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RESEARCH ARTICLE

Aggression regulates monogyny in non-mutilating *Diacamma* ants

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Abstract In queenless species of ants, colonies consist of workers with equivalent reproductive potentials. Aggressive interactions regulate fertility and sexual activity. The genus *Diacamma* is unusual, because monogyny is regulated by mutilation (i.e., removal of a pair of tiny innervated thoracic appendages) of all young workers. One exception is the 'nilgiri' population from south India, where only 6 % of workers were mutilated in ten field colonies (range 2.8–12.5 %). Nonetheless, all colonies were monogynous. To investigate the behavioural mechanisms underlying the replacement of the 'gamergate' (mated reproductive worker) in 'nilgiri', we experimentally divided colonies in two. In the groups lacking the gamergate, aggression soon started among the younger workers. One of these workers exhibited a dominant posture after 1–2 days, and this new alpha started ovipositing and sexual calling within 2–3 weeks. When she was confronted with the original gamergate, olfactory recognition occurred immediately, and this sometimes led to a characteristic dominance behaviour ('sting smearing'). The fate of 85 young workers of known age was studied: they were usually the target of aggression from either gamergates or new alphas. Their gemmae elicited attention, although these were seldom removed. A small change in the gemma pheromone apparently caused an evolutionary switch from mutilation (as occurs in the very closely related *D. ceylonense*) to a reversible regulation of reproduction in 'nilgiri'.

Keywords Reproduction · Gamergate · Gemma · Dominance · Ponerinae

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Introduction

Queenless ant species have workers that are all potentially able to store sperm and produce female offspring, leading to intense reproductive conflicts in colonies. Short periods of aggressive interactions determine who will become the gamergate(s): only one or a few workers have active ovaries and get an opportunity to mate with foreign males. There are both monogynous and polygynous queenless species (Monnin and Peeters, 2008), but in all of them, gamergates cannot disperse alone to start new colonies, unlike in species with winged queens. Hence, new opportunities for direct reproduction always involve competition among closely related nestmate workers, either to replace an old gamergate, or after a colony divides by fission. Reproductive conflicts in queenless ants are arguably more extreme that in associations of founding queens, because the latter are usually unrelated (Bernasconi and Strassmann, 1999; Keller, 1995).

The ponerine genus *Diacamma* is queenless but it exhibits a unique regulatory mechanism: monogyny is mediated by an irreversible morphological alteration during adult life. A remarkable feature of *Diacamma* is that all workers emerge from cocoons with a pair of tiny innervated thoracic appendages, termed: "gemmae" (Peeters and Billen, 1991). In *Diacamma* sp. from Japan (Fukumoto et al., 1989), *D. australe* from Australia (Peeters and Higashi, 1989), *D. pallidum* from Malaysia (Sommer et al., 1993), *D. scalpratum* from Thailand (C. Peeters, unpublished data), *D. cyaneiventre*, *D. ceylonense* and *D. indicum* from India (André et al., 2001; Baratte et al., 2006; Viginier et al., 2004), only the gamergates retain their gemmae. They bite off the gemmae of newly emerged workers ('callows'). This mutilation leads to a permanent change in lifetime trajectory, because workers lacking gemmae never mate

(references above). Mutilation causes the degeneration of the neuronal connections between the sensory hairs on the gemma's surface and the central nervous system, and this may explain the irreversibility of modifications in individual behaviour (Gronenberg and Peeters, 1993). Moreover, in *Diacamma* sp. from Japan, the bursa copulatrix degenerates within days of mutilation, and copulation becomes impossible (Allard et al., 2005). When a gamergate is absent (death or after colony fission), the first worker that emerges retains her gemmae and proceeds to mutilate all subsequent callows. Eventually she mates with one foreign male and becomes gamergate.

In a southern Indian population of *Diacamma*, referred to as 'nilgiri', the gamergate does not mutilate her nestmates and yet monopolises reproduction (Peeters et al., 1992, in which 'nilgiri' is incorrectly called *D. vagans*). This is a derived condition, because 'nilgiri' is highly related to *D. ceylonense* (Baudry et al., 2003), in which mutilations occur systematically. Although gene flow with neighbouring populations of *D. ceylonense* cannot be detected (Baudry et al., 2003), we are currently unsure whether 'nilgiri' is a separate species. Since 'nilgiri' callows keep their gemmae, they retain the potential to replace the gamergate. We experimentally divided nine colonies to study the pattern of aggressive interactions during gamergate replacement, and the influence of individual age. Our experimental divisions approximate fission events (dependent colony foundation, see Peeters and Molet, 2010), which are obligate in all queenless ants. We compare the resolution of reproductive conflicts in this non-mutilating species to that in other *Diacamma,* as well as queenless ants in other genera, and discuss the evolution of diverse behavioural mechanisms to regulate gamergate replacement.

Methods

Ant colonies and husbandry

Ten complete colonies of *Diacamma* 'nilgiri' were collected from southern India in November 1999, June 2000 and March 2002: one from Masinagudi (state of Tamil Nadu), three from Trimbakpur (state of Karnataka), and six along the road between Gundlupet and Bandipur (Karnataka). Colonies contained 273 ± 99 workers (mean number $\pm$ SD, range 147–418) and 49 ± 38 cocoons (range 16–130). All workers were checked for the presence of gemmae and then individually marked with paint. Many of the 488 field-collected cocoons were destroyed during transport, or males emerged. Only 94 cocoons yielded workers in the laboratory, and the date of emergence was noted for 85 of these (callows are easily identified, because their tarsal segments remain pale for several days following eclosion). Colonies were main-

tained in plaster nests (28×27 cm) with a glass roof that allowed observations. Temperature (25 °C) and humidity were controlled. Ants were fed three times per week with live mealworms or small crickets.

Colony divisions and replacement of reproductives

In all *Diacamma* species studied (see "Introduction"), fission of the monogynous colonies creates an opportunity for a new gamergate to differentiate; colony splitting is widely used to simulate a natural queen turnover event, e.g., Gill and Hammond (2011). Except for one colony that was much smaller than the average (88 workers in the laboratory), we divided the remaining nine colonies in two equal groups (117 ± 42 workers, mean $\pm$ SD, range 64–189). This was done less than 10 days after field collection, and care was taken to respect the normal age structure among adults, i.e., both groups had the same ratio of workers active inside and outside laboratory nests. Cocoons were distributed evenly. Since colonies are monogynous (Peeters et al., 1992), our divisions created nine 'orphaned' groups lacking a gamergate (Fig. 1). Because 'nilgiri' gamergates cannot be identified by their retention of gemmae (unlike other *Diacamma* species), orphaned groups were recognised post hoc, which was easy due to an unambiguous start in aggression among workers.

After 27 ± 7 days (mean $\pm$ SD, range 19–41), we removed seven of the nine newly differentiated alpha workers (future gamergates) from the orphaned groups, they were dissected, and again we studied their replacement (Fig. 1). This time schedule was based on data from *D. ceylonense*, where a future gamergate begins sexual calling after 17 days and egg-laying after 19 days (Cuvillier-Hot et al., 2002).
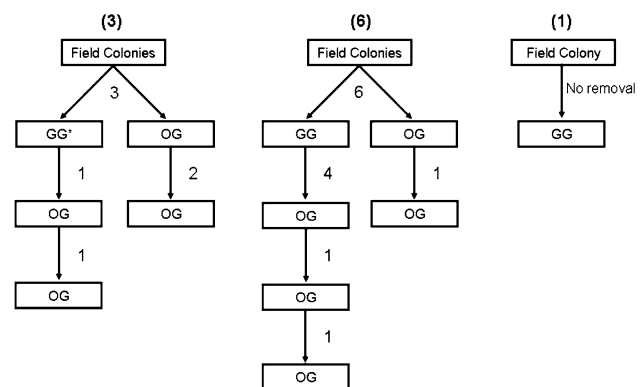

Fig. 1 Experimental protocol to divide nine colonies and study the differentiation of 20 new alphas. Another colony was not divided, because very few workers were alive. *Vertical arrows* indicate removal of a reproductive (gamergate or new alpha); *numbers* give sample sizes. Gamergates and wild-caught alphas could only be distinguished at the end, once dissected. *GG* gamergate group, *GG** wild-caught alpha group, *OG* orphaned group
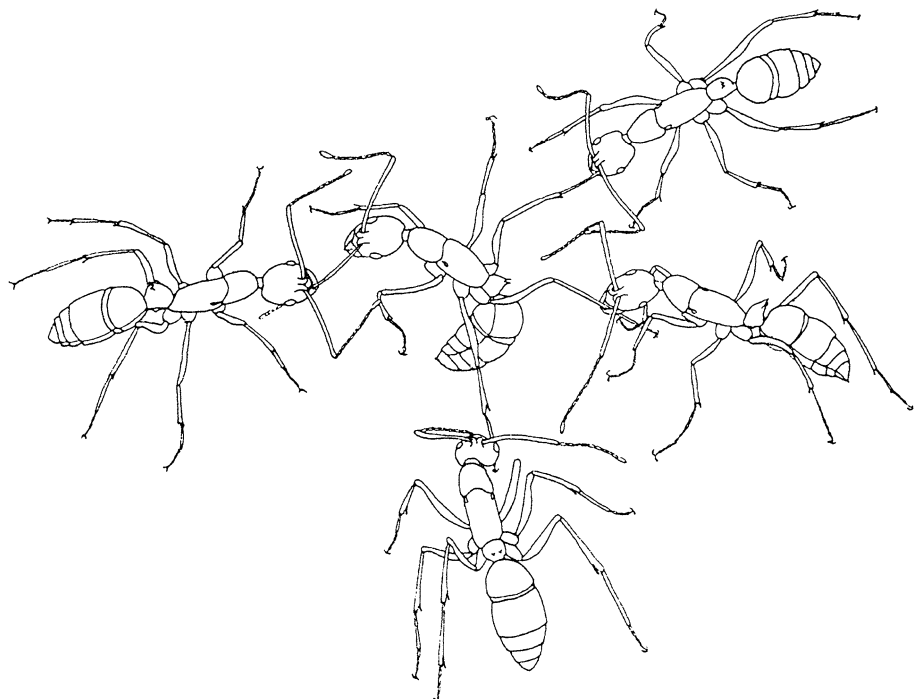
We also removed the gamergate from five of the parent groups after 1–4 months (70 ± 36 days, mean ± SD, range 24–121 days), and this triggered the differentiation of five new alpha workers (Fig. 1). In total, we studied 20 replacement events, of which 6 can be argued to be non-independent values. We justify this pseudoreplication, because colony genotype is less important when studying the importance of individual age.

Observation of social interactions

We observed 4 gamergate groups (including the undivided colony) for a total of 54 h, and 20 orphaned groups for 178.5 h, using 30-min observation periods. Gamergate groups were each observed for 15 ± 2 h (mean ± SD, range 12–16.5) over 42 ± 16 days (mean ± SD, range 30–65) and orphaned groups for 9 ± 4 h (mean ± SD, range 5–15.5) over 25 ± 8 days (mean ± SD, range 13–40). The following interactions and behaviours were recorded.

– Antennal boxing, and biting of legs sometimes followed by jerking. Both yield the same directional information, and we refer to it as aggression. Attackers and victims were identified in all groups but frequency of aggressive acts was only recorded for 16 new alphas.
– Inspection of gemmae: antennation and licking.
– Immobilization: several workers holding another worker for hours or days (Fig. 2). All participants were identified.

– Sting smearing: one worker bends her gaster and exposes the sting, then attempts to rub it on another worker; afterwards, she quickly escapes. This behaviour (similar to that described by Monnin and Peeters, 1999; Monnin et al., 2002) sometimes led to the immobilization of the targeted worker.

Egg-laying activity and sexual calling

To determine the onset of oviposition, we recorded the presence of newly laid eggs (i.e., bright white). We also observed egg-laying, which is a conspicuous behaviour, because the gaster is flexed underneath the thorax and the sting is extruded. After some time an egg appears, and it is then briefly carried in the mandibles. The duration of oviposition was timed whenever possible. After removals or completion of the behavioural studies, we dissected 6 field-collected reproductives and 14 new alphas, as well as 30 nurses from six colonies. The remaining four field-collected reproductives and six new alphas could not be dissected. Ovarian activity was described using Cuvillier-Hot et al.'s (2001) scale for *D. ceylonense*. Examination of spermathecae revealed that three field-collected reproductives were not mated, and they are hereafter termed 'wild-caught alpha' (Fig. 1).

We also recorded the beginning of sexual calling: the hind legs are repeatedly rubbed against the gaster, thus releasing secretions from the metatibial gland (Hölldobler et al., 1996).

Fig. 2 Immobilization behaviour in *Diacamma* '*nilgiri*'. One worker is continuously held for hours or days by several nestmates (the latter change over time)

Confrontation between reproductive individuals

Seven new alphas (from 7 orphaned groups out of 20) were taken to their corresponding gamergate group ($n = 3$). Another three new alphas were taken to groups with a wild-caught alpha ($n = 3$). All these ten new alphas had started laying eggs or sexual calling. We immediately recorded interactions between the reproductives during 30–60 min, after which, we returned the new alphas to their group of origin.

Results

Mutilations

All ten colonies of 'nilgiri' were examined less than 10 days after field-collection, and 6.1 % (mean number) of the workers were mutilated; this ranged from 2.8 to 12.5 % across colonies. Half of the mutilated workers (83 out of 170) had no gemmae left, the other half (87) retained one gemma.

Gamergate groups

After colony division, no widespread aggression was observed in the groups with a gamergate ($n = 3$). This was also the case in the undivided colony, and these were all used as a comparison for the 'orphaned' groups. The gamergates were rarely aggressive (0.67 ± 1.92 attacks per 30 min, mean $\pm$ SD, $n = 139$ obs., 4 gamergates) (Fig. 3), and they seldom walked around (32 ± 37 s per 30 min, mean $\pm$ SD, $n = 104$ obs., 4 gamergates observed for a total of 25 h). However, when callows emerged, they were bitten by the gamergate (recorded for 17 out of 19 callows). Their gemmae were inspected, this region of the thorax was sometimes scraped with the mandibles but mutilation rarely occurred (3 callows out of the 17 that had been bitten).

Gamergates were observed to lay 13 eggs, and oviposition lasted for 5 min on average (5.1 ± 0.8 mean $\pm$ SD, range 3.4–6.3). Dissections revealed that gamergates ($n = 3$) had very developed ovaries (state 5; Cuvillier-Hot et al., 2001). Another three presumed gamergates were found to be 'wild-caught alphas' because their spermatheca was empty; their ovaries were less developed (two were state-4, the other was state-3), although yellow bodies indicated some eggs had been laid. Unlike the confirmed gamergates, wild-caught alphas exhibited sexual calling and were thus likely to mate if foreign males had been present. We lack dissection data for the remaining four gamergates, but they behaved like the other three, i.e., no sexual calling and quick oviposition.
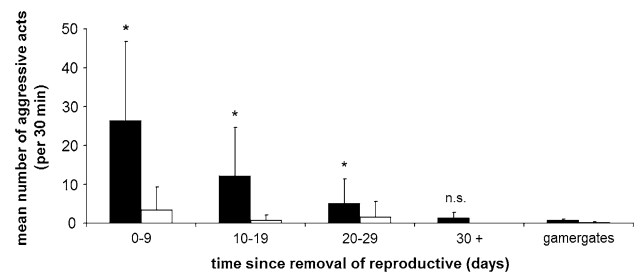

Fig. 3 Temporal shift in aggression (antennal boxing, biting of limbs) performed by new alphas (*filled*) after colony division, and in the cumulated aggressions of all other workers (*non-filled*). Gamergates ($n = 4$) are included for comparison. Observations were grouped as follows: 0–9 days, $n = 131$ observation periods, 16 alphas; 10–19 days, $n = 98$ obs., 15 alphas; 20–29 days, $n = 46$ obs., 9 alphas; 30+ days, $n = 27$ obs., 3 alphas; for four gamergates, $n = 139$ obs. When the same individual was observed in successive time windows, the mean value of her scores was used. *Error bars* show standard deviation. *Asterisks* indicate significant differences ($p < 0.05$; permutation tests) in comparisons between the gamergate group and each temporal group of alphas

Orphaned groups

Colony division or removal of a reproductive triggered a lot of aggressive interactions among workers that were no longer in contact with the reproductive (Fig. 3): biting was common, there were a few sting smearings and immobilizations. After 1–8 days, one worker per orphaned group exhibited a conspicuous dominance behaviour ($n = 20$ replacements): she walked around high on her legs, mandibles opened, and bit many of the workers encountered. This 'patrolling' inside the nest was frequent during the first 9 days (7 new alphas did this for 7.5 ± 3.8 min over 30 min, mean $\pm$ SD, $n = 51$ observations), but it declined over time (3.8 ± 1.6 min on days 20–29, $n = 26$ obs., 4 new alphas), and so did aggression (Fig. 3). The new alpha eventually monopolised aggression (Fig. 3), even though she had sometimes been initially aggressed and immobilised (Table 1). All callows were aggressed by the new alpha (66 out of 66), gemmae were inspected and sometimes scraped, resulting in a few mutilations (7 callows out of 66).

Egg-laying began 15 ± 3 days (mean $\pm$ SD, range 10–21, $n = 17$ orphaned groups) after division. Ovipositions ($n = 16$) lasted three times longer than for gamergates: it took 16.2 ± 2.5 min (mean $\pm$ SD, range 10.9–20.2, 5 alphas). In two orphaned groups that originated from colonies with a wild-caught alpha, another worker laid an egg soon after the new alpha had differentiated, and the wild-caught alphas ate this egg on both occasions. Dissections of 14 new alphas, 27 ± 11 days (mean $\pm$ SD, range 15–55) after division or removal of a reproductive, revealed that ovaries were active (12 were state-4, 2 were state-3; Cuvillier-Hot et al., 2001), unlike other workers (8 were state-2, 22 were state-1). Alpha was also the only ant to perform sexual calling near

Table 1 Temporal shift in the behaviour of newly differentiated alphas. The same individuals were observed for different durations; decreasing sample sizes reflect removals or death

	Time window after removal of the reproductive (days)			
	0–9	10–19	20–29	30+
Number of alphas	20	20	11	4
Aggressed (%)	14.3 (3)	0 (0)	0 (0)	0 (0)
Immobilised (%)	23.8 (5)	0 (0)	0 (0)	0 (0)
Performing 'gemmae inspection' (%)	57.1 (12)	20.0 (4)	27.3 (3)	0 (0)
Laying eggs (%)	0 (0)	80 (16)	100 (11)	100 (4)
Performing sexual calling (%)	0 (0)	45.0 (9)	81.8 (9)	100.0 (4)

Number of alphas observed in each case is indicated between brackets

the nest entrance (Table 1). Sexual calling began after 20.1 ± 4.9 days (mean ± SD, range 12–29, $n = 14$ new alphas). This confirms that alpha is the future gamergate, and she can mate as soon as a foreign male visits the nest.

Alpha was generally a young worker: she was less than 20 days old in seven out of nine orphaned groups in which there were callows of known age. As workers of known age became older, they were less likely to be attacked or immobilised, and instead they performed immobilizations (Table 2).

Confrontations between reproductives

When a new alpha was introduced in her gamergate group (7 alphas, 3 groups), the gamergate attacked her and alpha always behaved submissively (crouching, escape). The gamergate did sting smearing against five new alphas that were already laying eggs; one of these reacted with an attempted sting smearing against the gamergate. The other two confrontations involved new alphas that had not started ovipositing yet, and the gamergate did not do sting smearing. This difference indicates that sting smearing is a dominance behaviour used only in a highly specific context.

When introduced in groups with a wild-caught alpha ($n = 3$), the new alphas were soon attacked. Two of them became submissive and were targets of sting smearing, even though they did not yet lay eggs. The third new alpha was an active egg-layer and she dominated the wild-caught alpha by successfully performing sting smearing against her (hence, the wild-caught alpha was soon immobilised by other nestmates).

Discussion

Instead of systematic mutilations by the gamergate observed in seven *Diacamma* species, monogyny and the replacement of senescent gamergates in 'nilgiri' are regulated by aggressive interactions among nestmates. The aggressive behaviours were similar to that in other ponerine and even leptothoracine genera (Cuvillier-Hot et al., 2004; Monnin and Peeters, 1999; Gill and Hammond, 2011). In 'nilgiri', as soon as the reproductive was removed from the colonies, aggression began and one of the younger workers became dominant. This new alpha started laying eggs and performed sexual calling after 2–3 weeks. By then, alpha was only infrequently aggressive, and this temporal shift in behavioural profile is extremely similar to that exhibited by the future gamergate of *D. ceylonense* (unmutilated young worker in this species) (Cuvillier-Hot et al., 2002), except that the 'nilgiri' alpha does not routinely mutilate new callows.

Although they are not systematically removed, the gemmae of 'nilgiri' remain the focus of short-lived attention. Gamergates and new alphas use their antennae to touch the thorax and gemmae of newly emerged callows, but this is seldom followed by scraping with mandibles (scraping is needed to disarticulate the gemmae in *D. ceylonense*; Baratte et al., 2006). Histology of the 'nilgiri' gemmae revealed that they contain about 50 exocrine cells, much fewer than in *D. australe* (about 500) (Peeters et al., 1992); these cells connect to minute pores opening to the outside (Peeters and Billen, 1991). When cocoons were transferred between colonies of *D. ceylonense* and 'nilgiri', newly

Table 2 Behavioural profile of workers of known age ($n = 85$) during the first week after removal ($n = 20$) of a reproductive

	Age at the time of the experimental removal of a reproductive (days)				
	1–10	11–20	21–30	31–50	50+
Numbers of replacement events	12	6	7	9	7
Numbers of workers in age class	52	24	27	32	52
Immobilised (%)	32.7 (17)	33.3 (8)	11.1 (3)	18.8 (6)	3.8 (2)
Aggressed by alpha worker (%)	73.1 (38)	58.3 (14)	55.6 (15)	31.3 (10)	19.2 (10)
Aggressive (%)	13.5 (7)	16.7 (4)	7.4 (2)	40.6 (13)	11.5 (6)
Performing immobilizations (%)	0 (0)	12.5 (3)	3.7 (1)	37.5 (12)	13.5 (7)
Becoming the new alpha (%)	7.7 (4)	12.5 (3)	7.4 (2)	0 (0)	0 (0)

Number of workers observed is indicated between brackets

emerged *D. ceylonense* workers were immediately mutilated by a '*nilgiri*' gamergate, but the converse was not true (Ramaswamy et al., 2004). Thus, the absence of mutilation does not result from a behavioural shift of '*nilgiri*' gamergates, but rather from altered characteristics of the '*nilgiri*' callows. Both histological and behavioural data indicate that an olfactory signal continues to be emitted by the gemmae of '*nilgiri*', but we speculate it has become somewhat modified from that in *D. ceylonense*, causing a reduced motivation to mutilate. Field colonies of '*nilgiri*' included on average 6 % of mutilated workers, and half of these retained one gemma. Such asymmetric mutilations have never been reported in orthodox *Diacamma* species. This and occasional mutilations of '*nilgiri*' in the laboratory suggest individual variability in the gemma signal. Nonetheless, mutilation in '*nilgiri*' has similar behavioural consequences as in *D. ceylonense* and other orthodox species, because '*nilgiri*' workers without gemmae never became dominant.

Following removal of reproductives, alpha was responsible for most of the aggressions. Other workers initiated aggression too infrequently to allow us to determine a hierarchy, and in most colonies, a beta worker having a better probability of replacing the gamergate could not be identified. This is unlike the linear hierarchies documented in several queenless ants (Ito, 1993a; Ito and Higashi, 1991; Monnin and Peeters, 1999; Cuvillier-Hot et al., 2004). However, age has a major effect on the probability to become gamergate, as in other queenless ants (Ito, 1993b; Higashi et al., 1994; Monnin and Peeters, 1999). We studied the fate of 85 callows (all emerged in the laboratory) for more than a month, and new alphas belonged to the younger age classes (Table 2). This is consistent with the observation that callows were a privileged target of aggression from the gamergates or new alphas. The importance of age is similarly striking in orthodox *Diacamma* species, because it is the callows that are systematically mutilated, and the future gamergate is always young. In *D. ceylonense*, 0–4 days old callows have distinct blends of cuticular hydrocarbons (Cuvillier-Hot et al., 2001), and we speculate that '*nilgiri*' callows are recognised by these cues.

By engineering confrontations between the original gamergate and a daughter/sister that had become dominant and egg-layer, we provided support for the olfactory detection of a signal associated with the onset of ovarian activity. This has been shown in other queenless ants (e.g., Gobin et al., 1999; Liebig et al., 1999; Kawabata and Tsuji, 2005) and is probably the result of an altered chemistry of the cuticular hydrocarbons (e.g., Cuvillier-Hot et al., 2004; Peeters and Liebig, 2009). Indeed in *D. ceylonense*, the relative proportions of several long-chained hydrocarbons vary according to the degree of egg-laying activity (Cuvillier-Hot

et al., 2001). The gamergates of '*nilgiri*' have the same cuticular profile as in *D. ceylonense* (unpublished data), and the profiles of '*nilgiri*' foragers and reproductives are similarly distinct. Just like in *D. ceylonense* and other queenless ants, aggression is a rare event in gamergate colonies of '*nilgiri*', and thus, a fertility signal seems sufficient for nestmate workers to refrain from reproductive challenges. In all cases of re-introduction (except one involving a wild-caught alpha), the new alpha lost her dominant position. There was more or less aggression depending on whether an alpha was laying eggs or not (there was also a difference between gamergates and wild-caught alphas). Alpha in '*nilgiri*' is guaranteed to mate, and subordinates should refrain from producing their own sons; the same conflict involves the unmutilated worker (future gamergate) in *D. ceylonense* (Cuvillier-Hot et al., 2002).

In *D. ceylonense*, replacement of the gamergate is straightforward: the first callow to emerge keeps her gemmae and mutilates all subsequent callows. However, if cocoons are not available in laboratory colonies, the mutilated (and thus permanently virgin) workers establish a hierarchy. One worker accedes to the alpha rank, but a few subordinates continue to fight for weeks or months while laying male-destined eggs (alpha often eats these) (unpublished data). Thus, the hierarchy does not become stable in *D. ceylonense*, and there is only a weak link between dominance status and ovarian activity (see Peeters and Tsuji, 1993). In contrast, the '*nilgiri*' alpha monopolises egg-laying, and she begins sexual calling. We speculate that, because alpha lacks gemmae and can thus never mate in *D. ceylonense*, other high-ranking mutilated workers behave selfishly and increase their fitness by producing their own sons.

The absence of mutilation has only been found in one population ('*nilgiri*') of *Diacamma*, which makes it difficult to do a comparative analysis of the evolution of mutilation behaviour. The possibility that '*nilgiri*' represents the ancestral condition in the genus (i.e., innervated gemmae have evolved, but they are not mutilated) has been shown not to be true (Baudry et al., 2003), because '*nilgiri*' is highly related to *D. ceylonense*, and they both belong to the derived *vagans*-group of species. It appears that a small difference in the gemma pheromone of '*nilgiri*' has led to a major shift in the behavioural mechanism regulating reproduction. Unlike orthodox *Diacamma*, subordinates in '*nilgiri*' retain the ability to mate and replace senescent reproductives, similarly to all other queenless ants.

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