**1.1 ± 0.2**		
19	Anabaseine					25.6 ± 9.5	34.5 ± 10
19.4	3MeC15	2.2 ± 0.2	1.2 ± 0.1	**7.8 ± 1.8**	**1.4 ± 0.4**		
19.5	Unidentified alkaloid					17.2 ± 5.5	11.6 ± 2.7
19.8	C16:1			0.8 ± 0.1	0.4 ± 0.2		
20	C16	3.4 ± 0.7	1.6 ± 0.2	1.6 ± 0.2	1.5 ± 0.5		
20.3	8MeC16			0.5 ± 0.1	0.6 ± 0.2		

20.5	3,7MeC15			0.3 ± 0.2	0.3 ± 0		
21.2	5Me C16			0.5 ± 0.2	1.5 ± 0.2		
21.5	4MeC16			0.4 ± 0.2	1.5 ± 0.6		
21.5	C17:2		1.2 ± 0.1	2.7 ± 0.7	2 ± 0.7		
21.1-21.4	C17:2					3.1 ± 2.1	2.6 ± 1.1
21.75	3MeC16	2.7 ± 0.3	3.3 ± 0.6	1.4 ± 0.2	3.4 ± 0.7		
21.6-21.9	C17:1					1.7 ± 0.5	2.2 ± 1.1
22.2	C17	2.8 ± 0.3	4.6 ± 2.7	8 ± 1.3	8.6 ± 5.6	2.2 ± 0.8	1.3 ± 0.5
23.2	7MeC17		1.2 ± 0.1	1 ± 0.2	1.5 ± 0.5		
23.3	5MeC17			0.7 ± 0	0.3 ± 0		
23.6	C18:2	3.1 ± 0.3	4.6 ± 2.8	1.6 ± 1.1	1 ± 0.5		
23.75	Unidentified HC 2					0.7 ± 0.1	2.3 ± 1.1
23.8	C18:1		1.3 ± 0	0.6 ± 0.1	0.8 ± 0		
24.3	C18		2.4 ± 0.7	1.2 ± 0.5	1.3 ± 0.5	0.8 ± 0.3	4 ± 1
24.6	Unidentified HC 3					1.2 ± 0.4	0.8 ± 0.4
24.6	4MeC18			0.4 ± 0.1	0.4 ± 0.1		
25.2	Hexadecanal	2.1 ± 0.3	2.8 ± 0.7	0.8 ± 0.4	0.7 ± 0.4		
25.8	C19:2	6.3 ± 1.6	13 ± 6.8	6.4 ± 1	14.3 ± 2.6	4.1 ± 1.1	4.5 ± 1.4
25.9	C19:1		1.2 ± 0.1	18 ± 4.7	12.2 ± 3.1	5.9 ± 3.4	1.8 ± 0.7
26.3	C19			2.9 ± 1.9	0.6 ± 0.1	0.8 ± 0.3	0.7 ± 0.3
27.2	9MeC19	6.6 ± 0.2	8.6 ± 5.1	2.5 ± 1.7	0.4 ± 0		

Effects of queen-laid eggs on larval fate

Out of 15 larvae initially including in the group, 6.9 ± 0.7 survived until the pupal stage in the QL-egg treatment. Larval survival was significantly lower in the QR treatment (5.2 ± 0.7; $Z = 2.9$, $P = 0.004$). The QL treatment did not differ significantly from the two others (QL-egg vs QL: 6.9 ± 0.7 vs 5.8 ± 0.5, $Z = 1.861$, $P = 0.063$; QR vs QL: 5.2 ± 0.6 vs 5.8 ± 0.5, $Z = 1.05$, $P = 0.294$).

The queens laid a mean of 24 ± 9.6 eggs per day, showing large interindividual variation. The addition of queen-laid eggs to QL groups neither inhibited nor delayed queen production (Fig. 3.5). At least one larva developed into a queen in 44.4% of the QL-egg groups. Their probability of producing at least one queen did not differ from that of the QL groups (Z = -0.237, P = 0.813), in which queens were produced in 38.9% of the time. As expected, the production of queens in the QR groups was low (13.9%) and differed significantly from the QL-egg groups (Z = 2.344, P = 0.019).

The time to queen production did not differ between the QL and the QL-egg treatments (20 ± 1 vs 23 ± 2 days respectively; t_{13} = 1.556, P = 0.144). However, they both produced queens significantly earlier than the QR treatment (35 ± 2 days to the first queen; QL-egg vs QR, t_{13} = 4.811, P < 0.001; QL-egg vs QR, t_{13} = 3.926, P = 0.002), since queens started to be produced 10 days after removing the MQ at the end of the treatment.

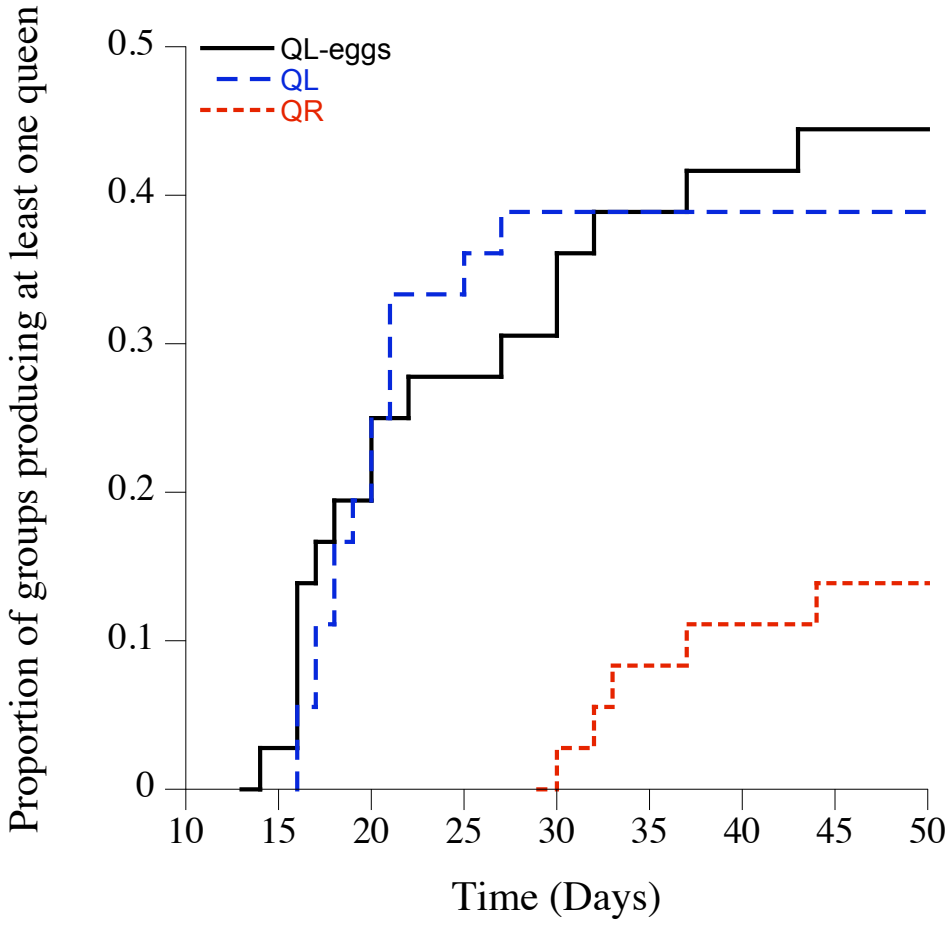


Figure 3.5: Time to queen production when queen-laid eggs were present (QL-eggs, solid black line), the queen was present (QR, larger dashed red line), or the queen was absent (QL, smaller dashed blue line). For each treatment, n=36.

Discussion

Ant queens advertise their presence by producing pheromones that have profound effects on the social harmony, reproduction, and resource allocation of their colonies. Depending on the species, the presence of a mated queen may inhibit worker reproduction and/or the development of larvae into future queens. So far, most studies point to long-chain cuticular HCs acting as the main ant queen pheromones. These compounds were shown to correlate with her fertility and are recognized by workers, or affect their reproduction (Monnin et al. 1998; Liebig et al. 2000; D'Ettorre et al. 2004b; Endler et al. 2004; Holman et al. 2010). However, the non-volatility of these compounds at ambient temperatures raises the question of their transmission within the colony. Queen-laid eggs, which are transported by workers to various nest chambers, have been hypothesized to vehicle the queen signal. However, our results show that in *Aphaenogaster senilis*, larvae still develop into queens despite the biochemical similarity between the queen and her eggs.

The analysis of cuticular HC secretions highlighted several compounds that may be part of the queen signal. Queens' cuticles contained great amounts of long dimethylalkanes, 3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane, that were almost absent in workers. A recent study showed that these compounds are also present in virgin queens, but in lower proportions than in mated queens (Chapter 4). These dimethylalkanes are associated with caste and mating status. They may reflect an individual's fertility. In this case, they would be expected to increase in orphan egg-laying workers. A similar pattern was observed in the ponerine ants *Harpegnathos saltator* and *Pachycondyla inversa*, in which egg-laying individuals had higher amounts of respectively 13,23-dimethylheptatriacontane and 3,11-dimethylheptacosane than non-reproductive workers (Liebig et al. 2000; D'Ettorre et al. 2004b). Our results also indicate caste dimorphism in Dufour secretions, with the queen's gland containing a higher proportion of 5- and 3-methylpentadecane. This result corroborates Smith et al.'s (2012a) findings that the *Aphaenogaster cockerelli* queen's Dufour gland contained more methylalkanes than those of the workers. Given that the proportion of Dufour gland compounds did not differ between virgin and mated queens, the Dufour gland may be the source of caste-related cues rather than fertility signals (Chapter 4). The composition of the poison gland did not differ between castes, indicating that this gland is unlikely to be involved in queen signalling. Finally, other

compounds with higher polarity such as small peptides that are not extracted in dichloromethane might also be involved in queen signalling.

Cuticular and Dufour gland HCs were present on the eggs surface. As when the queen was compared to the workers, queen-laid eggs had more dimethyl alkanes than worker-laid eggs. The proportion of one of the two queen-specific cuticular HCs, 3,11+3,9+3,7-dimethylnonacosane, was greater in queen-laid eggs than in worker-laid eggs. However, HC composition differed between the queen and her eggs. Although the composition of cuticular HCs of *C. floridanus* adults and their eggs diverged somewhat, Endler et al (2004) also found some similarities in their profiles, as was the case here with *A. senilis*. Several queen-specific compounds were present on the surface of queen-laid eggs, but absent from worker-laid eggs. Our results also showed that proportions of two Dufour gland compounds in worker and queen differ significantly. However, their eggs have similar proportions of Dufour gland-deriving HCs. If a signal of caste or fertility is present in the Dufour gland, it is absent from the eggs. The queen did not mark her eggs with the poison gland, implying that the opening of the abdominal glands is independent in queens of *A. senilis*.

Daily egg addition to the experimental QL-egg groups did not inhibit queen production. In contrast, the control QR groups from which the eggs were removed every second day did not produce any queens until day 35. A previous study showed that emission of queen pheromones prevents the development of the larvae into queens (Boulay et al. 2007). Additionally, the queen's body shape or queen's behaviour toward workers or larvae might also prevent the production of queens. Vargo and Passera (1991) showed that adding queen-laid eggs to QL groups did not inhibit queen production in the ant *Linepithema humile*. They suggested that the queens produced could have developed from the added eggs. This proposition is unlikely in the present study, since the first queen pupa appeared at day 16 and egg incubation in *A. senilis* lasts about 30 days (Boulay et al. 2009). The presence of eggs, however, affected larval survival. More larvae reached the pupa stage in the groups to which queen-laid eggs were added than in the QR groups. Cannibalism of eggs could affect brood survival, as it can provide additional nutrition (Brian and Rigby 1978). Several lines of evidence supported oophagy by larvae in a recent study of the effect of worker-laid eggs on larval development (Chapter 2).

Our results, like those of Vargo and Passera (1991), suggest that queen-laid eggs do not bear the queen signal that inhibits larvae from developing into queens. However,

in *C. floridanus,* queens' eggs were shown to limit worker ovarian development even in absence of the queen (Endler et al. 2004), possibly through queen-specific hydrocarbons. We did not test whether the eggs inhibited worker reproduction in *A. senilis*. If we had done so, we might have found that the amount of queen-specific HCs on eggs was sufficient to limit worker ovarian development but not enough to affect larval development. Alternatively, two different signals may affect worker reproduction and larval development, and the former might be absent from the eggs. Queen-laid eggs bear queen pheromones affecting reproductive decisions in *C. floridanus* but not in *A. senilis* (our results; Endler et al. 2004), which belong to different subfamilies. It may also differ between species of the same subfamily. Transmission of queen pheromones could additionally depend on the chemical nature and properties of the queen signal or on the life history traits of each species. The mean colony size of *C. floridanus* is larger than that of *A. senilis,* which limits contact between queen, workers, and brood. Additionally, the production of queens is a crucial process in *A. senilis* colony life since it allows the colony to survive after queen disappearance (Chéron et al. 2009). Therefore, the queen signal in this species may have been selected to disappear rapidly after queen death in order to transmit up-to-date information on the presence of a reproductive queen and to allow the colony to proceed more quickly. Direct transmission by the queen would reduce the time required for message transmission. As sociality evolved, the formation of colonies and the increasing group size may have selected for efficient transmission of information, such that the colony could rapidly and adequately adjust to its current state. The use of chemical communication in organizing colonial activities has been found to be prevalent; however, much remains to be understood about the transmission of pheromones utilized in colonies of social insects.

Chapter 4

Recognition of caste and mating status maintains monogyny in the ant Aphaenogaster senilis

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Abstract

In social insects, queens typically monopolize reproduction. In monogynous fission-performing species, several potential conflicts are expected between the mother queen and potential future queens (totipotent larvae and new queens), which may challenge reproductive monopoly. Conflicts might be resolved by signalling the presence of a mated queen. Pheromonal signals are generally involved, but their chemical nature and effects are largely elusive. The aim of the study was to determine whether conflicts between queens over the head of the colony are resolved by signalling the queen mating status in the ant *Aphaenogaster senilis*. We contrasted same age newly mated and virgin queens with respect to their releaser and a primer pheromone effects on workers and brood, as well as their cuticular and abdominal gland chemistry. Virgin queens were less effective than newly mated queens in inhibiting the development of the larvae into queens. Yet, potential challenger queens are recognized and heavily aggressed independently of mating status. Chemical analyses showed that mating status was associated with changes in cuticular hydrocarbon and poison gland composition, but not in Dufour gland compositions. Two dimethyl alkanes are singled out as queen specific constituents that signal both caste and mating status. We hypothesised that pheromone emission by virgin queens did not reach the threshold needed to fully inhibit larval development into queens but was sufficiently high to stimulate overt aggression by mated queens. These results provide evidence of the complexity of chemical communication in social insects in which a small number of signals may have a multitude of effects depending on the recipient.

Keywords: Hymenoptera, Age, Queen pheromone, Cuticular hydrocarbons, Dufour gland, Poison gland.

Introduction

In social insects, queens typically monopolize reproduction, while workers do not mate, and rarely reproduce. In many species, colonies are headed by a single queen (monogynous species) and her death generally gives rise to a hopeless situation for the colony, unless queen replacement is possible (e.g., honeybee (Winston et al. 1989), *Monomorium pharaonis* (Edwards 1987), and *Aphaenogaster senilis* (Chéron et al. 2009)). Caste determination (the fate of totipotent larvae to develop either to queen or worker) comprises a potential conflict between the brood, the queen and workers. Kin selection theory predicts that totipotent larvae obtain greater inclusive fitness by developing as queens rather than as workers, since it is more related to its own progeny than that of any other nestmate female (Hamilton 1964; Bourke and Ratnieks 1999b; Nonacs and Tobin 1992). The result of such selfish larvae is supernumerary queens, which is disadvantageous to the colony members, queens and workers alike. It is therefore predicted that either the queen or workers will evolve means to regulate the number of queens that develop in the colony. In species that reproduce by fission, whereby new queens are produced while the mother queen is alive (Peeters and Ito 2001), the conflict may be accentuated since queen and larvae reared as queens are present concomitantly.

Overt conflicts between the newly emerged queens and the mother queen are also predicted in fission-performing species. First, since the daughter queens mate near the natal nest, they may have the opportunity to usurp the mother queen. Second, the new nest is generally established in the vicinity of the mother nest. Since the workers are sisters, they bear similar heritable recognition cues, and thus may not recognize each other as aliens. This provides an opportunity for colony merging and consequently overt conflict between the mother queens and the newly emerged queens. Queens should, therefore, be selected to be aggressive even towards their daughter queens as well as trigger aggression by workers towards the potential usurper queens. Since any such conflict challenges reproductive monopoly, elucidating the mechanisms maintaining monogyny seems important for our understanding of the evolution and maintenance of eusociality.

The stability, efficacy, and complexity of animal societies often rely on an integrated communication system. Recognition of relatedness, sex, caste, reproductive state, and social rank allow individuals to adjust their behavioural and physiological

responses according to the group composition (Komdeur and Hatchwell 1999, Brennan and Kendrick 2006). Efficient communication might, for example, mitigate the above described conflicts over reproductive monopoly (Vargo and Passera 1991, Wenseleers et al. 2003). In the monogynous ant *Aphaenogaster senilis,* both field and laboratory studies indicate the existence of a primer queen pheromone (pheromone that induces physiological modifications and generally generate a delayed behavioural response) that inhibits the development of almost all diploid larvae into new queens (Boulay et al. 2007); new queens develop only when the mother queen disappears or just before colony reproduction by fission (Chéron et al. 2009; Chapter 1). However, the mechanism by which such an inhibition is achieved remained elusive. According to the "honest signalling" theory, the queen pheromone is predicted to reliably signal both her mating status and reproductive potential, and the brood and/or worker behavioural and physiological responses is expected to be proportional to the queen's quality (Kocher et al. 2009). The importance of signalling the mating state in the competition between virgin and newly mated queens over who heads the colony (Tarpy et al. 2004, Boulay et al. 2010), is corroborated by several studies that have shown the occurrence of important changes in pheromone composition to accompany the post-mating physiological modifications (Hora et al. 2008, Kocher et al. 2008, Castella et al. 2009). In the present study, we investigated whether, in *A. senilis*, signalling of the mating status resolves or at least mitigates the conflicts over who will head the colony.

The chemistry of ant queen pheromones was little studied and focused mainly on cuticular hydrocarbons (CHCs) (Monnin 2006; Le Conte and Hefetz 2008). For example, 3,11 dimethyl heptacosane mediates the recognition of egg-layer individuals in *Pachycondyla inversa* (D'Ettorre et al. 2004b); 3-methyl hentriacontane mediates the regulation of worker sterility in *Lasius niger* (Holman et al. 2010); Pentacosane elicits worker policing in queenright colonies of *Aphaenogaster cockerelli* (Smith et al. 2009). Although queen pheromones may have multiple origins (Vargo and Husley 2000), less attention was drawn to the role of queen glandular secretions other than CHCs in queen regulation of social behaviour. In the well studied fire ant *Solenopsis invicta* the poison gland is the source of primer and releaser queen pheromones (pheromones which have short-term effects on the recipient behaviour) (Vander Meer et al. 1980; Vargo 1997), which, among other functions, was shown to elicit the execution of non-timely sexual larvae (Klobuchar and Deslippe 2002). Caste specific secretion was also described in Dufour gland of at least in some ant species, e.g. *A. senilis* and *A. cockerelli*, (Boulay et

al. 2007, Smith et al. 2012a).

In the present study we examined the releaser and primer effects of putative queen pheromones on larval development and worker behaviour, by comparing newly mated queens with virgin queens. As mated queens are generally older than virgin queens, the results may be confounded by age differences. Therefore, we disentangled mating state from age-related signals by contrasting same age virgin and mated queens. We first tested the hypothesis that a queen primer pheromone preventing the development of larvae into queens is present in mated, but not virgin queens. We then tested the hypothesis that, in the presence of their mother queen, both the queen and workers recognize and aggressed mated queens irrespective to relatedness, but are more tolerant towards virgin queens. Finally, we explored the chemical differences between same-age newly mated queens and virgin queens in CHCs and two abdominal gland secretions, the Dufour and the poison glands in order to identify candidate chemicals acting a queen primer and releaser pheromones.

Methods

Colony collection and maintenance

Stock colonies of *A. senilis* were collected in July 2011 in the Doñana National Park. In the laboratory, they were housed in ø2x20 cm test tubes, the bottom of which was filled with water retained by a cotton plug. These tubes were kept in 28x18x11 cm Fluon-lined (to prevent the ants from escaping) containers that served as foraging arena.

Production of virgin (VQ) and newly mated (NMQ) queens in fenced field conditions

On the 8th of August 2011, 13 stock colonies containing abundant brood including totipotent 1st instar larvae were each divided depending on the initial colony size, into either two, three or four groups comprising at least 400 workers. The thirteen groups containing the mother queen (MQ) were kept in the laboratory for the upcoming experiments. In order to generate virgin and newly mated queens, 27 queenless groups (the daughter colonies) were returned to Doñana National Park. They were placed in circular nest-boxes (ø10 cm and 14 or 27 cm high), half-filled with moist sand. These nest boxes were buried about 40 cm deep in the ground to protect the ants from extreme ground surface temperature (>60ºC at midday), and connected by a 20 cm tubing to a

ø23 x 24 cm circular foraging arena placed on the ground surface. Daughter colonies were provided with mealworms and biscuits every second week, and kept under these conditions for almost two months to allow them to rear at least one new queen. As *A. senilis* queens bear short wings, they cannot fly out of the foraging arena for mating, and relied on males from surrounding wild colonies that could fly in and mate with them. The foraging arenas of 19 daughter colonies were left open to allow such males to reach the newly emerged queens. To obtain same-age virgin queens, the foraging arenas of eight colonies were covered with a fine mesh preventing males from entering.

On the 29th of September all the nests were brought to the laboratory, and the queens mating status was diagnosed by the absence of wings (this was verified a posteriori by examining the dissected spermatheca). Nine alate VQs and 13 wingless NMQs were found in 7 and 13 daughter colonies, respectively. The remaining seven daughter colonies either died or did not produce queens. As the development from 1st instar larvae to adulthood is about one month (Ruel, unpublished data), queen age was less than 20 days when returned to the lab.

Effect of queen mating status on the development of the larvae into new queens

To determine whether mated, but not virgin queens emit a primer pheromone that inhibits larval development into queens, 7 daughter colonies containing a VQ and 8 colonies containing a NMQ, were each divided into a QL and a QR group of equal size (between 96 and 200 workers per group). Two of the 7 VQ daughter colonies could not be split because they did not have enough workers. Nevertheless, we created two additional QL groups from another, new, VQ daughter colony . All the brood present in these colonies was removed and replaced by 20 1st instar larvae from their respective mother colonies, to standardize queen production. All groups were placed each in ø2 x 20 cm test tubes containing a water reservoir on its bottom and connected to a ø10x10 cm circular Fluon-lined box.

The colonies were monitored thereafter three times a week during 40 days to record the development of queen pupae. The occurrence of worker- and queen-laid eggs was also monitored on the 13th day of the experiment, which was previously shown to be the time frame for egg laying by orphaned workers (Chapter 1).

Are NMQs more aggressed than VQs?

In order to test whether worker and queens recognized daughter-queens' mating status, we examined the interactions between workers as well as MQs, and NMQs or VQs.

In November 2011, sixteen groups of 150 workers were created from ten daughter colonies and their respective six mother colonies containing the MQ (Table 4.1). Five of the daughter colonies contained a NMQ and five contained a VQ. They were daughters of the respective MQ, and workers were sisters (the time-frame of the experiment was too short to allow the NMQ offspring developing to adulthood). All individuals from the MQ groups were marked with a dot of paint on the head or abdomen to identify their origin.

A total of 15 aggression tests (Table 4.1) were conducted between one the six MQ groups and:

- either a VQ group (n = 5), the VQ was the MQ's daughter
- a related NMQ group (n = 5), the NMQ was the MQ's daughter
- or an alien NMQ group (n = 5; positive control), the two groups were not related (kin).

Table 4.1: Repartition of the six different colonies (each represented by a letter from a to f) between the 15 aggression tests. The tests were performed between a MQ group (MQa to MQf) and a group with either a daughter VQ (VQa to VQe), a related or an alien NMQ (NMQa to NMQd and NMQf).

Mother queen	Virgin queen	Related newly mated queen	Alien newly mated queen
MQ a	VQ a	NMQ a	NMQ b
MQ b	VQ b	NMQ b	NMQ c
MQ c	VQ c	NMQ c	NMQ d
MQ d	VQ d	NMQ d	
MQ e	VQ e		NMQ f
MQ f		NMQ f	NMQ a
	5 tests	5 tests	5 tests

Each MQ and NMQ group was tested only once per day. Bioassays were conducted by gently placing two groups in the same 31 x 20 x 6 cm Fluon-lined box, and observing aggressions between MQ, and NMQ or VG, and their respective workers. The assays were stopped after 30 min or when too many workers aggregated to form an aggressive mass around the queens.

We recorded the time elapsed to first aggression between queens. Additionally, the number of worker aggressions towards queens were recorded every 3 min on 3-level scale: 1: opening the mandibles; 2: bending the abdomen; 3: biting and pulling the appendages. The encounter between workers from different groups was also recorded by video, and workers-workers aggressions were observed on a ø9 cm area during the first 3 minutes, for 10 out of the 15 aggressions tests (3 between non-related, 7 between related workers),

Queen chemical profiles as a function of the mating status

The composition of the CHCs, and Dufour and poison gland secretions of seven VQs and eleven NMQs from the daughter colonies were compared. NMQs and VQs were collected one week after the bioassays, frozen at -20ºC and subsequently dissected to separate the thorax, Dufour and poison glands. The degree of ovarian development, the presence of yellow bodies and a full spermatheca served to determine the queens' reproductive and mating status. Thoraces, Dufour, and poison glands were extracted in 50µL dichloromethane and stored at -20ºC until chemical analyses. Samples were injected into a gas chromatograph equipped of a Flame Ionization Detector. For long chain thoracic hydrocarbons oven temperature was programmed from 130ºC to 240ºC at 15ºCmin^{-1}, then from 240 to 300 at 3ºC min^{-1}. For glandular extracts, temperature was programmed from 60ºC to 210ºC at 5ºC min^{-1}, then 210ºC to 300ºC at 15ºC min^{-1}. Twenty nanograms of eicosane were added to each sample as an internal standard. Peaks were verified by combined gas chromatography/mass spectrometry (AutoSpec Premier, Waters) using the same chromatographic conditions as described previously.

Statistical analyses

All data were analysed with the R-package software v. 2.7.2 (R Core team, 2011). To test the effect of queen mating status on the development of the larvae into new queens and the presence of eggs, we performed three generalized linear mixed models (lme4 package). They were fitted to compare the probability and number of larvae developing into queen, and the probability of egg laying between VQ, NMQ and QL groups. The response variable was the binomial production of queens (binomial distribution), the number of queens produced (Poisson distribution), and the presence of eggs on the 13^{th} day of experiment (binomial distribution). Another generalized linear mixed model was fitted to compare the probability of aggression when workers of different nests

encountered. The response variable was a table containing the number of passive and aggressive interactions observed (binomial distribution). For all the models, the colony was included as a random factor. The significance of each factor levels was assessed by contrast analysis.

The time before an aggression, as well as its intensity, were compared according to the mating status of the queens by performing Mann-Whitney tests. The same tests were used for the chemical analyses to compare the total amounts as well as the relative quantity of each compound between VQs and NMQs. The sequential Holm-Bonferroni correction was used to control family-wise error in multiple comparisons (Tables 4.2 and 4.3).

After calculating the proportion of each compound by comparing peak areas, the relative amounts were standardized by subtracting the mean proportion of a compound across samples to each individual proportion and dividing by the result by standard deviation of the mean (see Boulay et al. 2007). By so doing, all the variables have a zero mean and unit standard deviation. Multivariate analyses were performed to examine the divergence between groups based on their chemical profiles. To that end, we first selected a subset of major compounds. We used thirteen, eleven and nine compounds (respectively for thoracic CHC, the Dufour and the poison glands), each one representing more than 2%, 1% and 0.4% of the total quantity in at least half of the samples of the VQ or NMQ groups. A hierarchical cluster analysis by complete linkage method was performed in order to determine whether NMQ and VQ clustered in two different groups based on the proportion of the previously defined major compounds. The mean proportion of these compounds from 10 workers from foreign colonies was used as an out-group. The Bray-Curtis distance matrix was used to compare the chemical distances between NMQs and VQs (NMQ-VQ) and among NMQs or among VQs (NMQ-NMQ and VQ-VQ) by performing two permutation tests.

Results

Effect of queen mating status on the development of the larvae into new queens

Dissections confirmed that all alate individuals classified as VQs had empty spermatheca, and undeveloped ovaries, whereas a full spermatheca, growing oocytes, and yellow body were observed in all wingless individuals classified as NMQs. Thus,

the presence of wings in queens born in the field is a good diagnostic of the mating status.

Queens were reared in less VQ groups (43%) than in QL groups (80%), and in more VQ groups than in NMQ groups (0%). However, the probability of producing at least one queen in the VQ treatment did not differ significantly neither from that of the QL (Z = 1.674, N_1 = 7, N_2 = 15, P = 0.094),), nor from the NMQ treatments (Z = -0.377, N_1 = 7, N_2 = 8, P = 0.706). On the other hand, the production of queen was significantly higher in the QL than in the NMQ treatment (Z = 2.148, N_1 = 15, N_2 = 8, P = 0.032).

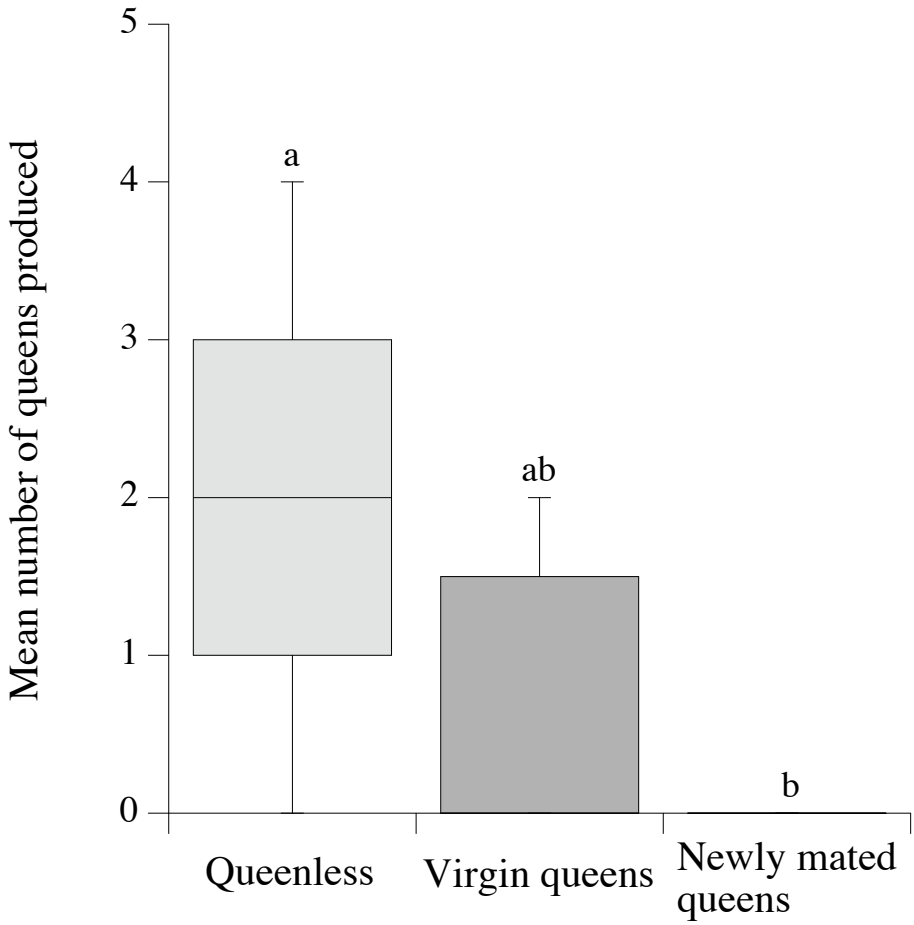


Figure 4.1: Number of queens produced in the queenless, virgin queens and newly mated queens treatments. The different letters denote statistical significant differences.

Less queens were reared in the VQ groups than in the QL groups, but more were reared in the VQ groups than in the NMQ groups (Fig. 4.1). The number of queens produced in the VQ group was neither significantly different from the QL treatment (Z = 1.898, N_1 = 7, N_2 = 15, P = 0.058), nor from the NMQ treatment (Z = -0.752, N_1 = 7,

$N_2 = 8$, $P = 0.452$). The number of queens in the QL group (mean ± SE: 1.8 ± 0.35) was significantly higher than in the NMQ treatment ($Z = 3.054$, $N_1 = 15$, $N_2 = 8$, $P = 0.002$).

All the NMQ groups contained high amounts of eggs before the 13th day. These eggs were most probably laid by the NMQs, judging from their ovarian statuses. The probability to have eggs before the 13th day did not differ between the NMQ treatment and the VQ ($Z = -0.603$, $N_1 = 8$, $N_2 = 7$, $P = 0.547$) and the QL treatment ($Z = -0.653$, $N_1 = 8$, $N_2 = 15$, $P = 0.514$). Eggs were present in 57% and 53% of the VQ and QL groups, respectively ($Z = 0.037$, $N_1 = 7$, $N_2 = 15$, $P = 0.97$), but in much lower quantities than in the NMQ groups (pers. obs). Unlike the NMQ treatment, these eggs were worker-laid, since VQ had undeveloped ovaries and no yellow bodies.

Are NMQs more aggressed than VQs?

Physical contacts between queens were always followed by overt aggression (10 aggressions on 10 physical contacts). Queens bit each other and rubbed their abdomen onto their challenger's body. VQs were attacked by MQs in 4 out of 5 tests, while aggressions between NMQs and MQs were observed in 6 out of 10 tests (3 for daughter and 3 for alien NMQs). Although none of the queens attacked workers, 83.3% of the queens were aggressed by workers. The intensity of aggression by workers did not depend on mating status (Fig. 4.2; aggression index: daughter VQs 13.8 ± 7.7 vs daughter NMQs 11.2 ± 5; $U = 13$, $N_1 = 5$, $N_2 = 5$, $P = 1$).

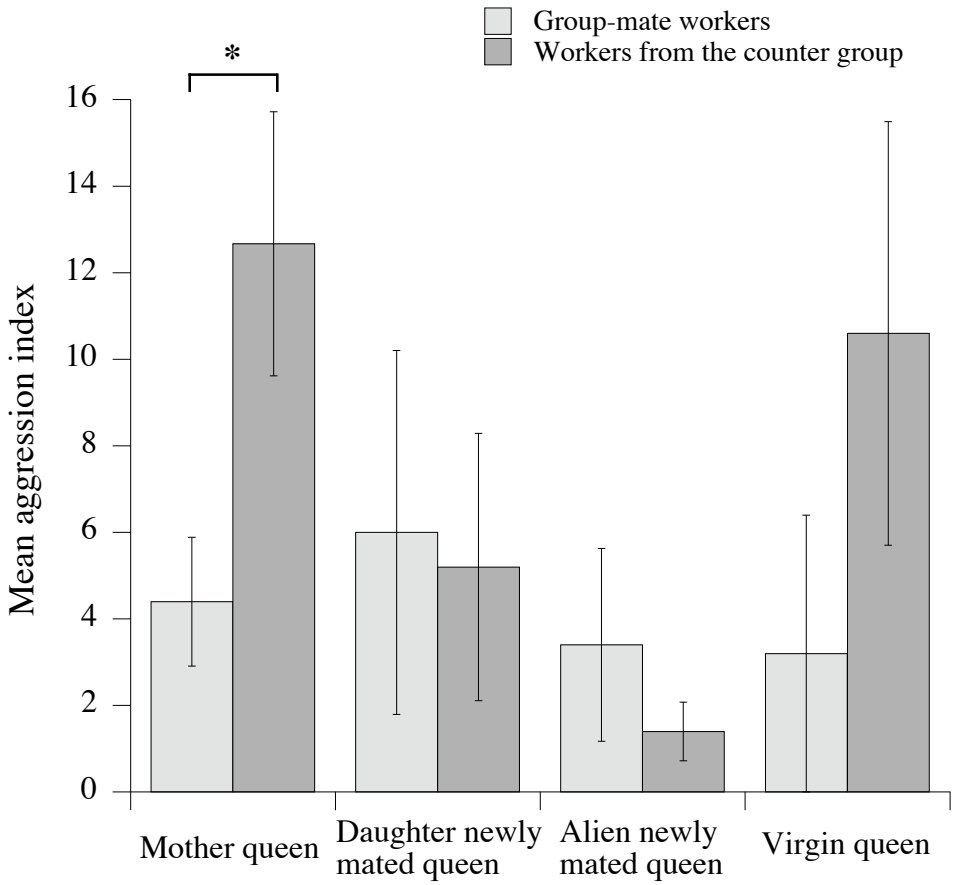


Figure 4.2: Intensity of aggression (Mean ± SE) by nestmate and non-nestmate workers to mother queens, daughter newly mated queens, alien newly mated queens and virgin queens. The star denotes significant difference between the two aggression indices.

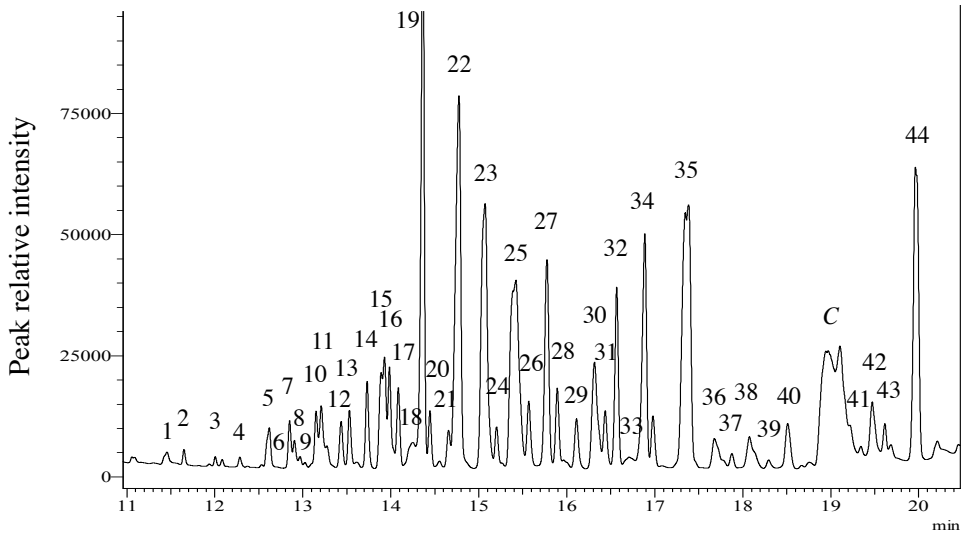
Since colonies were split at the beginning of the study, several encounter involved related (kin) queens and workers from different nests. We tested whether aggressions were based on relatedness or nest membership cues. The intensity of aggression by workers did not depend on relatedness (aggression index: daughter NMQs 11.2 ± 5 vs alien NMQs 4.8 ± 7.7; $U = 15$, $N_1 = 5$, $N_2 = 5$, $P = 0.671$). However, it depends on the nest they were from. Non-nestmate workers attacked more rapidly (8.9 ± 1.6 min vs 12.2 minutes ± 1.7; $U = 606$, $N_1 = 30$, $N_2 = 30$, $P = 0.018$) and were more aggressive (9.2 ± 1.9 vs 4.3 ± 1.1; $U = 318.5$, $N_1 = 30$, $N_2 = 30$, $P = 0.042$) than nestmate workers. Indeed, MQs were more attacked by non-nestmate than by its own workers (Fig. 4.2: $U = 66$, $N_1 = 15$, $N_2 = 15$, $P = 0.048$), but it was not significant for VQs and NMQs (VQs: $U = 8$, $N_1 = 5$, $N_2 = 5$, $P = 0.347$; daughter NMQs: $U = 12$, $N_1 = 5$, $N_2 = 5$, $P = 1$; alien NMQs: $U = 13$, $N_1 = 5$, $N_2 = 5$, $P = 1$).

Out of 256 worker-worker interactions only 6.6% were aggressive. Aggressive interactions between non-related workers were significantly more frequent than between related workers (12.8% vs 3.9% respectively; $\chi^2_1 = 4.236$, $N_1 = 3$, $N_2 = 7$, $P = 0.04$).

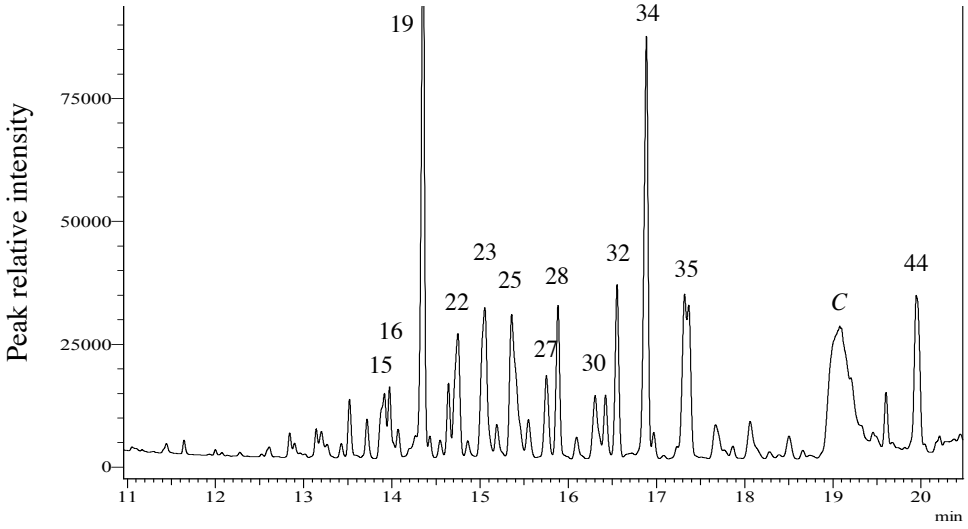
Queen chemical profiles as a function of the mating status

A total of 44 saturated hydrocarbons (HCs) were identified in the queens' thoracic extracts (Fig. 4.3). Two peaks representing a mixture of dimethyl alkanes, 3,11+3,9+3,7DiMeC29 and 3,9+3,11DiMeC31 (peaks 35 and 44 in Fig. 4.3) were queen-specific. They were present both in NMQ and VQ, but almost absent in workers.

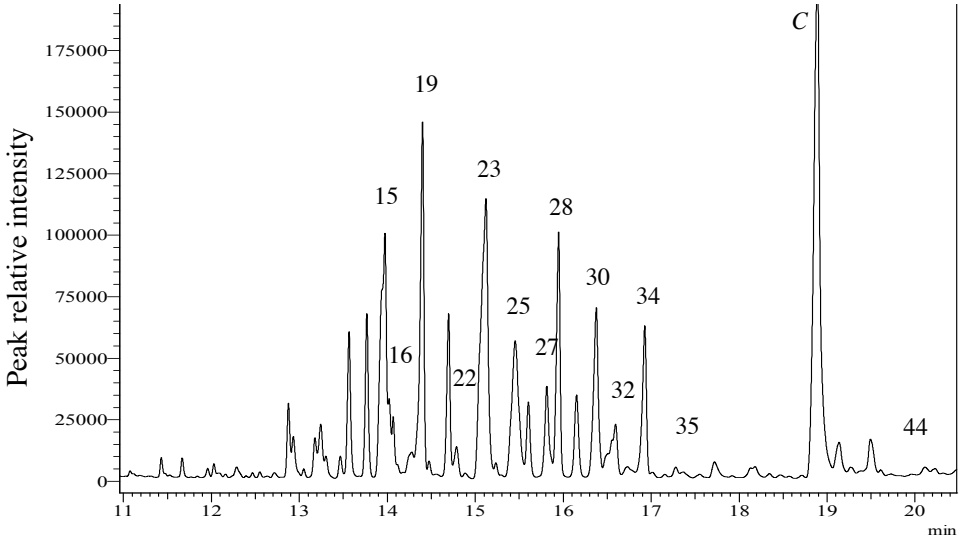
a) Newly mated queen's thorax



b) Virgin queen's thorax



c) Worker's thorax



1	C25:1	10	10,12diMeC26	**19**	**3MeC27**
2	C25	11	8,12+6,10diMeC26	20	5,9diMeC27
3	7MeC25	12	4,8diMeC26	21	C28
4	3MeC25	13	C27	**22**	**3,7+3,9diMeC27**
5	C26	14	4,8,12triMeC26	**23**	**12,14+10MeC28**
6	3,7DiMeC25	**15**	**9+11+13MeC27**	24	6MeC28
7	10+12MeC26	**16**	**7MeC27**	**25**	**4MeC28**
8	8MeC26	17	5MeC27	26	6,10diMeC28
9	6MeC26	18	11,15+7,11diMeC27	**27**	**4,8diMeC28**

28	**C29**	37	6MeC30
29	4,8,12triMeC28	38	4MeC30
30	**11MeC29**	39	6,10diMeC30
31	7MeC29	40	4,12diMeC30
32	**5MeC29**	41	11MeC31
33	11,15+13,15diMeC29	42	11,19diMeC31
34	**3MeC29**	43	3MeC31
35	**3,11+3,9+3,7diMeC29**	**44**	**3,9+3,11diMeC31**
36	10MeC30	C	Cholesterol

Figure 4.3: Three gas chromatograms of cuticular hydrocarbons profiles of a newly mated queen's (a), a virgin queen's (b) and a worker's thorax (c). Only the major compounds (see the Methods) are denoted on the virgin queen and worker profiles. These compounds are in bold in the legend. The cholesterol was not included in the analysis.

The queens separated into two main groups, VQs and NMQs, as revealed by the cluster analysis based on HCs relative proportions (Fig. 4.4a). The Bray-Curtis aggregation distance between NMQs and VQs was significantly greater than that among NMQs or among VQs (NMQ-VQ 0.21 vs VQ-VQ 0.12, $Z = 5.066$, $N_1 = 77$, $N_2 = 21$, $P < 0.001$; NMQ-VQ vs NMQ-NMQ 0.09, $Z = 8.23$, $N_1 = 77$, $N_2 = 55$, $P < 0.001$). Queens' CHC profiles were characterized by a higher proportion of dimethyl alkanes (Table 4.2). Four of the five major peaks comprising dimethyl alkanes (peaks 22: 3,7+3,9diMeC27, 27: 4,8diMeC28, 35: 3,11+3,9+3,7diMeC29 and 44: 3,9+3,11diMeC31) were significantly in higher relative amounts in NMQs than in VQs, while 12,14diMeC28 (in mixture with the inseparable 10MeC28) did not. By contrast, the proportions of all linear alkanes and particular that of nonacosane were lower in NMQs compared to VQs. The total amount of HCs did not differ significantly between queens according to their mating status (Fig. 4.5a; $U = 51$ $N_1 = 7$, $N_2 = 11$, $P = 0.285$). Of the 13 major HCs, only nonacosane was more abundant on VQs than on NMQs thoraces (Table 4.3).

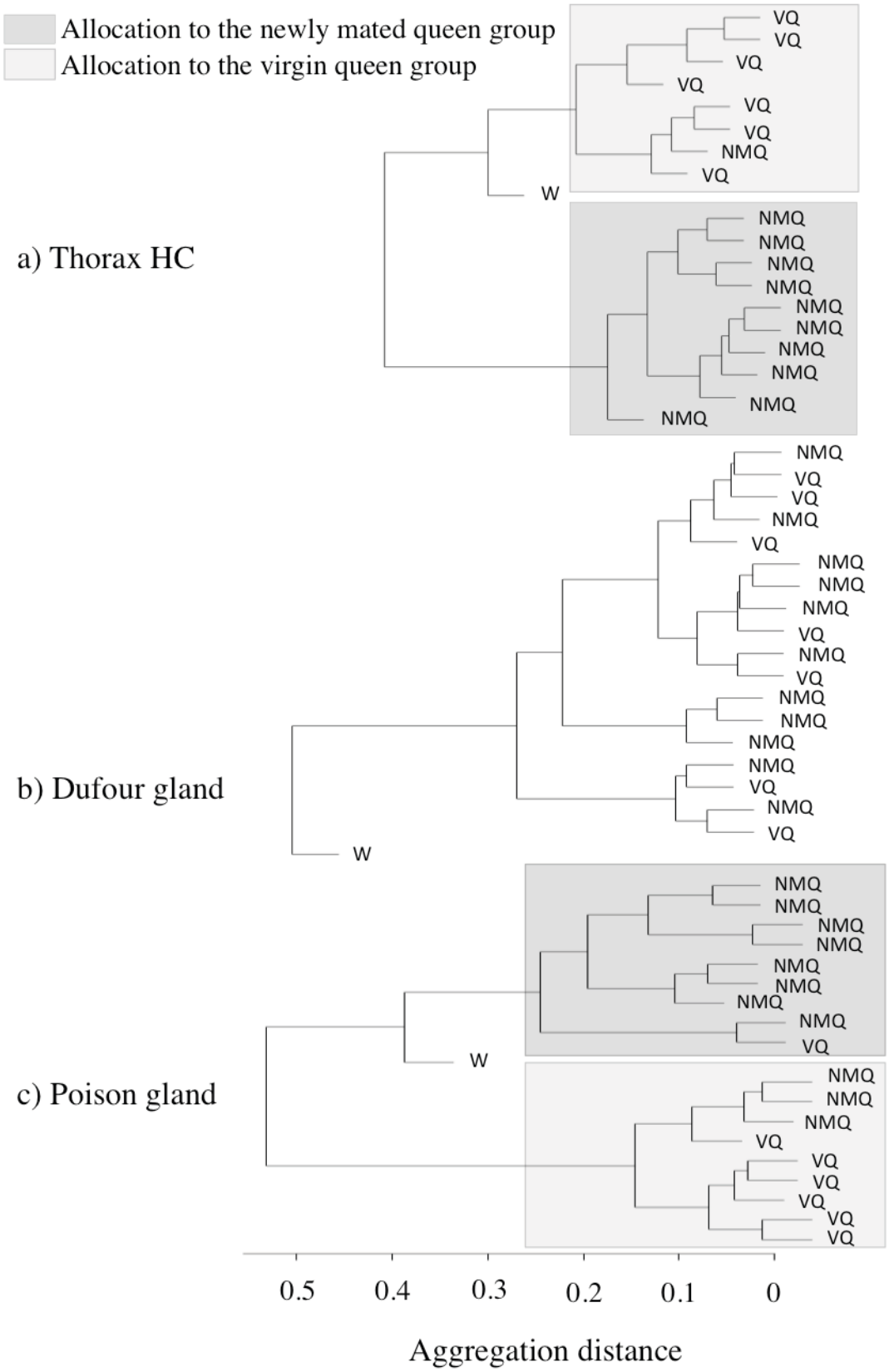
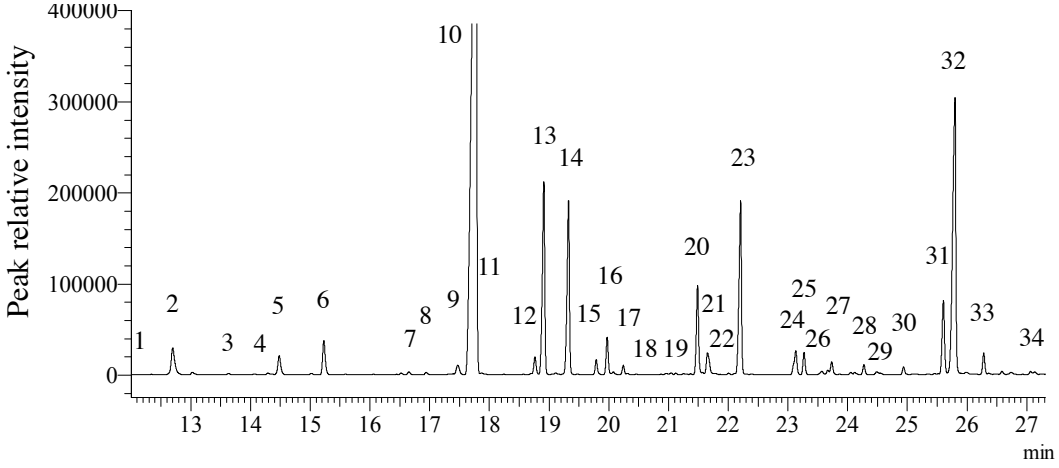


Figure 4.4: Dendrogram of chemical distances based on a) CHC profile, b) Dufour gland profile, and c) poison gland profile between newly mated queens (NMQ) and virgin queens (VQ). The mean profile of 10 workers was added as an out-group (W). Light squares include chemically close individuals, and are by a majority virgin queens. Dark squares include chemically close individuals, and are by a majority newly mated queens.

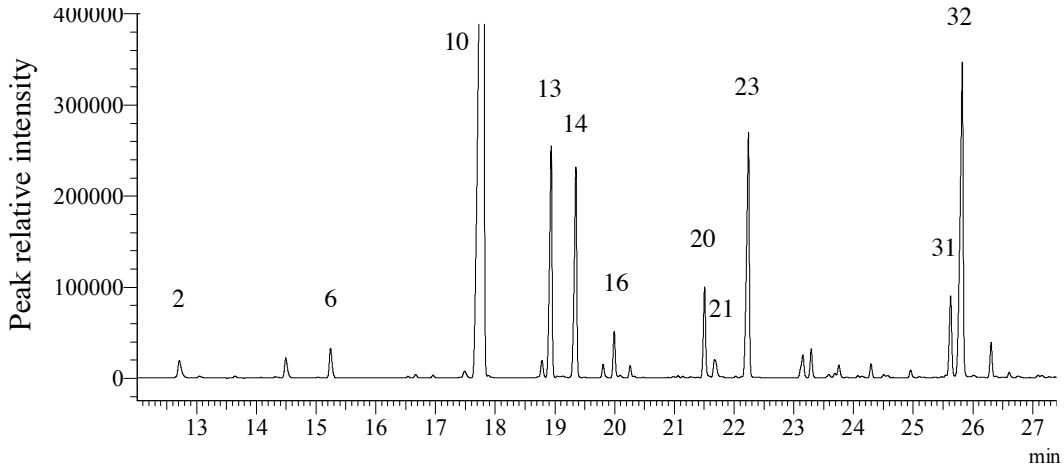
Table 4.2: Proportion of the major compounds on the thorax, in the Dufour and poison glands for virgin queens and newly mated queens (n = 7 and n = 11). (Mann-Whitney test with Holm-Bonferroni correction)

Gland	Peaks number on Fig. 4.3, 4.6, 4.7	Compounds	Virgin queens	Newly mated queens	P_{α}
	28	C29	4.3 % ± 0.4	1.7 % ± 0.1	< 0.004
		Total linear alkanes	*10.5 % ± 0.9*	*5.3 % ± 0.3*	*< 0.017*
	15	9+11+13MeC27	3.3 % ± 0.3	2.7 % ± 0.1	ns
	16	7MeC27	2.4 % ± 0.2	1.7 % ± 0.1	< 0.007
	19	3MeC27	15.1 % ± 0.7	11.6 % ± 0.5	< 0.008
	25	4MeC28	6.9 % ± 0.5	6.8 % ± 0.3	ns
	30	11MeC29	3 % ± 0.2	2.6 % ± 0.1	ns
Thorax	32	5MeC29	3.8 % ± 0.1	3.5 % ± 0.1	ns
HC	34	3MeC29	9 % ± 0.6	5.8 % ± 0.3	< 0.004
		Total methyl alkanes	*62.4 % ± 1.1*	*51.2 % ± 1.3*	*< 0.05*
	22	3,7+3,9diMeC27	5.8 % ± 0.5	9.2 % ± 0.4	< 0.006
	23	12,14diMeC28+10MeC28	8.1 % ± 0.6	6.4 % ± 0.5	ns
	27	4,8diMeC28	2.9 % ± 0.1	3.8 % ± 0.1	< 0.006
	35	3,11+3,9+3,7diMeC29	5.7 % ± 0.8	11.2 % ± 0.6	< 0.005
	44	3,9+3,11diMeC31	2.9 % ± 0.8	7.9 % ± 0.7	< 0.005
		Total dimethyl alkanes	*24.7 % ± 1.5*	*41.3 % ± 1.4*	*< 0.025*
	2	C13	0.9 % ± 0.2	1.7 % ± 0.2	ns
	6	C14	1 %	1.3 %	ns
	10	C15	45 % ± 1.8	41.1 % ± 2.1	ns
	13	5MeC15	5.9 % ± 0.6	7.2 % ± 0.5	ns
	14	3MeC15	5.4 % ± 0.5	6.7 % ± 0.5	ns
Dufour	16	C16	1 %	1.5 % ± 0.1	ns
	20	4MeC16	3 % ± 0.4	3 % ± 0.3	ns
	21	C17:2	1 % ± 0.2	1.3 %	ns
	23	C17	7.4 % ± 0.3	6.4 % ± 0.3	ns
	31	C19:2	3.5 % ± 0.4	3.4 % ± 0.5	ns
	32	C19:1	19.4 % ± 2	15.3 % ± 1.4	ns
	1	C13	0.5 % ± 0.2	1.7 % ± 0.3	< 0.006
	3	Unknown1	0.4 % ± 0.3	1.4 % ± 0.3	ns
	6	Anabasine	4.2 % ± 0.5	3 % ± 0.8	ns
	7	Anabaseine	84 % ± 5.5	69 % ± 3.5	ns
	8	Unidentified alkaloid	7.2 % ± 4.4	18.1 % ± 1.9	ns
Poison	10	C17:2	1.3 % ± 0.3	0.9 % ± 0.3	ns
	14	Unknown2	0.3 %	0.6 %	ns
	17	C19:2	1.2 % ± 0.7	0.9 % ± 0.2	ns
	18	C19:1	0.2 %	1.5 % ± 0.6	ns

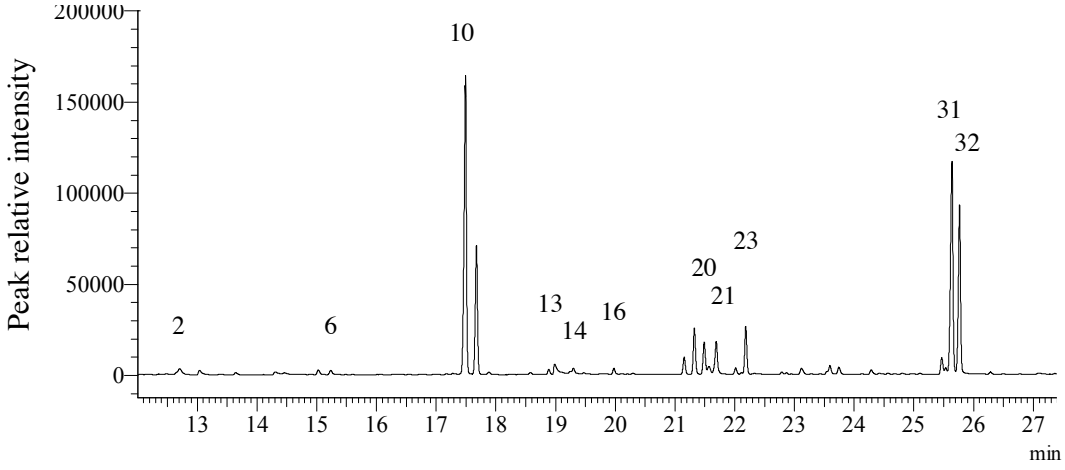
a) Newly mated queen's Dufour gland



b) Virgin queen's Dufour gland



c) Worker's Dufour gland

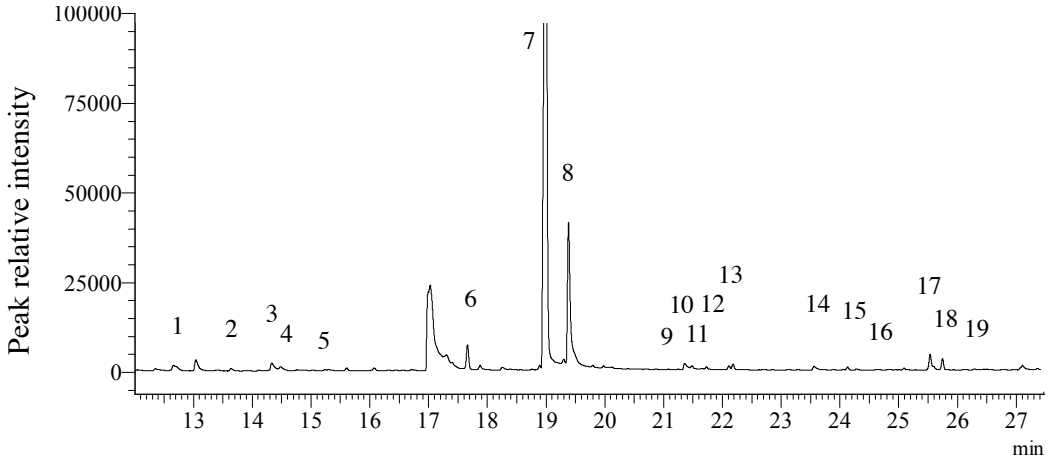


1	C13:1	**10**	**C15**	19	5Me C16	28	C18
2	**C13**	11	Terpene	**20**	**4MeC16**	29	4MeC18
3	5MeC13	12	7MeC15	**21**	**C17:2**	30	Hexadecanal
4	Unknown1	**13**	**5MeC15**	22	3MeC16	**31**	**C19:2**
5	3MeC13	**14**	**3MeC15**	**23**	**C17**	**32**	**C19:1**
6	**C14**	15	C16:1	24	7MeC17	33	C19
7	3MeC14	**16**	**C16**	25	5MeC17	34	9MeC19
8	C15:2	17	8MeC16	26	C18:2		
9	C15:1	18	3,7C15	27	C18:1		

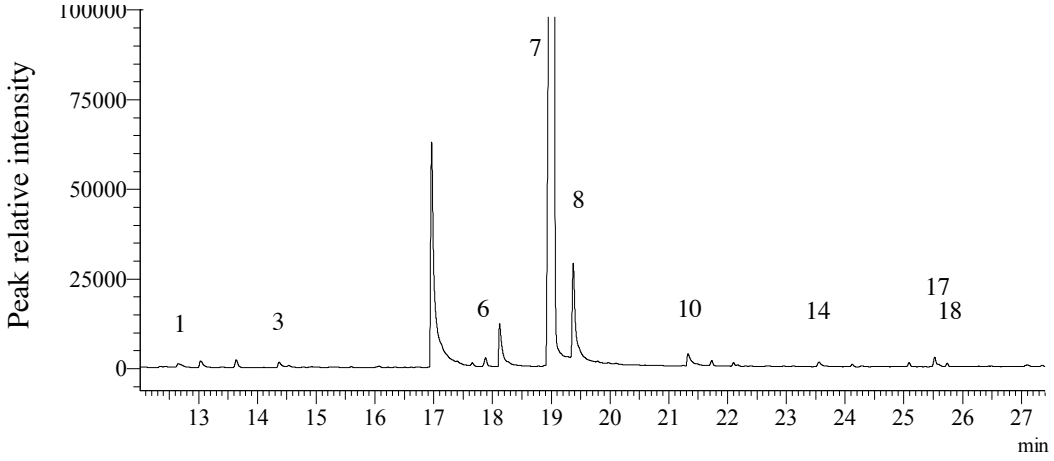
Figure 4.6: Three examples of gas chromatograms of Dufour gland profiles of a newly mated queen's (a), a virgin queen's (b) and a worker's thorax (c). Only the more represented compounds were annotated on the virgin queen and worker profiles. Most compounds were identified and published previously (Boulay et al, 2007).

Dufour glands contained 32 HCs that were accompanied by small amounts of hexadecanal and a yet unidentified compound (Figure 4.6). The relative proportions of the major compounds did not allow differentiation between queens according to their mating status (Fig. 4.4b). The aggregation distances between NMQs and VQs did not differ from those among NMQs or among VQs (respectively 0.13 vs 0.11, $Z = 1.147$, $N_1 = 77$, $N_2 = 21$, $P = 0.247$; 0.13 vs 0.13, $Z = -0.069$, $N_1 = 77$, $N_2 = 55$, $P = 0.943$). This was confirmed by the lack of significant differences in the proportions of the major HCs (Table 4.2). VQs' Dufour glands were more copious than those of NMQ (Fig. 4.5b; $U = 73$, $N_1 = 7$, $N_2 = 11$, $P < 0.001$). Out of the 11 major compounds, 6 were in larger quantities in VQs (Table 4.3). Queens that were previously used in the aggression tests had lower amount of secretion in Dufour gland than those that were not (273.1 ng ± 113.2 vs 601.8 ng ± 96.8 respectively), although this was not statistically significant ($U = 6$, $N_1 = 5$, $N_2 = 6$, $P = 0.126$).

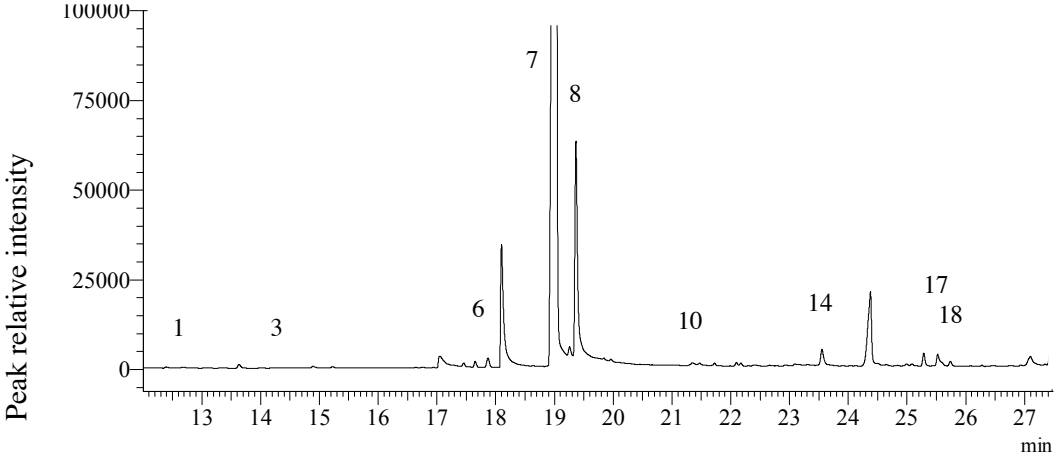
a) Newly mated queen's poison gland



b) Virgin queen's poison gland



c) Worker's poison gland



1	**C13**	**8**	**Unidentified alkaloid**	15	C18
2	5MeC13	9	C17:2	16	Unknown3
3	**Unknown1**	**10**	**C17:2**	**17**	**C19:2**
4	C14:1	11	C17:1	**18**	**C19:1**
5	C14	12	C17:1	19	C19
6	**Anabasine**	13	C17		
7	**Anabaseine**	**14**	**Unknown2**		

Figure 4.7: Three examples of gas chromatograms of poison gland profiles of a newly mated queen's (a), a virgin queen's (b) and a worker's thorax (c). Only the more represented compounds were annotated on the virgin queen and worker profiles. Most compounds were identified and published previously (Lenoir et al, 2011).

Nineteen compounds were identified in the poison gland including three alkaloids, 13 HCs and three yet unidentified compounds (Figure 4.7). Cluster analysis revealed two groups containing mostly NMQ and VQ, respectively (Fig 4.4c). The distances between NMQs and VQs were significantly higher than among NMQs or among VQs (respectively: 0.23 vs 0.15, $Z = 2.558$, $N_1 = 77$, $N_2 = 21$, $P = 0.009$; 0.23 vs 0.16, $Z = 4.29$, $N_1 = 77$, $N_2 = 55$, $P < 0.001$). In terms of relative quantities only tridecane was significantly higher in NMQs than in VQs (Table 4.2). VQs had greater amounts of secretion in their poison gland than NMQs (Fig. 4.5c; $U = 71$, $N_1 = 7$, $N_2 = 11$, $P = 0.002$). VQs poison glands contained significantly more anabasine, anabaseine and heptadecadiene than NMQs (Table 4.3). Queens used in the aggression tests had greater amount of secretion than those that were not (respectively 187.6 ng ± 22.2 vs 53.7 ng ± 9.4, $U = 30$, $N_1 = 5$, $N_2 = 6$, $P = 0.004$).

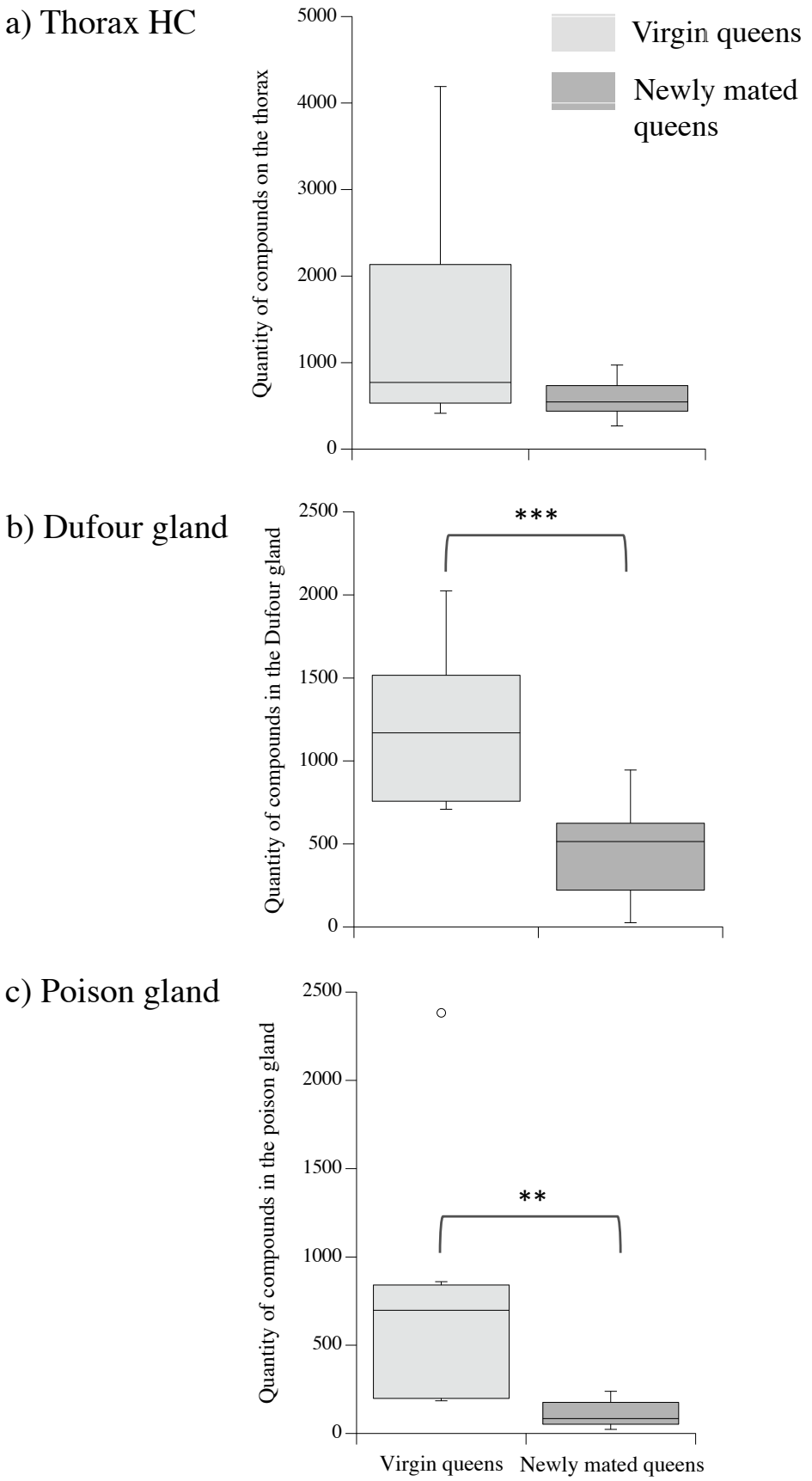


Figure 4.5: Total amounts of compounds (in nanograms) a) on the thorax, b) in the Dufour and c) in the poison glands in virgin queens (light box, n = 7) and newly mated queens (dark box, n = 11). Significant differences are denoted as followed: ** $P < 0.005$ and *** $P < 0.001$.

Table 4.3: Quantities of compounds on the thorax, in the Dufour and poison glands that differ between virgin queens and newly mated queens (n = 7 and n = 11). (Mann-Whitney test with Holm-Bonferroni correction)

Gland	Compounds	Virgin queens	Newly mated queens	*U*	*P-value*	P_{α}
Thorax HC	C29	61.1 ng ± 20	10.3 ng ± 1	77	< 0.001	0.003
Dufour	C15	538.5 ng ± 82.4	200.2 ng ± 43.2	68	0.006	0.0063
	5MeC15	66.1 ng ± 7.7	31.4 ng ± 6.8	68	0.006	0.0071
	3MeC15	60.8 ng ± 7.1	28.5 ng ± 5.8	70	0.003	0.0056
	C16	12.1 ng ± 1.9	5.9 ng ± 1.2	67	0.008	0.0083
	C17	87.4 ng ± 12.5	29.5 ng ± 5.7	75	< 0.001	0.0045
	C19:1	248.4 ng ± 57.5	70.6 ng ± 13.3	72	0.001	0.005
Poison	Anabasine	29.4 ng ± 9	12.8 ng ± 0.4	77	< 0.001	0.0056
	Anabaseine	690.5 ng ± 280.9	81 ng ± 16.7	73	< 0.001	0.0063
	C17:2	8.2 ng ± 2.6	1.4 ng ± 0.7	71	0.002	0.0071

Discussion

Social insects' queens are predicted to continuously advertise their mating status in order to maintain their reproductive monopoly. In *A. senilis*, mated queens inhibit the development of other queens (Ledoux and Dargagnon 1973; Boulay et al. 2007), but whether they do so by affecting worker nursing behaviour or directly influencing larval development is still unknown. The present study shows that newly mated queens are also able to inhibit queen production in 80% of the groups, but VQs did so in only 43% of the groups, lending credence that after mating queens acquire this inhibitory capacity. However, when potential challenger queens are introduced into a QR group, they are rapidly recognized and greatly aggressed, irrespective of their mating status, by both the workers and the resident queen. Chemical analysis has hinted on a possible source for caste and mating status signal, since the mated queen CHCs are affluent with a series of dimethyl alkanes, two of which are almost absent in workers, and are present in lesser amounts in VQs.

The production of queens was low. Limiting the investment in sexuals might be an adaptive strategy in monogynous fission-performing species, whereby queen survival is high during colony foundation and competition between queens is costly as it leads to the death of all but one individual. Few extra individuals may serve as "life insurance" in case the first new queen should accidentally die (Chéron et al 2009).

Workers and MQs discriminated the two castes but might not discriminate between mated and unmated daughter queen challengers. When two distinct groups of

A. senilis were put in contact, queens as well as workers rapidly attacked the opponent queen, irrespective of her mating status. Interestingly, the aggression was specifically targeted at the queens whereas aggression towards workers of the opposing group was low. In a recent study on *Aphaenogaster cockerelli,* queens specifically attacked egg-laying, but not sterile workers (Smith et al. 2012a) indicating that aggressions of workers were based on fertility signals. Why are infertile VQs of *A. senilis* aggressed while infertile worker of *A. cockerelli* are not? Aggressions could be based on two different signals in *Aphaenogaster* ants, a caste signal for queens and a fertility signal for egg-laying workers. Alternatively, the emission of fertility signals, which is correlated to egg laying in workers, could precede ovarian development in the queen caste.

As age was controlled for, chemical differences between VQs and NMQs were only due to physiological changes that accompany mating and egg laying. NMQs exhibited a higher proportion of dimethyl alkanes and a lower proportion of linear and monomethyl alkanes than VQs. Two mixtures of long-chain dimethylalkane that dominated the NMQs profiles were virtually absent in workers and at intermediate levels in VQs, rendering them possible good candidates of both caste and mating status signal. Their greater proportion in NMQs may also constitute a reliable fertility signal. These hypotheses are now waiting for further experimental test. There is growing evidence in other ant species that CHCs correlate with egg laying and serve as fertility signals (Monnin and Peeters 1997, Liebig et al. 2000, Cuvillier-Hot et al. 2001, Hannonen et al. 2002, Heinze et al. 2002, Dietemann et al. 2003, Smith et al. 2008, Holman et al. 2010). For example, 3,11-dimethylheptacosane and 13,23-dimethylheptatriacontane were found in higher quantities in egg-layers of *Pachycondyla inversa* (D'ettorre et al. 2004b) and *Harpegnathos saltator* (Liebig et al. 2000).

Assuming that the queen specific CHCs constitute the basic signal that directs both larval fate and aggression toward a challenging queen, we propose a "threshold hypothesis" for explaining the results obtained in this study. The response to the queen specific signal is threshold dependent, being lower for eliciting aggression than for affecting larval development. We propose that the average amount of these dimethyl alkanes measured in VQs was just at the threshold level. Only queens that were above average (43%) were able to inhibit larval determination into queens. In contrast this average quantity was well above the aggression threshold so that all queens, irrespective of their mating status were aggressed. The MQs and the workers could also recognize

challenging queens by their cuticular dimethylalkanes. Their sensitivity threshold would be low in this context and even the lesser amount present in VQs would be sufficient for eliciting an aggressive response. The same pheromones could have both releaser and primer effects, but the sensitivity threshold of the recipient (workers, queens or larvae) in a given context (recognition or larval development) would determine their effect.

Dufour and poison glands were more copious in VQs than in same-age NMQs. The glandular content could serve as a sex pheromone, explaining its diminution in mated queens (Vander Meer et al. 1998, Buschinger 2003). Alternatively, but not exclusively, mated queens could regularly discharge their abdominal glands during contests as well as other marking-related behaviours (Vander Meer and Morel 1995, Bhadra et al. 2007). On several occasions, MQs and NMQs were observed rubbing their abdomen on the opponent queens presumably marking it. Although we could not detect differences in the secretion amounts between queens used in the aggression tests and queens that were not, these might have been masked by natural variations of the gland amount. It is worthwhile noting that the poison gland is unlikely involved, since the amount of secretion was greater in queens used in the aggression tests compared to queens that were not. Marking an opponent would be another mechanism involved in supernumerary queen elimination. Gamergates (worker-like reproductive females) of the ponerine ant *Dinoponera quadriceps* as well as queens of *A. cockerelli*, mark with Dufour's gland secretion challenging workers with developed ovaries, eliciting their elimination by nestmate workers (Monnin et al. 2002; Smith et al. 2012a). The differences in Dufour gland composition between castes of *A. senilis* (Boulay et al. 2007) suggest that it may be the source of caste-related as well as dominance-related signals. The negligible differences in the secretion composition of the poison gland between VQs and NMQs, exclude the likelihood of it being involved in queen signalling.

Several characteristics define a queen and distinguish her from workers. Our study showed that the mated queen informs the colony of both her caste and mating status in order to maintain her reproductive monopoly. A mated queen inhibit the development of the larvae into queens, she announce intruder queens as potential challenger by actively marking, which in turn triggers the workers to attack the challengers. Our results suggest that the CHCs could be the source of the queen signals. The abdominal glands could also play a role in queen signalling, probably with different functions, like marking the challengers to stimulate worker aggression. Future efforts to

characterize the queen pheromones should be extended to include a wider range of chemicals. In the evolution of sociality an increased need of a precise intra-colonial recognition system arose, including detecting multiple levels of individual identity such as caste, mating status or dominance. Complex mixture of secretions has evolved to signal the presence of a functional breeder and generate an adequate response of the other members of the colony. Pheromone communication between breeder and non-breeder mitigate intra-colonial conflicts and maintain colony harmony.

Chapter 5

Differential ontogeny between castes of cuticular and Dufour gland hydrocarbons in the ant Aphaenogaster senilis

Camille RUEL, Xim CERDÁ, Raphaël BOULAY

In preparation.

Abstract

In social life, signaling the reproductive potential is particularly important for conflict resolution over reproduction among colony members. In ants, queens monopolize reproduction, and should therefore constantly advertise their presence. In the ant *Aphaenogaster senilis*, mated queens emit pheromones that inhibit the development of totipotent diploid larvae into queens. Dimethylalkanes had been hypothesized to be the queen primer pheromone in this species. However, whether they reflect fertility, mating status, caste or age is still unclear. The aim of this study was to investigate whether virgin queen of different ages emit the queen primer pheromones. Virgin queens and workers had different amounts of dimethylalkanes, indicating that these hydrocarbons reflect caste. Then we investigated how cuticular and Dufour hydrocarbons vary with age. We found a temporal dynamic of the amount and composition of cuticular and Dufour gland hydrocarbons. The amount of dimethylalkanes increased in queens with age, while their proportions decreased in workers. However, these divergences between castes were never associated with the emission of the primer queen pheromone that inhibits the development of the larvae into queens. If dimethylalkanes are the queen primer pheromones, they do not reach the threshold in virgin queens needed to fully inhibit the development of the larvae into queens. Even if mating is a key mechanism controlling the emission of queen primer pheromone, our study suggests that age should also be controlled when comparing individuals of different castes or mating status.

Keywords: Social insects, Queen Pheromone, Hydrocarbons, Age, Caste.

Introduction

In groups' lifestyles, up-to-date communication among members is the key to a well functioning group, because it enables the organisms to respond adequately to a changing environment. Rapid emission and extensive sharing of informative signals is usually achieved through chemical communication in social insects. While sex, colony or caste membership remain constant over time, individual state changes over the course of season, according to resource intakes (Den Boer and Duchateau 2006), after mating (Kocher et al. 2008), while laying eggs (Vargo 1999), or switching tasks, etc. Pheromones secretion is therefore expected to adjust to an individual state.

Signaling its reproductive potential is particularly important in queens for conflict resolution over reproduction among colony members and to maintain social cohesion. For example, the honeybee queen mandibular pheromones, 9-oxo-2-decenoic acid and other minor products, inhibit worker ovarian development and new queens rearing (Winston et al. 1989; Hoover et al. 2003). Mating is an important factor regulating the emission of queen pheromones in bees (Strauss et al. 2008; Kocher et al. 2008). Additionally, the age was shown to influence the amount and composition of the mandibular gland compounds (Simon et al. 2001; Wossler et al. 2006; Rhodes et al. 2007). In ants, although the amount and composition of cuticular and post-pharyngal gland hydrocarbons was shown to vary within a short range of days after hatching in workers (Soroker et al. 1995; Dahbi et al. 1998; Lenoir et al. 2001a; Cuvillier-Hot et al. 2001), little is known about the effect of age on pheromones emission in the queen caste. Most studies focused on the correlation between cuticular hydrocarbon profile and ovarian activity (Monnin et al. 1998; Liebig et al. 2000; Oettler et al. 2008) or mating (Hora et al. 2008; Oppelt and Heinze 2009). Disentangling mating state and egg laying from age-related signals is important to understand the production of queen pheromones in ants.

Queen primer pheromones (that induce physiological modifications and generate a delayed behavioural responses in the recipient) are practically unknown in ants. In *Lasius niger*, the queen monopolizes reproduction by secreting a cuticular hydrocarbon, 3-methyl-hentriacontane, that regulates worker sterility (Holman et al. 2010). Little attention was drawn to the role of ant queen glandular secretions, other than cuticular hydrocarbons, in queen signalling. Yet, at least in some species, e.g. *Aphaenogaster senilis* and *A. cockerelli*, important queen-worker differences exist in the composition

of Dufour gland secretion (Boulay et al. 2007; Smith et al. 2012a). In *Aphaenogaster senilis*, monogyny is maintained by the presence of a mated queen. A primer queen pheromone inhibits the development of almost all diploid larvae into new queens (Boulay et al. 2007). However, if the MQ disappears workers rapidly rear emergency replacement queens that are in competition during a few weeks (Chéron et al. 2009; Chapter 2). Replacement of lost queens is important in species that disperse through colony fission, whereby new queens found colonies with a group of workers (Peeters and Ito 2001). Recent studies suggest that dimethylalkanes could be involved in queen signaling (Chapter 3 and 4). Their proportion was greater in queens than in workers, and queens possessed two queen-specific dimethylalkanes. These compounds correlated with caste. However, mating and/or egg laying as well as differences in age could also explain this pattern.

In the present study, we investigated whether virgin queen of different ages emit the queen primer pheromones. We first characterized the dynamic of cuticular hydrocarbons and Dufour gland profiles of virgin queens and workers with age. The evolution of cuticular hydrocarbons profile from birth to 20 and 60 days had already been studied in workers (Lenoir et al. 2001a; Ichinose and Lenoir 2009b). The only study comparing functional queens and workers did not control for age (Chapter 3). We predicted that the amount of cuticular hydrocarbons and abdominal gland compounds should increase and their composition should vary with age. If dimethylalkanes reflect caste, virgin queens should have higher proportions than same age workers. Second, we tested whether the presence of virgin queens of variable ages inhibited the production of new queens. A recent study on *A. senilis* showed that the presence of a 2- to 3-months-old virgin queen inhibited the development of the larvae into new queens in several, but not in all groups (Chapter 4).

Methods

Colony collection and virgin queens (VQs) production

Colonies of *A. senilis* were collected between June 2010 and March 2011 in Doñana National Park. In the laboratory, they were housed in ø2x20 cm test tubes. These tubes were kept in 28x18x11 cm Fluon-lined containers.

In order to obtain VQs, two small groups of about 100 workers and at least 20 young totipotent larvae were separated from each mother colony and left queenless

(QL). Each source group was monitored every two days. Newborn VQs were marked with a dot of colour oil-based paint (Mitsubishi Pencil UniPaint®) on the thorax to record their birthdate. In this species, the impossibility to exchange physical contacts between nestmate workers for two weeks or more is known to provoke the divergence of colony-specific CHCs (Lenoir et al. 2001b). In order to limit such effect between the queenright (QR) colony and the respective QL groups, half the workers from the QL groups were returned to the mother colony every three weeks and replaced by the same number of QR workers.

Chemical profiles of VQs and workers

The chemical profiles of 32 VQs and 24 workers from seven colonies were analyzed. VQs were classified in five age cohorts of one day (n = 4), 20 days (n = 6 thoraces and 7 Dufour glands), one month (n = 5), two months (n = 5 and 4), three months (n = 5 thoraces and 4 Dufour glands), and four months (n = 6). The workers were classified in three age cohorts of one day (n = 7 and 5 for thoraces and Dufour glands), 20 days (n = 8 and 2), and three months (n = 8 and 9). VQs and workers were collected and frozen at -20ºC and subsequently dissected to separate the thorax and the Dufour gland. Ovarian development (developed vs undeveloped), and the presence of yellow bodies were also recorded. Chemical compounds of the thoraces and Dufour glands were extracted in 50µL of dichloromethane and the extracts stored at -20ºC until chemical analyses. 1µL of each sample was injected into a gas chromatograph equipped with a Flame Ionization Detector. For long chain thoracic hydrocarbons oven temperature was programed to run from 130ºC to 240ºC at 15ºCmin^{-1}, and then from 240ºC to 300ºC at 3ºC min^{-1}. For glandular extracts, temperature was programed to run from 60ºC to 210ºC at 5ºC min^{-1}, and then from 210ºC to 300ºC at 15ºC min^{-1}. Twenty nanograms of eicosane were added to each sample as an internal standard.

Data were analyzed with the R package software version 2.7.2 (R Development Core Team, Vienna, Austria). The total amounts and relative quantities of each compound were compared between VQs and workers of different ages. The sequential Holm-Bonferroni correction was used to control family-wise error in multiple comparisons. To reduce the number of variables, we only considered 14 and 12 major compounds of the thorax and the Dufour gland, representing each one at least 4% and 3% of the total amount of compounds on the thorax and in Dufour gland, respectively, in at least half the ants in one group.

Effect of VQs on queen production

A total of 25 groups of 200 workers were formed from eight mother colonies in order to determine if VQs of variable ages emitted the pheromones inhibiting queen production. Seven groups contained a one-week-old VQ from the source group (NVQ), seven contained a one-month-old VQ (MVQ), four contained a two-months-old VQ (OVQ), and seven were left QL. All groups were placed in ø2 x 20 cm test tubes containing a water reservoir on its bottom and connected to a ø10x10 cm circular Fluon-lined box. They were provided with 20 1st-instar larvae from the respective mother colony, and the development of larvae was monitored thereafter four times a week until all larvae reached the pupal stage.

Two generalized linear mixed models (hereafter GLMM; lme4 package) were fitted to compare brood survival and development either castes as a function of the presence and age of the VQs. Response variables were the total number of pupae and the number of queens produced (Poisson distributions). Colony was included as a random factor and the date the experiment started was added as a covariable. The significance of the explanatory variables was tested by analysis of variance based on the Akaike Information Criterion (AIC), and the significance of each factor levels was assessed by contrast analysis. A mixed model fitted by nlme package for R was fitted to compare the time until the production of the first queen. The groups that did not produce any queens were not included in the analysis.

Results

Chemical profiles of VQs and workers

Dissections showed that all VQs had undeveloped ovaries. Though 21.7% workers, all of 3-months-old, had yellow bodies, indicating previous egg laying, none had growing oocytes, suggesting they were not reproductively active at the time of the experiment.

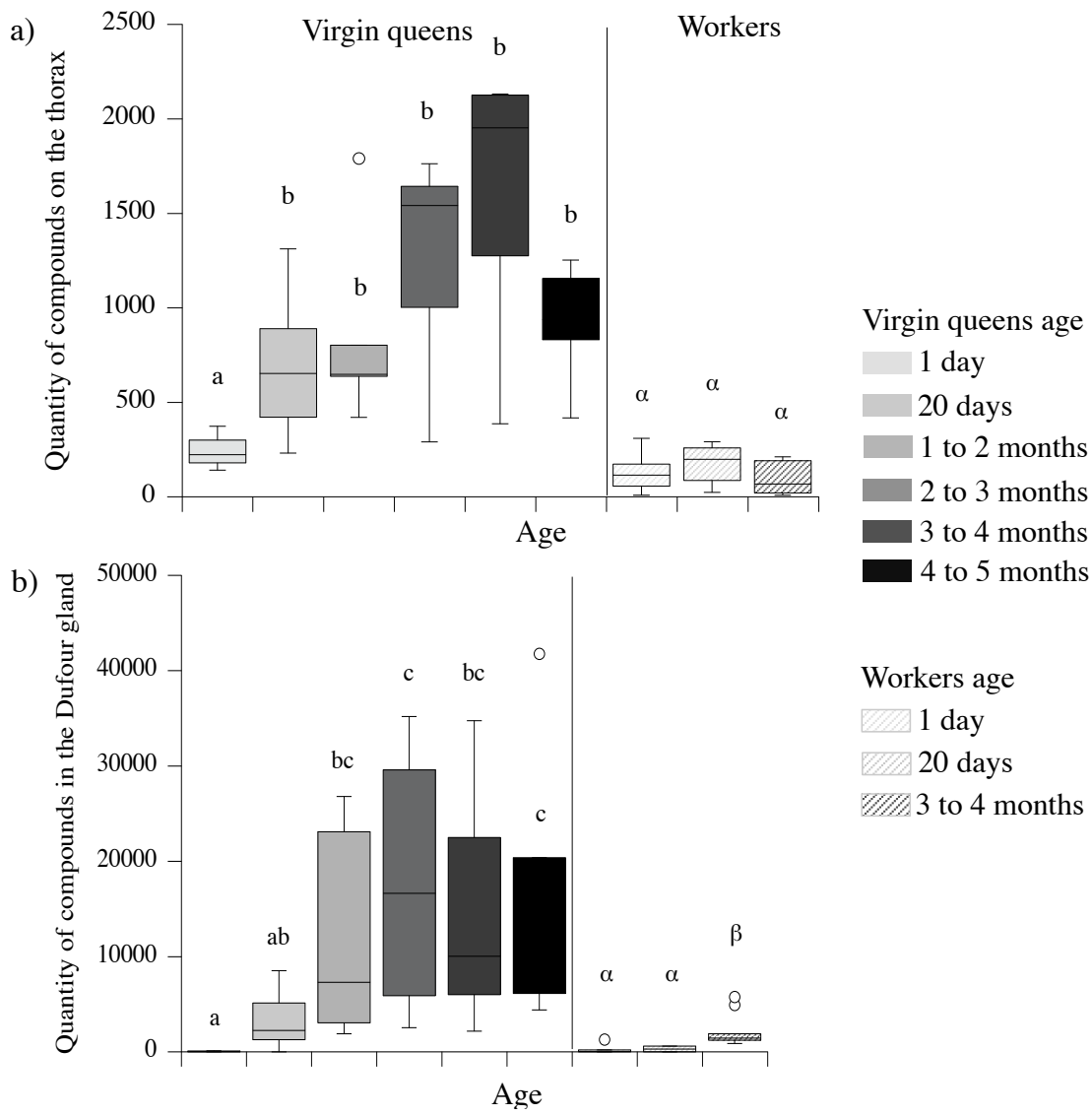


Figure 5.1: Amount of compounds (in nanogram) on (a) the thoraces and (b) in the Dufour glands of VQs and workers of different ages from one-day-old to four-months-old. Significant differences are indicated by letters a, b or c between VQs, and α or β between workers.

A total 44 saturated hydrocarbons, from 25 to 31 carbon chain-lengths, were detected and identified in the females' thoracic extracts. The amount of cuticular hydrocarbons (CHCs) in VQs, but not in workers, increased after birth and was stabilized at 20 days (Fig. 5.1a). At three months, VQs had significantly more CHCs than same age workers (mean ± SE 1574.4 ± 335.7 ng vs 98.4 ± 31.2 ng; Mann-Whitney test: $U = 0$, $N_1 = 5$, $N_2 = 8$, $P = 0.002$). Proportions of linear alkanes and dimethylalkanes respectively increased and decreased from one day to three months in workers (Fig. 5.2a; Mann-Whitney tests: $U = 0$, $N_1 = 7$, $N_2 = 8$, $P < 0.001$; $U = 48$, $N_1 = 7$, $N_2 = 8$, $P = 0.021$) but remain constant in VQs (Fig. 5.2b; Mann-Whitney tests: $U = 18$, $N_1 = 4$, $N_2 = 5$, $P = 0.063$; $U = 9$, $N_1 = 4$, $N_2 = 5$, $P = 0.905$). Consequently, three-

months-old VQs had significantly greater proportions of dimethyl alkanes and lesser of linear alkanes than same age workers (Fig. 5.2). Greater proportion of 3,7+3,9-dimethylheptacosane, 3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane in VQs compared to workers explained differences in dimethylalkanes (Table 5.1). The two former HCs were almost absent in workers. VQs had less octacosane than workers, explaining differences in linear alkanes between castes. One and 20-days-old VQs and workers had same proportions of linear and dimethyl alkanes. Methylalkanes did not differ between workers and VQs the same age.

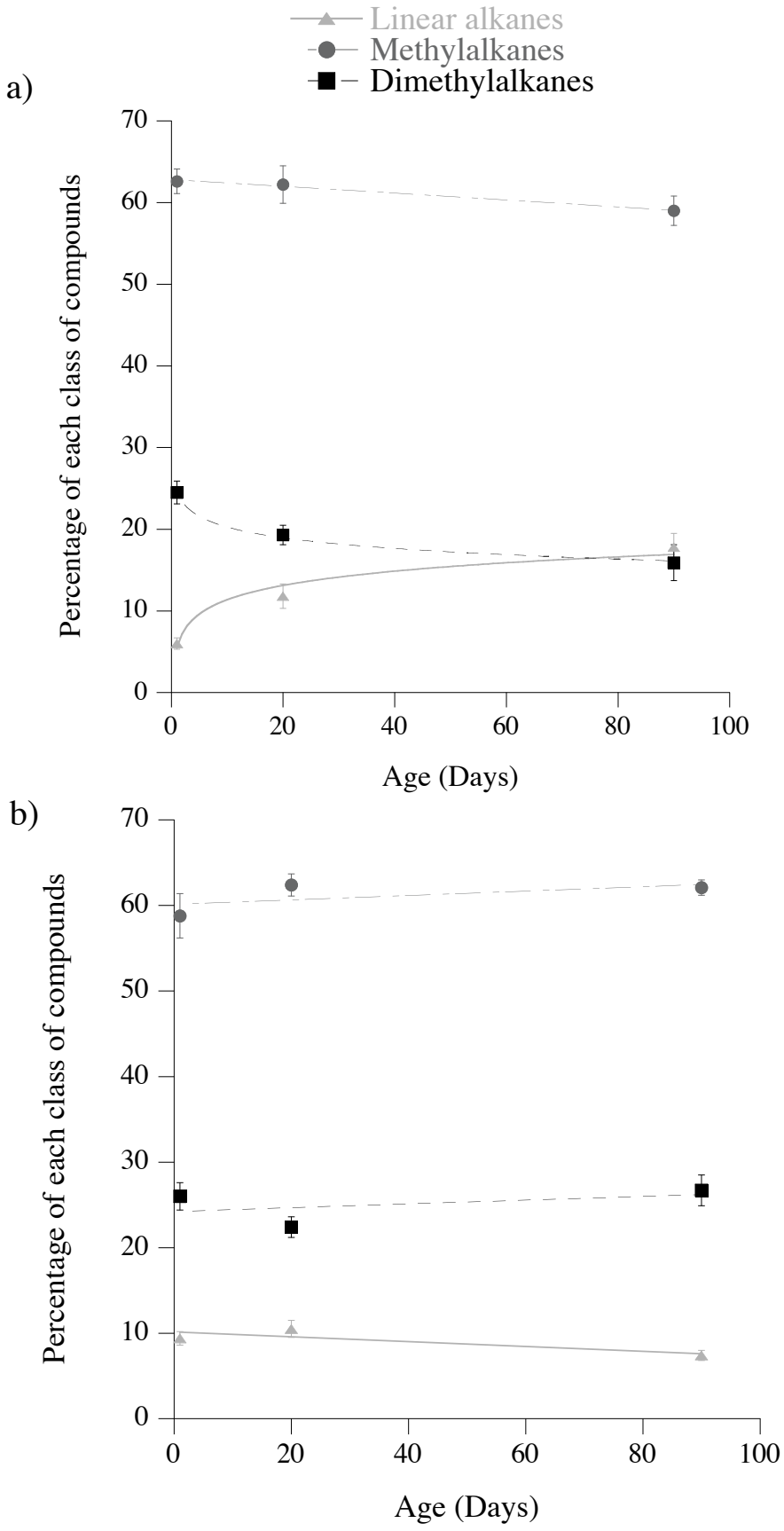


Figure 5.2: Percentages of linear (triangle, light grey line), methyl (circle, grey line) and dimethyl (square, black line) alkanes on the thorax of a) workers and b) VQs of one-day-old, 20-days-old and three-months-old.

Table 5.1: Percentages of the major linear alkanes and dimethylalkanes on thoraces of three-months-old workers and VQs (n = 8 and n = 5). Significant results are shown in bold. ns: non significant. (Mann-Whitney test with Holm-Bonferroni correction)

Class of compounds	Compounds	Workers	Virgin queens	P_{α}
Linear alkanes	C28	**4.9 % ± 0.7**	**1.2 % ± 0.1**	**< 0.017**
	C29	7.5 % ± 1.2	4.1 % ± 0.3	ns
Dimethylalkanes	3,7+3,9diMeC27	**1 % ± 0.1**	**4.8 % ± 0.6**	**< 0.008**
	6,10diMeC28	2.7 % ± 0.2	2 % ± 0.1	ns
	3,11+3,9+3,7diMeC29	**0.6 % ± 0.1**	**6.3 % ± 0.5**	**< 0.01**
	3,9+3,11diMeC31	**0.5 % ± 0.1**	**3.7 % ± 0.6**	**< 0.013**

Dufour glands contained 32 HCs, ranging from C13 to C19 carbon chain-length, accompanied by small amounts of an aldehyde, hexadecanal, and an unidentified HC. The amount of Dufour compounds in VQs increased the first month before stabilizing at 1- to 2-months-old (Fig. 5.1b). The amount of workers' Dufour gland compounds increased after 20 days. At 3- to 4-months-old, VQs had significantly more copious Dufour glands than same age workers (14,254.65 ± 7080.9 ng vs 2264.7 ± 597.9 ng; Mann-Whitney test: $U = 2$, $N_1 = 4$, $N_2 = 9$, $P = 0.011$). Three- to 4-months-old VQs were characterized by a high proportion of pentadecane compared to workers (40.1 % ± 1.6 vs 8.8 % ± 1.2). They also had greater proportions of heptadecane and 5- and 3-methylpentadecane, and lesser proportions of nonadecene and 4-methylcetane than workers (Table 5.2). Pentadecene was almost absent in VQs compared to workers (0.2 % ± 0.1 vs 24.4 % ± 2.7).

Table 5.2: Percentages of the major compounds in Dufour glands of three-months-old workers and VQs. Significant results are shown in bold. ns: non significant. (Mann-Whitney test with Holm-Bonferroni correction)

Compounds	Workers	Virgin queens	P_α
C13	2.3 % ± 0.5	0.7 % ± 0.3	ns
C15:1	**24.4 % ± 2.7**	**0.2 % ± 0.1**	**< 0.004**
C15	**8.8 % ± 1.2**	**40.1 % ± 1.6**	**< 0.005**
Unidentified terpene	0.3 % ± 0.1	0.2 % ± 0.1	ns
5MeC15	**1.1 % ± 0.1**	**4.7 % ± 0.4**	**< 0.005**
3MeC15	**1.4 % ± 0.2**	**4.3 % ± 0.4**	**< 0.006**
4MeC16	**4.3 % ± 0.7**	**0.3 %**	**< 0.007**
C17:2	2.3 % ± 0.8	5.1 % ± 0.3	ns
C17	**3.7 % ± 0.5**	**6.5 % ± 0.6**	**< 0.008**
C18:2	1.1 % ± 0.1	0.5 % ± 0.2	ns
C19:2	**20.7 % ± 1**	**5.4 % ± 0.5**	**< 0.006**
C19:1	16 % ± 1.5	22.6 % ± 1.5	ns

Effect of VQs on queen production

71.2 ± 4.0% of the larvae reached the pupal stage. The presence of VQs did not affect larval survival (Table 5.3, Model 1: $\chi^2_3 = 1.023$, $P = 0.796$). Very few male pupae were produced (three in two groups).

Table 5.3: Results of the model selection (GLMM) testing the effect of the presence of VQs and the date the experiment started on the number of larvae reaching the pupal stage (Model 1 - Poisson distribution), and the number of queens produced (Model 2 - Poisson distribution). Significant results are shown in bold.

	Models	*df*	AIC	χ^2	$\chi^2 df$	P
	Larval survival					
	Virgin queen + Time	8	39.512	1.023	3	0.796
Model 1	Time	5	34.535	7.08	3	0.07
	1	2	35.615			
	Number of queens produced					
	Virgin queen + Time	8	31.535	0.762	3	0.859
Model 2	Time	5	26.298	2.161	3	0.54
	1	2	22.459			

At least one larva per group developed into queen in all the QL groups. Queens were found in 85.7% of the NVQ and MVQ groups. 100% of the OVQ group produced queens. The presence of VQs did not affect the number of queens produced (Table 5.3,

Model 2: $\chi^2_3 = 0.762$, $P = 0.859$). The QL control produced on average 2.6 ± 0.4 queens. In the presence of VQs of increasing age cohort (one-week, one month, two months), 2.4 ± 0.5, 2.1 ± 0.6 and 2.3 ± 0.3 queens were produced (Fig. 5.3).

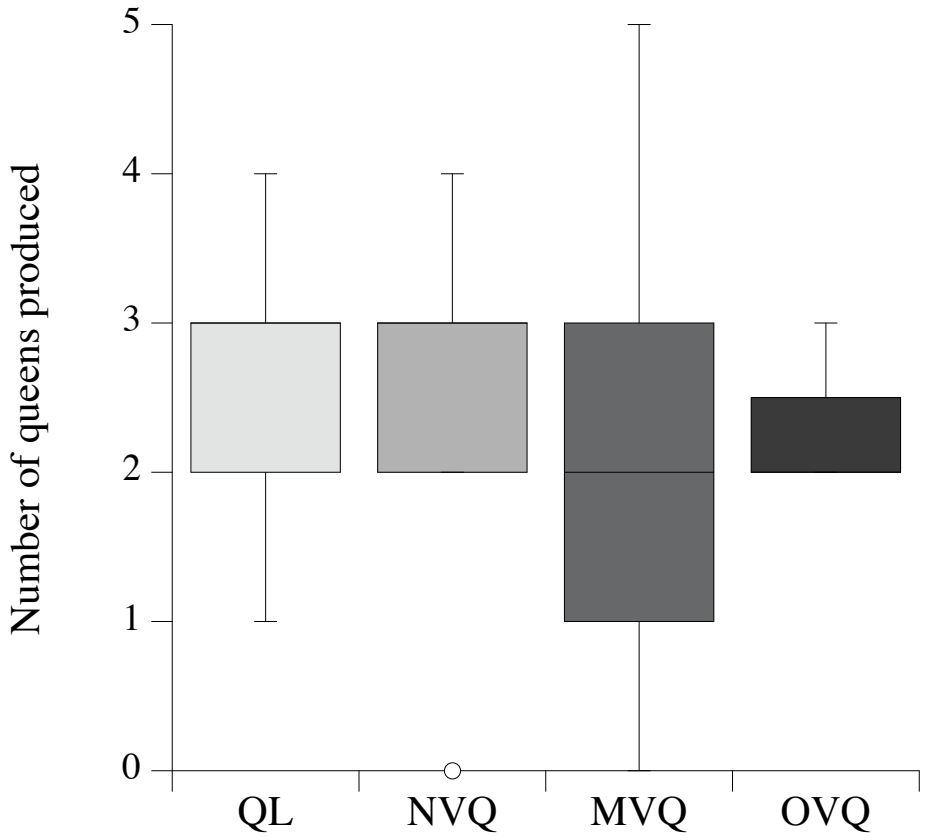


Figure 5.3: Queen production in the QL control (QL, light grey box) compared to the presence of VQs of known ages (NVQ one week, grey box; MVQ one month, dark grey box; OVQ two months, black box). There were no differences in the number of queens produced between each treatment.

Although queens were observed earlier in the QL and NVQ treatments (16.6 ± 1 and 16.5 ± 1.1 respectively) than in groups with older VQs (17.7 ± 1.4 for MVQ groups and 19.25 ± 1.4 for OVQ groups), the time before queen production was not significantly affected by the presence of VQs ($F_3 = 0.704$, $P = 0.573$).

Discussion

In most social insect species, the queen advertises her presence and reproductive potential through pheromones that influence most of reproductive decision and collective or individual behaviours in the colony. Long-chain HCs, particularly branched alkanes, correlate with caste or egg laying in ants (Liebig et al. 2000), and are

thought to be involved in fertility signaling of the queen (Monnin 2006). In *A. senilis*, mated queens emit greater proportions and amount of dimethylalkanes than workers, and some dimethylalkanes are queen-specific (Chapter 3). These CHCs had been hypothesized to be the queen primer pheromone. If it reflects caste, rather than mating status, VQs should produce enough dimethylalkanes to inhibit the development of the larvae into queen. In the present study, we investigated CHCs variation during ontogeny and we tested whether VQs of different ages produced pheromones inhibiting the development of the larvae into queens. We found temporal dynamic of the amount and composition of cuticular and Dufour gland HCs. Three-months-old VQs had more CHCs and a greater proportion of dimethylalkanes than workers. They produced the two queen-specific dimethylalkanes. However, the production of these compounds did not inhibit the development of the larvae into queens, since the presence of a VQ did not prevent the larvae from developing into queens.

Since age and mating status were controlled in our study, physiological caste-related differences explained the divergence in absolute and relative quantities of dimethylalkanes between old VQs and same age workers. Particularly, the worker caste lacked almost completely 3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane, while they were significantly represented in VQs. Additionally, VQs had greater amount of CHCs, suggesting that the amount of dimethylalkanes emitted by VQs is much higher than in workers. In Chapter 4, we showed that mating and/or egg-laying also affected the production of CHCs, since mated queens produced 1.7 times more dimethylalkanes than VQs, 3,11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane were respectively 2 and 2.7 times greater in mated than in virgin queens. In other ant species, branched alkanes are likely to serve as caste or fertility signals (Liebig et al. 2000; D'Ettorre et al. 2004b; Cuvillier-Hot et al. 2001; Johnson and Gibbs 2004; Holman et al. 2010). In *A. senilis*, dimethylalkanes and particularly the queen-specific dimethylalkanes are good candidates of both caste and mating status signals. The contents of abdominal glands increased during the first days after hatching. At maturity, Dufour glands were more copious in VQs compared to workers. Proportions of some alkenes, methyl- and linear alkanes depended on caste, corroborating previous results and suggestion that Dufour gland may be the source of caste-related cues (Boulay et al. 2007; Chapter 3).

CHCs proportions varied with age according to the caste. Composition per class of compounds did not differ in young VQs and workers. But when age increased, strong

differences were observed between castes. Old VQs had 1.7 times more dimethylalkanes than same age workers and 2.4 times less linear alkanes. These differences were only due to changes in the composition of workers CHCs profile, since composition of CHCs remained constant in VQs. Because of different melting temperatures, linear alkanes are thought to confer less permeability to the cuticle (Gibbs 2002; Jonhson and Gibbs 2004) and should thus be involved in the resistance to desiccation, while branched alkane are likely to be involved in communication functions (Dani et al. 2001; Johnson and Gibbs 2004; Monnin 2006). In workers, increasing linear alkanes with age might be an adaptation for task switching from intranidal tasks to foraging activities. In fission-performing species, such as *A. senilis*, queens spend very short time outside for mating and colony founding. Consequently, selection pressures for low cuticular permeability should be weak. Signaling its presence to achieve or maintain monogyny might favor a constant high production of queen signals that may be dimethylalkanes.

Although dimethylalkanes or Dufour gland HCs could be the queen primer pheromones since they correlated with caste, our results showed that the presence of VQs did not affect larval fate, survival and development time. Additionally, the age was not a factor inducing enough production of primer pheromones in VQs. Therefore, if dimethylalkanes are the queen primer pheromones, they do not reach the threshold needed to fully inhibit the development of the larvae into queens in VQs from 1-week- to 3-months-old. This threshold would be reached after mating, since mating and/or egg laying are correlated to a greater proportion of dimethylalkanes. In other ant species, *Monomorium pharaonis* and *Linepithema humile,* the presence of VQs does not influence larval development (Berndt and Nitschmann 1979; Vargo, and Passera 1991). In contrast, in the fire ant, *Solenopsis invicta* virgin wingless egg laying queens produce pheromones that inhibit the larvae from developing into queens (Vargo 1988). The production of these pheromones is probably linked to ovary development in this species, which also could be the case in *A. senilis*, but could not be tested here since VQs do not develop their ovaries.

The presence of an adult VQ does not limit the production of other competitor VQs. But behavioural mechanisms resolve the conflicts between VQs (Chéron et al. 2009; Cronin and Monnin 2009). They interact aggressively and the firstborn is always dominant. Workers also attack the VQs, leading usually to the death of the supernumerary VQ. The production of VQs is reduced in this species (Boulay et al.

2007), which limit competition and the cost of an overproduction of queens for the colony. The production will stop when no totipotent larva remains, or when one queen is mated and starts producing the primer queen pheromone. Dimethylalkanes are good candidates for the queen signal because their proportion depends both on caste and mating status. Overall composition of CHCs is vey plastic and is strongly related to age, caste, and mating status and/or egg laying. Without the effect of mating, chemical profile diverges differently for each caste when aging. These results suggest that age should be controlled when comparing individuals of different castes or mating status.

Chapter 6

General discussion

6.1. Social factors in caste differentiation

In light of our results, we confirmed that the differentiation of the brood was strongly regulated by social factors.

The presence of a mated queen, conveyed by pheromonal cues and behavioural interactions, fully inhibited the development of larvae toward queens. It is the most determinant factor in the investment in sexuals in *A. senilis*.

Our data also demonstrated that the number of workers constrained reproductive decisions. The production of queens correlated with foraging activities, which were reduced in small- compared to large groups. The entire process from foraging to larval feeding is probably determinant for larval development. Food processing is likely less efficient in small groups, which might reduce the quality and/or quantity of food provided to the larvae. Further studies could investigate how limitation in food processing affects larval caste fate.

Our results stress the importance of collective organization in social life. The production of queens depends on the realization of tasks such as food processing at the group level. This may benefit to the colony as a whole. Small groups might not be able to survive and would benefit from investing in worker instead of queen production in order to increase group size.

The number of larvae developing as queens was always low, as expected in fission-performing species. We thus predict a conflict between the totipotent larvae over their fate. This conflict should become stronger with a number of larvae increasing. It might be worthy investigating if all the larvae are equally totipotent, and which larvae are chosen to become queen. Additionally, month of production is hypothesized to be an important factor of queen success. Larvae developing in queens before summer should have higher probability to mate, because male production in the field occurs mostly in June.

The physiological mechanism by which a larva develops toward worker or queen was not investigated in this thesis. Looking at the effect of the queen, the number of workers and the amount of food on juvenile hormone level in larvae and its association to genes expression might shed light on the mechanisms of caste differentiation in ants (Nijhout and Wheeler 1982).

6.2. Nature of the queen signal

An interesting finding of the current study is the potential implication of dimethylalkanes in the queen signal. The queen caste had greater amount of CHCs than the worker caste, and the proportion of dimethylalkanes increased after mating in queens (Fig. 6.1a). Additionally, we found two queen-specific dimethylalkanes, 11+3,9+3,7-dimethylnonacosane and 3,9+3,11-dimethylhentriacontane (Fig. 6.1b).

We demonstrated a strong link between caste and mating status, the amount and relative amount of dimethylalkanes, and the behavioural and physiological responses of the other members of the colony. High amounts of dimethylalkanes in queens correlated with aggressive interactions by queens and workers. Higher amounts in mated vs virgin queens were associated with the development of all larvae in workers (Fig. 6.2).

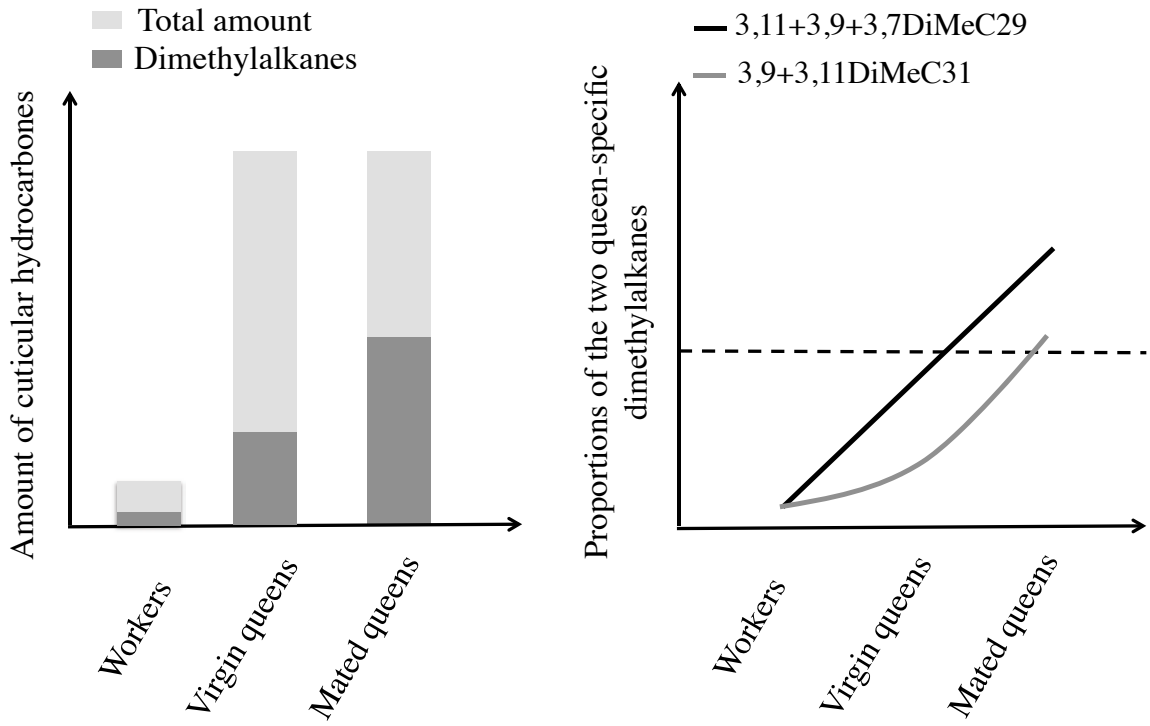


Figure 6.1: Hypothetical models representing variations in a) the amount of CHCs and b) the relative amount of the two queen-specific dimethylalkanes on the cuticule of workers, virgin and mated queens. The dashed line is the hypothetical threshold to reach for the inhibition of queen production. These graphics resume our data, but do not represent real values.

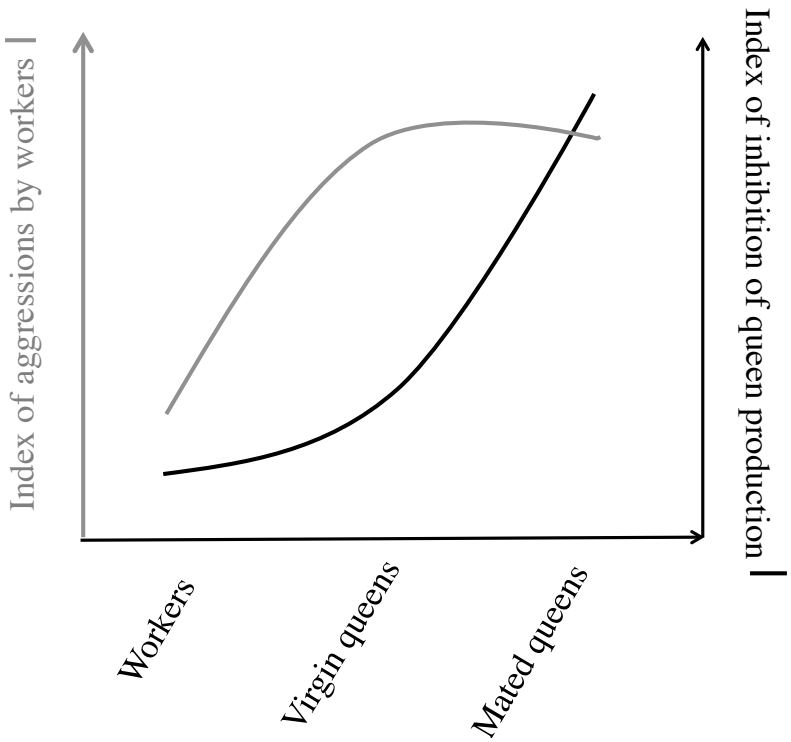


Figure 6.2: Intensity of aggression and inhibition of queen production by workers, virgin and mated queens. This graphic resumes our data, but does not represent real values.

Mating and ovary development trigger changes in CHCs profile in many ant species (Liebig et al. 2000; Dietemann et al. 2003; Hora et al. 2008; Oppelt and Heinze 2009). Mating and/or egg laying was one mechanism leading to high emissions of dimethylalkanes in queens of *A. senilis*, while linear and methylalkanes decreased. Branched alkanes are thought to be used as signals while linear alkanes more likely prevent desiccation (Dani et al. 2001; Johnson and Gibbs 2004; Monnin 2006). Since the presence of mated queens compared to VQs fully inhibited the production of queens, we hypothesized these compounds are a signal of mating status and/or egg laying.

We also showed that the production of CHCs is linked to the age of the individuals. Old VQs were expected to inhibit the production of queens. However, the increasing amount of dimethylalkanes with age in VQs was not sufficient to trigger the development of larvae into worker instead of queens. Yet, virgin and mated queens are discriminated from workers, since they are more aggressed than workers. We hypothesized that dimethylalkanes are a signal of caste. Different amounts of dimethylakanes could inform both of caste and mating status.

We propose a "threshold hypothesis" for explaining our results. The response to the queen-specific signal would be threshold dependent (Fig. 6.1b). If workers recognized the queen caste by their dimethyl alkane cuticular constituents, we must assume that their sensitivity threshold is rather low. Even the lesser amount present in VQs was sufficient for eliciting an aggressive response. This hypothesis also explains our results in the fourth chapter. The lesser amounts of dimethylalkanes in VQs would have just reached the threshold of worker or larvae sensitivity, thence only in part of the groups the inhibition of queen-rearing would come into effect.

This hypothesis suggests that one type of compounds in different amounts would inform of the queen caste, mating status and/or egg laying, and maybe dominance (see chapter 4 and discussion below). The emission of one molecule with different functions would be less expensive than various compounds each one fulfilling one function, but it would be more prone to response errors.

Queen pheromones are predicted to reliably signal her reproductive potential (Keller and Nonacs 1993, Liebig et al. 2000). We give a new insight showing that queens and workers recognized castes, irrespective of their mating status or egg laying. We did not test whether chemical divergences between individuals reflected their fertility. Further studies could investigate whether oogenese and egg laying rate correlate with variations in dimethylalkanes. If these hydrocarbons reflect fertility, their amount would be expected to increase in orphan egg-laying workers. We could not test this hypothesis on VQs, since none was observed laying eggs.

Why dimethylalkanes would be produced in higher proportions in queens than in workers? Higher dimethylation enzymatic activity may be partly linked to oogenese. Comparing the amount of dimethylalkanes in laying and non-laying workers would shed light to this hypothesis. Comparison to close related species and genus might provide further insights into the role of dimethylalkanes in queen signalling in ants and might inform of the evolution in time of such signals.

These results contribute to substantial amount of works showing the important role of CHCs in queen-worker communication (Monnin 2006; Le Conte and Hefetz 2008). The role of the Dufour gland in queen signalling might have been overlooked. We found caste dimorphism in Dufour secretions but no difference between virgin and mated queens, which suggests the Dufour gland might be the source of caste-related cues rather than fertility signals. Additionally, queens of *Aphaenogaster cockerelli* were shown to mark with Dufour's gland secretion challenging workers with developed

ovaries, eliciting their elimination by nestmate workers (Smith et al. 2012a). As *A. senilis* queens were observed rubbing their abdomen onto a challenger, Dufour gland secretions might also serve as dominance-related signals in our species. As in *A. cockrelli*, the queen signals may have various origins including cuticular and Dufour gland hydrocarbons, each with different functions. Further studies are needed to clarify the role of the Dufour gland in queen signalling.

Most of our data suggest that the poison gland is not involved in the inhibition of the development of larvae into queens. In workers, the poison gland is the source of the trail pheromone (Lenoir et al. 2011). Trail marking is not expected to evolve in queens. More studies are needed to explain why the queen caste retained its poison gland.

My Ph.D. thesis provide solid basis for further studies on queen pheromone in *A. senilis*. Next studies should focus on testing the effect of queen extracts and synthetic dimethylalkanes, especially the queen-specific dimethylalkanes, on the development of larvae in bioassays (like in our studies) and on worker behaviour by behavioural experiments combined with electroantennography (see D'Ettorre et al. 2004b). Extending our research to a larger range of compounds, which are not extractable in dichloromethane, using other solvents or other techniques (e.g. absorbing the volatile compounds by trapping them from the vapour phase with an absorbent; see Moritz and Crewe 1991), might reveal new candidate molecules.

6.3. Transmission of the queen signal

If cuticular dimethylalkanes are part of the queen pheromone, they should be efficiently transmitted to most colony members. The non-volatility of long chained HCs at ambient temperatures raises the question of their transmission within the colony.

We tested whether queen-laid eggs are used as vehicles to distribute the queen signal within colonies and prevent larvae from developing into future queens. Our results showed that even though the queen-laid eggs were chemically similar to the queen, their addition to QL groups did not prevent the development of new queens. The amount of queen primer pheromone might not have reached the threshold needed to inhibit the development of the larvae into queens. Nevertheless, oophagy is very likely to occur (see chapter 2) and queen-laid eggs play a role in larvae survival. The presence

and effect of trophic eggs should be tested, as they might provide an important nutrition source for larvae in QL as well as QR conditions.

How the queen signal is transmitted in *A. senilis* colonies remains unknown. As in honeybees, messenger workers could redistribute the queen signal throughout the nest (Seeley 1979; Naumann et al. 1991). This can be tested putting QL workers caring for totipotent larvae in contact with QR workers in absence of the queen. Besides, the production of queens is a crucial process in *A. senilis* colony life since it allows the colony to survive after queen disappearance (Chéron et al. 2009). The queen signal in this species may have been selected to disappear rapidly after queen death. The queen's body shape or queen's behaviour toward workers or larvae might be more important than previously thought to prevent the production of queens. More observations of the queen behaviour toward workers and larvae are needed to understand the effect of the presence of the queen on caste differentiation.

6.4. What's new about fission?

Our study brings new insight on the evolution and maintenance of reproduction by colony fission in *A. senilis*.

The success of colony reproduction should depend on colony size. Queen pheromones in very large colonies were hypothesised to be diluted, leading to the production of new queens and eventually fission event (Boulay et al. 2007). In addition, large colony size might increase colony efficiency, which results in larval rearing into queen rather than workers. The number of larvae seems of lower importance since few larvae are needed to produce at least one queen. Therefore, past queen fertility (determining the number of workers) is a stronger factor influencing the success of colony reproduction than short-term egg laying.

In the field, VQs were found both in QR and QL situations (pers. obs.). Whether new queens are produced before and/or after fission remains to be investigated. If they are produced before fission, totipotent larvae and adult VQs will be in contact with the MQ. Therefore, the emission of queen pheromones should be lowered during colony reproduction events. We hypothesized that totipotent larvae and VQs could escape the MQ when the colony is larger. There also could be seasonal variation of pheromone

emission. The response of workers and larvae to queen pheromones may vary with season too. Adult VQs could be accepted after birth. However, after a 3-month separation (such as a fission event), VQs are strongly attacked. This suggests that nestmate recognition cues override relatedness signal. Therefore, colony merging after colony separation for fission would be more likely restricted to the first weeks.

6.5. Concluding remarks

By using a model system like *A. senilis*, we have been able to assess the importance and the mechanisms by which workers and queen influenced caste differentiation. The collective decision-making over the investment in sexuals depended mainly on whether the workers and the larvae assessed the presence and reproductive state of the queen. Behavioural interactions and pheromonal communications were more relevant than physiological mechanisms in determining the production and survival of new queens. The evolution of sociality might have favoured complex and accurate pheromonal signals between nestmates, which make laborious the study of queen pheromones. Yet, studying chemical communication between queen and workers is crucial to understand the evolution and maintenance of social behaviour.

Conclusions

1) *Aphaenogaster senilis* provides a useful model for investigating conflict resolution and pheromonal communication in ants. We analysed variation in queen pheromones emission, and their effects on reproductive decisions. We also found significant results on the factors influencing caste differentiation.

2) The number of workers constrained reproductive decisions. Larval fate depended on the realization of food processing tasks by the workers at the group level.

3) Our results confirmed that mated queens fully inhibit the development of larvae toward queens.

4) The queen signals her caste and mating status to the colony, which maintains her reproductive monopoly. Both chemical and behavioural cues are involved.

5) Dimethylalkanes are good candidates of the queen primer pheromones since they correlate with both caste and mating status.

6) Although queen-laid eggs are chemically similar to the queen, they do not transmit queen primer pheromones. Further studies on queen pheromone transmission are needed.

7) The production of cuticular hydrocarbons is linked to the age of the individuals.

8) The amount of dimethylalkanes increased with age in virgin queens, but the presence of virgin queens did not inhibit queen production.

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In social insects, only one or a few individuals of a colony mate and reproduce. The production of reproductive queens and non-reproductive workers arises from a developmental switch at the larval stage, and is generally regulated by environmental factors. Workers are expected to influence the production of both castes through the control of larval development. Kin selection theory predicts that the current queen, the workers and the larvae have increasing interest in the production of new queens, suggesting potential intracolonial conflicts. These conflicts are likely resolved by signalling the presence of a mated queen. Pheromones are generally involved in conflict resolution, but their chemical nature and effects are largely elusive.

The present thesis aims at characterizing the social factors regulating caste differentiation in ant colonies. We investigated behavioural, physiological and pheromonal mechanisms that influence the production of queens. The monogynous ant *Aphaenogaster senilis* provides a useful model. The queen prevents the production of new queens by means of pheromonal communication. However, if she disappears, a few replacement queens are reared from the totipotent diploid larvae.

Overall, our results confirmed that the workers and the queen constrain colonial reproductive decisions. In chapter two, we showed that the production of queens correlated with the number of workers. Larval fate depended on the realization of food processing tasks by the workers at the group level. The third and fourth chapters confirmed that the mated queen fully inhibits the development of larvae toward queens. She signals her caste and mating status. Chapters three, four and five pointed at cuticular dimethylalkanes, and especially two queen-specific dimethylalkanes, as good candidates for the queen signal. These results contribute to substantial amount of works showing the important role of cuticular hydrocarbons in queen-worker communication. The third chapter asked for the transmission of the queen pheromones. We showed that queen-laid eggs do not transmit queen primer pheromones. Chapter four investigated the role of the queen's behaviour to maintain her reproductive monopoly. We conclude that collective decision-making over the production of sexuals is mainly shaped by the presence and reproductive state of the queen.

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