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Regulation of reproduction in a queenless ant: aggression, pheromones and reduction in conflict

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In the monogynous queenless ant *Diacamma ceylonense*, the future reproductive (future gamergate) is very aggressive towards infertile workers during the first days of her adult life. Overt aggression disappears at about three weeks, when the future gamergate begins to lay male-destined eggs and is ready to mate. Over the same period, her cuticular hydrocarbon profile alters, changing from a chemical signature similar to that of a sterile worker towards that of a gamergate. In nature, these behavioural and chemical changes will coincide with a reduction in conflict within the nest: faced with a virgin future gamergate, infertile workers have an interest in producing male-destined eggs; however, once the gamergate produces female eggs, they have an interest in rearing her offspring. This demonstration of a shift from physical inhibition to chemical signalling is interpreted in terms of sociogenetic theory, the role of cuticular hydrocarbons as an indicator of fertility in insects and the fact that the regulation of reproduction in *Diacamma* involves mechanisms redolent of both queenless and queenright ant species.

Keywords: reproductive conflict; chemical signalling; cuticular hydrocarbons; *Diacamma*; gamergate

1. INTRODUCTION

In the eusocial Hymenoptera, reproductive conflicts are particularly complex because virgin females can lay unfertilized, male-destined eggs, creating diverging interests between colony members. However, when a mated reproductive is present, virgin relatives always benefit from raising her diploid (female) offspring (review in Bourke & Franks 1995). The question of how workers detect the presence of a reproductive individual lies at the heart of the behavioural bases of sociality.

In many social insects, queens produce a pheromone that ensures that workers do not lay haploid eggs (Vargo 1998). These 'queen pheromones' were long thought to act directly on the physiology of workers, inhibiting egg laying. However, Keller & Nonacs (1993) predicted that if queen pheromones were honest signals rather than physiological inhibitors, then workers should suppress their own reproduction when queens produce both sexes, but not when queens produce only males. Such differential repression would not occur if queen pheromones were inhibitory. In most ant species, no such effect can be observed because queens always mate before they start laying eggs. However, in most queenless ants, future reproductives start to oviposit before they mate and thus have an initial phase of laying male-destined eggs before producing both females and males. This situation provides a unique opportunity for testing the nature and effect of signals produced by the reproductive individual.

In queenless ant species, workers have retained the ability to mate and store sperm (Peeters 1991). All female members of the colony are morphologically similar and

have apparently equivalent reproductive potentials. However, in many species, a single individual monopolizes reproduction. Dominance interactions regulate which ant becomes the 'gamergate' (i.e. inseminated and egg-laying worker) (Peeters 1993). Only dominant workers are sexually active and copulate when a foreign male visits the nest (Ito & Higashi 1991; Monnin & Peeters 1998; Gobin *et al.* 2001). Between the onset of sexual maturity and mating, the 'future gamergate' lays unfertilized male-destined eggs, as other workers could do. During this period, a sharp conflict thus exists between the future gamergate and ordinary workers. However, once the gamergate begins to produce female-destined eggs, the conflict is resolved: as in a queenright species, ordinary workers maximize their inclusive fitness by repressing their ovarian activity and by rearing the female-destined eggs produced by the reproductive. This article focuses on this change in reproductive conflict.

In the monogynous queenless ant genus *Diacamma*, as in other queenless ant species, the future gamergate produces exclusively haploid eggs for a variable period of time. However, the genus *Diacamma* is unique in that monogyny is determined by an irreversible behavioural mechanism, which strongly reduces conflict between workers and the reproductive. Workers eclose with two tiny innervated thoracic appendages ('gemmae'; Peeters & Billen 1991; Gronenberg & Peeters 1993), which are essential for mating (Fukumoto *et al.* 1989; Peeters & Higashi 1989). Soon after a worker ecloses, its gemmae are removed by the gamergate or future gamergate: these mutilated workers can never mate and produce females, although they retain their ability to produce males. In *Diacamma*, the conflict between the gamergate, who produces female-destined eggs, and the rest of the workers, who cannot, is thus similar to that in queenright species,

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and far less than in 'classic' queenless ants, in which dominant individuals retain their ability to mate and can replace the gamergate. The situation in *Diacamma*, combining aspects of both queenright and queenless species, makes it possible to explore whether the regulatory mechanisms involved in the reproductive division of labour vary according to the intensity of the conflict.

Studies of *Diacamma* species have suggested that there are different modes of regulation at different moments in the life of the gamergate. In *D. australe*, the future gamergate persistently harasses nestmate workers, whereas gamergates (that is, fertilized reproductives) are not aggressive, suggesting that mating is associated with a change in regulatory mechanism (Peeters & Higashi 1989). However, this study did not examine changes in the future gamergate's behaviour between eclosion and mating.

In *Diacamma* sp. from Japan, inhibition of worker egg laying requires direct contact with the gamergate, suggesting that she produces chemical signals (Tsuji *et al.* 1999). It is possible that a contact pheromone, perhaps composed of cuticular hydrocarbons, is involved, as is apparently the case in other queenless ants (Peeters *et al.* 1999; Liebig *et al.* 2000). In *D. ceylonense*, there is a striking correlation between cuticular hydrocarbon profiles and ovarian activity (Cuvillier-Hot *et al.* 2001), suggesting that ants may use variations in the blend of hydrocarbons to reveal the fertility of individuals.

In order to see whether there is a relation between regulatory mechanism, fertility, mating status and the nature and intensity of intracolony conflict, we measured changes in behaviour and cuticular hydrocarbons in *D. ceylonense* future gamergates between eclosion and mating.

2. MATERIAL AND METHODS

(a) Samples

Five whole colonies of *D. ceylonense* (231 ± 102 adult workers per colony) were collected from Bangalore (southern India) in October 1998 and April and June 2000. All workers were individually marked with paint, including those that eclosed in the laboratory. In *Diacamma*, the gamergate or future gamergate can be recognized visually because she alone retains her gemmae. The wild-caught gamergate was removed from four colonies. After removal of the gamergate, the first worker to eclose in each of these colonies took on the rank of future gamergate, because there was no reproductive present to remove her gemmae (this is what occurs naturally following the death of the gamergate or colony fission). To increase our sample size of future gamergates, the two largest colonies were divided into three groups of 50–100 mutilated workers together with cocoons and younger brood; 16 future gamergates were thus studied.

(b) Observations

The future gamergates were observed at irregular intervals from the time of eclosion until they were several weeks old, after which they were dissected to measure their ovarian development, using the ad-hoc scale of Cuvillier-Hot *et al.* (2001). During each 30 min observation period, we noted all aggressive events initiated by the future gamergate (antennal boxing followed by crouching of the target individual and forward lunges with biting). Mutilation attempts directed at callow workers were not considered in this study. The onset of egg laying was

determined by regularly checking for fresh eggs in the colonies; we also observed oviposition on a number of occasions. In each group, we recorded the first occurrence of 'sexual calling': this conspicuous behaviour, during which the future gamergate rubs her hind legs on her gaster, continues daily and ceases immediately after mating (Peeters *et al.* 1992; Nakata *et al.* 1998). During the period of behavioural observations, future gamergates were not allowed to encounter foreign males and thus remained virgin, artificially prolonging the period of potential conflict between the future gamergate and the rest of the workers. We also studied two colonies with a gamergate (one wild-caught, and the other mated in the laboratory) ($n = 10$ observations of 30 min each). The gamergates that had been studied were also dissected to verify that their spermathecae were filled with mobile sperm.

(c) Chemical analyses

Cuticular hydrocarbon profiles of living ants were measured with direct-contact solid-phase microextraction (SPME) and gas chromatography. Unlike destructive solvent extraction, SPME allows repeated observations of live insects. A polydimethylsiloxane SUPELCO fibre was rubbed for 2 min against the intersegmental membranes between the sixth and eighth abdominal tergites, after which the ant was returned to her nest (Cuvillier-Hot *et al.* (2001) provide full details of chemical analysis). Eleven future gamergates (10 of which were studied behaviourally) were measured either in the few days after emergence (0–4 days old; $n = 5$; FG1 group), or at several weeks old (25–66 days old; $n = 9$; FG2 group); three future gamergates were thus measured twice. Two future gamergates mated in the laboratory (one was used for behavioural observations, see above); they were each measured three times after mating, at various ages. In the analyses, we included data from Cuvillier-Hot *et al.* (2001) for other functional groups in the colony, i.e. gamergates (Gg, $n = 13$), young callows (C1, 0–4 days old, $n = 17$), nurse workers (C3, 16–42 days old, $n = 11$) and foragers (F, $n = 33$). Future gamergates belonging to the FG2 group were approximately the same age as the C3 workers (or were even older); however, C3 workers were all completely infertile, whereas all FG2 ants had begun to lay eggs. Two future gamergates (out of 16) did not perform sexual calling, yet they had begun to lay eggs. They were subsequently found dead outside the nests, aged 21 and 36 days old. In the days preceding their death, mutilated workers had frequently attacked them. These two individuals were excluded from the chemical analysis (see § 4).

The corrected areas of 19 identified hydrocarbon peaks were used as variables to perform a discriminant analysis. These peaks included the 16 peaks used in Cuvillier-Hot *et al.* (2001), together with two alkanes (n-C26 and n-C30) and an alkene (n-C27:1) chosen on a heuristic basis. Each of these three additional peaks makes up less than 2% of the cuticular hydrocarbons. The discriminant analysis clearly separated the three groups Gg, F and C1 (Wilk's $\lambda = 0.011$; approx. $F_{38,84} = 18.85$; $p < 0.000\ 01$; 100% correct classification; STATISTICA software), which are represented by their centroids. All the other groups were plotted on the graph as 'illustrative individuals' without being involved in the analysis.

3. RESULTS

In the first days of their adult life, future gamergates frequently patrolled and behaved aggressively towards

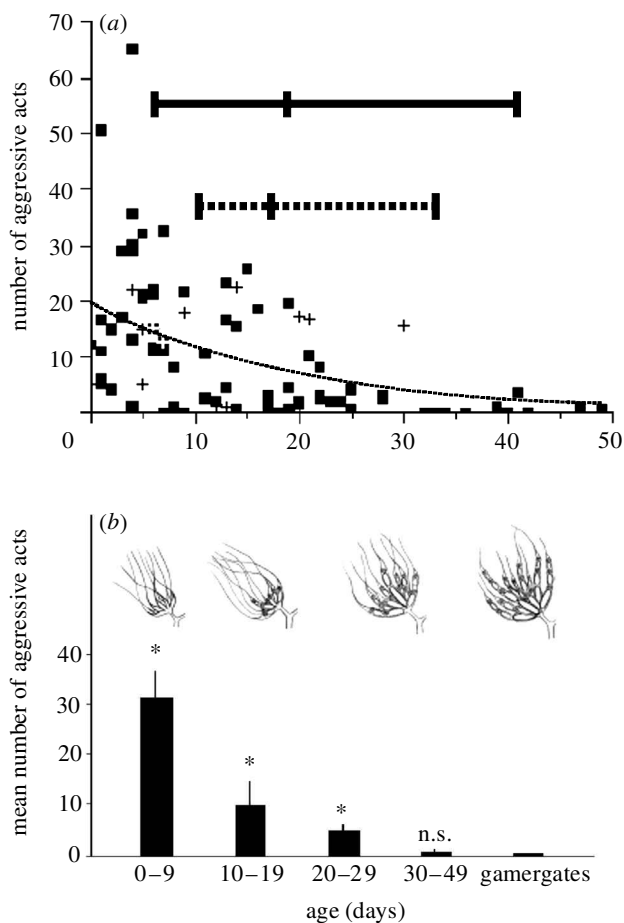


Figure 1. (a) Number of aggressive acts (antennal boxing and biting) performed by future gamergates (FG) at different ages. Each solid square corresponds to a single 30 min period of observation of a single FG ($n = 14$). A polynomial regression curve fits the decreasing trend. Crosses refer to the two FGs that never performed sexual calling and were finally found dead outside the nest (see § 2). The range of the age of onset of sexual calling (continuous line) and of egg laying (dashed line) of these future gamergates is given in the upper part of the figure. The central vertical line on each bar indicates the median value. All individuals remained virgin throughout the experimental period. (b) Data from (a) presented as the mean number of aggressive acts by future gamergates ($n = 16$) at different ages compared with the behaviour of gamergates ($n = 2$, 10 h of observation). Error bars show the standard error. Future gamergates were grouped according to age for statistical analysis. 0–9 days, $n = 15$ observation periods; 10–19 days, $n = 8$; 20–29 days, $n = 9$; 30–49 days, $n = 5$. When an FG was observed for several periods in a given age group, the mean value of her scores was used. Asterisks show significant differences in comparisons between the gamergate group and each age group of future gamergates ($p < 0.005$), as measured by permutation tests. Corresponding changes in ovarian development with age are shown in the upper part of the figure (only one ovary shown).

almost every worker they encountered (figure 1a,b). The latter crouched and never resisted. This aggression decreased progressively from ca. 10 days old, reaching the level typical of a gamergate (mean value = 0.4, $n = 10$ observations) after ca. 30 days. This decline in aggression

is significant (Page test, standardized $F = 2.634$, $p < 0.005$).

Future gamergates began to perform sexual calling at ca. 17 days old (median value; range 10–33; $n = 9$) and began egg laying at ca. 19 days old (median value; range 6–41; $n = 11$). Dissections indicated that all the future gamergates over 24 days of age ($n = 11$) had ovaries with mature eggs (state 4 according to Cuvillier-Hot *et al.* (2001)). Mature eggs can be present when the future gamergate is only 10 days old. There is thus a clear negative correlation between ovarian activity and aggressive behaviour: very young future gamergates cannot produce eggs and they attack nestmates, whereas this aggression stops around the time that egg-laying begins.

An important degree of variability in the initial level of aggression was observed (figure 1a,b). This can be accounted for by colony differences produced by the varying periods between the experimental removal of the previous reproductive from the colony and the appearance of a new future gamergate following eclosion of the first cocoon (0–44 days; median = 6). It takes ca. 13 days (median value, $n = 5$) before the ovaries of orphaned mutilated workers become fully active; as a result, in some colonies, mutilated workers had started to lay male-destined eggs before the new future gamergate eclosed. On six occasions, we observed a worker oviposit in the presence of a future gamergate. This situation is unusual, and all such ovipositions occurred when the future gamergate was less than 5 days old; in two cases, she immediately ate the egg. Dissections ($n = 87$) confirmed that mutilated workers generally have inactive ovaries (state 1 or 2; Cuvillier-Hot *et al.* 2001).

We have previously shown that the gamergates and infertile workers of *D. ceylonense* differ in their blends of cuticular hydrocarbons, and that the cuticular profile of young infertile workers changes with age to become identical to that of foragers (Cuvillier-Hot *et al.* 2001). In the present study, using 19 hydrocarbon peaks (see § 2), gas chromatography data indicated that the cuticular profiles of young future gamergates (FG1) are similar to those of young callows (C1 group; MANOVA, $F = 0.73$, p , n.s., JUMP software; see figure 2). Future gamergates that had begun laying eggs (FG2) had strikingly different profiles to those of infertile workers of the same age (C3). Finally, the ontogeny of the cuticular profile of two young gamergates mated in the laboratory shows a drift towards the group of older gamergates (figure 2b). Note that the FG2 group was not discriminated from the C1 group when only the 16 largest peaks used by Cuvillier-Hot *et al.* (2001) were considered (data not shown)—this can be taken to indicate the importance of small peaks in the cuticular signal. From a characteristic cuticular profile common to all callow workers (with or without gemmae), the profile of a future gamergate becomes similar to that of a gamergate. In parallel, her aggressiveness declines to virtually zero. Future gamergates and gamergates differ in fertility: the cuticle of the gamergates was sampled soon after field collection, when they exhibit maximum rates of egg laying (three eggs per day); in contrast, future gamergates (FG2) had only recently begun to oviposit (one egg per day). This probably explains the difference between FG2 and the gamergate group.

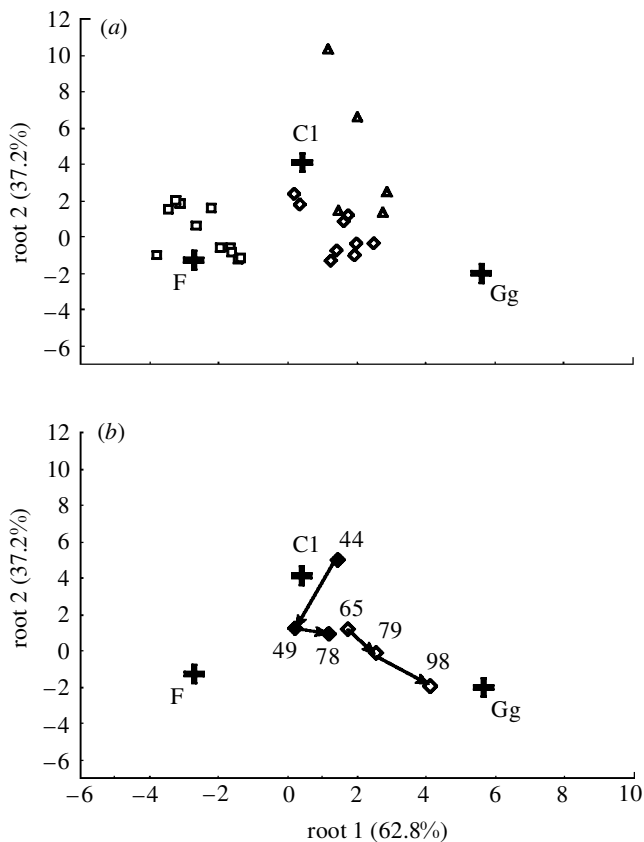


Figure 2. Ontogeny of future gamergate cuticular hydrocarbon profiles. (a) Future gamergates that have begun to lay eggs (FG2, open diamonds) compared with future gamergates that have yet to lay eggs (FG1, open triangles) and nurses (C3, open squares) of the same age as FG2. A discriminant analysis using gamergates (Gg), foragers (F) and young callows (C1, 0–4 days old) (Cuvillier-Hot *et al.* 2001) was used as a framework, as represented by the centroids of the distributions of these three groups; the FG1, FG2 and C3 data were superimposed on this. FG1, infertile future gamergates (0–4 days old, $n = 5$); C1, young callows (0–4 days old, $n = 17$); FG2, fertile future gamergates (25–66 days old, $n = 9$); C3, nurses (16–42 days old, $n = 11$); foragers (> 42 days old, $n = 21$); Gg, gamergates ($n = 13$). (b) Individual changes in the cuticular hydrocarbon profiles of two FGs that were inseminated in the laboratory. Each individual was measured at three different times after mating. The number next to each of the three points refers to the age, in days, of the FG when the measure was taken. Arrows show the direction of change with time.

4. DISCUSSION

After a colony of *D. ceylonense* is orphaned, the first worker to emerge becomes the future gamergate. She is initially very aggressive towards her nestmates, but this aggression decreases dramatically after 3–4 weeks (figure 1). The aggression shown by young future gamergates is exceptional because ant callows are normally timid (this is also true of mutilated *D. ceylonense* callows).

It is significant that the temporal change in the behaviour of the future gamergate and the development of a gamergate-like cuticular profile coincide with the onset of egg laying and of sexual calling (sexual maturity). We suggest that the two phases in her behaviour correspond to different underlying physiological states and relate to dif-

ferent types of conflict within the nest. In the first weeks of adult life, the future gamergate cannot lay eggs or mate; during this period, she uses aggression to subdue the other workers, all of whom could produce male-destined eggs and are thus in conflict with her.

With sexual maturity, which in nature will be rapidly followed by mating and the production of female-destined eggs, one level of conflict within the nest is resolved: ordinary workers now have an interest in rearing the gamergate's female offspring. At this stage, the gamergate stops being aggressive and produces chemical signals that reflect her reproductive state. The shift from physical inhibition to chemical signalling that we have demonstrated here thus coincides with a reduction in within-colony conflict. Artificially induced interqueen conflicts in polygynous ant species show changes between aggressive and chemical control that are associated with differences in reproductive activity (Ortius & Heinze 1999). Our study shows the importance of naturally occurring developmental changes in the regulation of reproduction, in relation to changing genetic conflicts at the colony level.

It might be expected that, because the decisive reduction in genetic conflict within the colony in fact occurs when the gamergate produces female-destined eggs following mating, ordinary workers should be able to detect this event through a putative 'fertilization signal', which is associated with sexual calling, the presence of a foreign male in the colony, mating or even the presence of fertilized eggs. However, by experimentally prolonging the delay between the onset of sexual calling and the production of female-destined eggs, we have shown that the stimulus that leads *D. ceylonense* workers to repress their fertility is an honest signal that the reproductive individual produces before and after mating. This signal reflects her fertility rather than her mated status. The fact that, in a number of cases, egg-laying future gamergates ceased being aggressive before the onset of sexual calling (see figure 1a) can be taken as proof that there is no 'fertilization signal' in this species. It should be noted (figure 1a) that the presence of a fertility signal does not necessarily and immediately mean that eggs are being produced: some individuals clearly use chemical signalling rather than aggression, but have yet to begin egg laying. We interpret this as meaning that the processes underlying the fertility signal are more rapidly effective than those involved in egg production, even though they both have the same physiological starting point.

Using fertility, rather than fertilization, as an indicator of the degree of conflict within the colony would have the advantage of also providing direct information about the quality of the reproductive in terms of productivity. However, if the future gamergate were not to mate, mutilated workers would no longer benefit from remaining sterile but would nevertheless be unable to detect this situation. Although there are no field data on the duration of the period between the onset of sexual maturity and mating, it seems highly improbable that this period will be very long. The stability of the system, and the very fact that *Diacamma* workers cannot detect whether a sexually mature future gamergate has mated or not, suggests that the selection pressure is extremely weak. Furthermore, males are produced throughout the year (R. Gadagkar and C. Peeters, unpublished data): for a future gamergate, the

limiting factor controlling the rapidity of mating will merely be the probability that a foreign male locates her nest.

The premature death of two future gamergates that never performed sexual calling (see §2), and the aggression they were subjected to, may provide us with some clues about the factors that would prevent workers from becoming the victims of such a fertility-based recognition system. We do not know how the workers detected these non-calling future gamergates, which died aged 21 and 36 days. Age was clearly not a factor: some future gamergates were observed for up to three months, during which time they continued to perform sexual calling and the low level of aggression in their colonies remained unchanged, with none of the mutilated workers laying eggs.

Variations in cuticular hydrocarbons are reliable markers of ovarian activity in other ponerine ants and in some wasps (Monnin *et al.* 1998; Liebig *et al.* 2000; Sledge *et al.* 2001). Experimental manipulation of social status leading to predictable changes in cuticular profiles provides even more convincing data of the link between ovarian activity and cuticular hydrocarbons. Our cuticular measures of *D. ceylonense* future gamergates show that their profile develops into one similar to that of a gamergate. We suggest that, as soon as the future gamergate's cuticular profile reveals that she is sufficiently fecund, nestmates recognize her egg-laying status through this signal and consequently suppress their own ovarian activity. As a result, sexually mature future gamergates no longer need to behave aggressively towards their nestmates.

Although bioassay data showing that the ants use cuticular hydrocarbons for recognition do not yet exist, chemical communication of ovarian activity has been demonstrated (Tsuji *et al.* 1999) and variations in cuticular hydrocarbons are a reliable indicator of ovarian activity, at least for human researchers. If the signals produced by the mature future gamergate are cuticular hydrocarbons, our data suggest that relatively small peaks may have a key role. In our previous study (Cuvillier-Hot *et al.* 2001), we analysed differences between *D. ceylonense* ants using 16 peaks. In this study, although these 16 peaks did discriminate FG2 and C3, they were not able to discriminate the FG2 and C1 groups. To obtain a satisfactory discrimination of these two groups, we had to include three additional peaks (n-C26, n-C27:1 and n-C30), which together make up less than 2% of the total cuticular hydrocarbons. Any selection of peaks is, by definition, heuristic—the cuticle of *D. ceylonense* carries up to 76 identified substances (Cuvillier-Hot *et al.* 2001), some, or all, of which are presumably involved in protection against desiccation, as well as distinguishing species, sex, social status and colony membership. The fact that minor peaks are apparently involved in distinguishing social groups is not surprising: the principle of minor substances contributing decisively to a chemical bouquet is well known. Furthermore, surprises may lurk in even the most well studied of chemical systems: small levels of hitherto ignored substances on the cuticle of female *Drosophila melanogaster* have recently been shown to have a fundamental role in mating in this species (Savarit *et al.* 1999).

In other queenless ants, dominant workers can replace the gamergate as the sole producer of female-destined

eggs, and queue to replace her as soon as her efficiency declines, either in terms of fertility or ability to dominate. In these species, in which conflict is particularly strong at the top of the hierarchy (Monnin & Ratnieks 2001), regulation of reproduction relies on both behavioural interactions and chemical communication. For instance, in *Dinoponera quadriceps*, the gamergate continues physical interactions, mainly with the second-ranking worker who has a high probability of replacing her (and subsequently mating) (Monnin & Peeters 1999). Such behaviour would make no sense in *Diacamma* species, in which the key conflict between the reproductive and the workers is irreversibly settled at eclosion, via the mutilation of the callow worker's gemmae by the gamergate or the future gamergate. As long as the gamergate's fertility is adequate, mutilated workers should self-regulate their oogenesis with no need for aggressive interactions. As we have shown, this is the situation for the majority of the time in a *Diacamma* colony.

The existence of a fertility signal made up of cuticular hydrocarbons as a regulator of social peace, generally coupled with aggression, may be widespread in primitively organized insect societies (Peeters *et al.* 1999; Liebig *et al.* 2000; Sledge *et al.* 2001). *Diacamma* species appear to represent a unique situation among queenless ants: the regulation of reproduction is irreversible (as in queenright species), and the role of aggression by the reproductive is minimal compared with that of chemical communication based on an honest signal that reveals the fertility of the reproductive. It seems possible that, in queenright species also, queen pheromones will turn out to be at least partly based on honest fertility signals, which provide workers with the essential information they need in order to repress their reproductive activity and thus maximize their inclusive fitness.

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