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Experimental Investigation of the Mechanism of Reproductive Differentiation in the Queenless Ant, *Diacamma* sp., from Japan

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Abstract

We test two hypotheses about regulation of the reproductive division of labour in the permanently queenless ponerine ant, *Diacamma* sp., from Japan. All workers emerge with gemmae (tiny innervated thoracic appendages), but only one individual keeps them in each colony, and she is the only mated reproductive worker (gamergate). The gemmae of all other workers are mutilated by the gamergate soon after their emergence, and they can never mate. In the presence of gamergate, mutilated workers have inactive ovaries and do not behave aggressively. Two possible consequences of mutilation are: 1. olfactory signal—a pheromone inhibiting the oogenesis of mutilated workers is no longer released by the gemmae; and 2. endocrine degeneration of its afferent neuronal connections interferes reproductive physiology of a gamergate. Gemmae of gamergates were coated with shellac (to prevent pheromone emission) or removed, and over three weeks we studied any changes in ovarian activity of the gamergates as well as nestmate workers. Coating of gemmae did not elicit worker oviposition, suggesting that gemmae pheromones do not have a regulatory function. Experimental mutilation of gamergates resulted in a slight increase in both the frequency of dominance interactions and the ovarian activity of mutilated workers, but this effect was much lower than in colonies where the gamergate was removed. This contrasts with the immediate change in the behaviour (aggressive to timid) of newly emerged workers following mutilation.

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Introduction

Ant workers are often able to lay unfertilized eggs (which develop into males) when there is no queen in their colonies (Bourke 1988; Choe 1988; Hölldobler & Wilson 1990). Information about the queen's presence is communicated by olfaction (Fletcher & Ross 1985), but the details of this remain fragmentary. In various species of the 'primitive' subfamily Ponerinae, workers are able to mate and lay diploid eggs, and the winged queen caste can be absent. Only a few workers reproduce sexually in each colony, however, and they are called 'gamergates' (Peeters 1991). Queenless ponerine ants are an interesting system to study the

regulation of worker reproduction, because in various species with small colonies, such as *Pachycondyla sublaevis* (Ito & Higashi 1991) and *Dinoponera quadriceps* (Monnin & Peeters 1998), aggressive interactions among a proportion of the workers lead to a dominance hierarchy, and subordinate workers are sterile.

The ponerine genus *Diacamma* is also queenless but exhibits another intriguing regulatory system: reproductive division of labour is mediated by social manipulation among adults. A unique feature of this genus is that all workers emerge from cocoons with a pair of tiny club-like thoracic appendages, termed 'gemmae' (Peeters & Billen 1991). In *Diacamma* sp. from Japan (formerly called *D. rugosum*) (Fukumoto et al. 1989), and *D. australe* (Peeters & Higashi 1989), only the gamergate retains the gemmae. She bites off the gemmae of newly eclosed workers soon after their emergence. This mutilation leads to an irreversible change in the behavioural trajectories of individuals because workers lacking gemmae can never mate. However, mutilated workers can lay haploid eggs if a colony becomes orphaned. When a gamergate is absent (after her death or through fission of colonies), one of the callow workers that subsequently emerge retains her gemmae and proceeds to mutilate all other sister callows. She also aggressively harasses mutilated workers, and these cannot produce eggs. Eventually she mates with a foreign male and becomes a gamergate (Fukumoto et al. 1989; Peeters & Higashi 1989). Once mated, she stops aggressing mutilated workers, but these remain sterile. While mutilation of new workers continues unaffected, the mediating mechanism of her regulation of worker oviposition appears to change from behavioural to olfactory, as a result of mating (Peeters & Higashi 1989). In orphaned colonies of *Diacamma* sp. from Japan, mutilated workers compete for oviposition by mutual aggression, which results in a distinct dominance hierarchy, and after a few weeks, the top-ranked worker begins to monopolize male production (Peeters & Tsuji 1993). Nevertheless, this dominant worker is mutilated and can never mate. Almost exactly the same system exists in *D.* sp. from Malaysia (Sommer et al. 1993), but not in *D. vagans* (from India) where regulation is not based on the systematic mutilation of the gemmae (Peeters et al. 1992).

A lot of questions arise about the complex link between mutilation and reproductive differentiation. Peeters & Billen (1991) discovered that a gemma is filled with about five hundred exocrine cells in *D. australe* (also in *D.* sp. from Japan; M. Obermayer pers. comm.). One suggested function was the production of sexual pheromones (Billen & Peeters 1991; Peeters et al. 1992). However, metatibial glands in the hind legs have now been found to be involved in the release of sexual attractants in at least two species of *Diacamma* (Hölldobler et al. 1996; K. Nakata et al. unpublished data). Another possibility is that gemmae produce a pheromone that inhibits oviposition of mutilated workers (Peeters & Billen 1991). This idea can be tested by blocking the release of the putative pheromone. Because secretions are released through minute pores opening on the outer surface of the gemmae, these can be coated with shellac. If mutilated workers begin to behave as in orphaned colonies (aggression and oviposition) after the gemmae of a gamergate are coated, gemmae might indeed secrete inhibitory pheromones. In contrast, if coating the gemmae causes no effect, other stimuli might be important for informing of the gamergate's presence.

Gronenberg & Peeters (1993) studied the anatomy of the afferent axons which connect sensory hairs on the gemma's surface with the central nervous system. These afferents branch extensively in the thoracic ganglia, and often terminate within the cell body rind of the ganglia. All axonal arborizations of the gemma degenerate completely after mutilation, and this may block transmission of neural stimuli that affect directly or indirectly (through alteration of endocrine conditions) a worker's long-term physiology and behaviour. Indeed, experimental mutilation of young virgin workers in *D. australe* and *D.* sp. from Japan causes a switch in individual behaviour (i.e. aggressive to 'timid') (Peeters & Higashi 1989; K. Tsuji, unpubl. data). Thus, workers that retain the gemmae have three characteristics: (a) ability to mate, (b) aggressive profile, and (c) active ovaries. We investigated whether experimental mutilation of gamergates affects (b) and (c): a gamergate would then lose her dominant status and stop oviposition. Mutilation of an already-mated worker is unlikely ever to occur under natural conditions, but it can give valuable insights into the physiological consequences of gemmae ablation.

In this study we manipulated (either by coating or by mutilation) gemmae of established gamergates to answer the above questions about the proximate mechanism connecting retention of gemmae and reproductive differentiation. The intensity of dominance interactions among mutilated workers was used to investigate the extent of gamergate control, and later their ovaries were dissected.

Materials and Methods

General Procedure

Diacamma sp. is the only species of this genus distributed in Japan (Morisita et al. 1989). We collected 24 colonies from three populations on the main island of Okinawa (Sueyoshi, Chinen and Nakijin) during 1993–1994. Each colony was transferred to a plastic container (41 × 31 × 8 cm) with a 2-cm-thick plaster floor and a cavity (10 × 6 cm, 1 cm deep) covered by a glass plate and serving as a nest. Ants were kept in the laboratory at room temperature (19–27°C) and uncontrolled light–dark cycles. They fed ad libitum on honey water, crickets and cockroaches every other day.

Fourteen colonies were used for the behavioural observations. Firstly, we marked all workers individually with colour enamel paints on the dorsal regions of thorax, petiole and gaster. One week prior to the beginning of observations, colony size was regulated by randomly removing a proportion of the mutilated workers. The number of eggs and larvae was not controlled, but all cocoons were removed because the presence of new workers affects the relative social status of older workers. Dominance ranks seem associated with the age of individual workers in this species (K. Nakata, pers. comm.), so that a drastic change in the colonial age structure may alter the frequency of dominance interactions.

Each colony was observed for 2 h almost every day, either directly or with a time-lapse video recorder. All ovipositions and aggressive interactions ('bite and jerk' an opponent's head or legs, Peeters & Tsuji 1993) were recorded and used for

the analysis. Less conspicuous dominance interactions, i.e. antennal boxing and avoidance (Peeters & Tsuji 1993) were not recorded. Daily observations continued for 8–14 d and provided baseline data about intracolony aggression.

Afterwards, each colony was randomly assigned to one of four different manipulation groups as follows.

1. Control: the thorax of the gamergates was partially coated with shellac, but the gemmae were left intact ($n = 3$ colonies).
2. Coated-gemmae: the gemmae of the gamergates were completely coated with shellac. Thus if gemmae produce a volatile pheromone, its release will be stopped ($n = 4$ colonies).
3. Removed-gemmae: the gemmae of the gamergate were surgically cut with tweezers, and her thorax was partially covered with shellac ($n = 4$ colonies).
4. Orphaned: the gamergate was removed from the colonies, which is the negative control ($n = 3$ colonies).

One day after the manipulation, the daily 2 h observations were resumed and lasted for 3 w. For each colony, we compared the frequencies of dominance behaviour before and after manipulation, so as to control for error owing to differences between colonies. A statistical analysis combining all colonies was also performed (see Results). To control for interpopulation differences, at least one colony from each of three populations was used for each manipulation (Table 1). The initial colony sizes (number of workers at the beginning of observation) were designed to fall within a similar range with almost equal mean and variance (Table 1). On the 21st day after the manipulations, all adult individuals were dissected to compare ovarian development.

Based on dissection data, we classified individual workers into one of four categories: mature ovaries, at least one mature oocyte (over 1 mm in length); developed ovaries, no mature oocyte but smaller developing yolky oocyte(s); less developed ovaries, only transparent and small nurse cells; and inactive ovaries, thread-like ovarioles without oocytes. To compare different treatments, a colony,

Table 1: Initial colony size (the number of workers including a gamergate at the beginning of behavioural observation) and the origin of fourteen *D. sp.* colonies

Manipulation	Colony code (size)	\bar{X}	SD
Control	Chinen 8 (39), Sueyoshi 2 (30), Nakijin 12 (80)	49.7	21.76
Coated-gemmae	Chinen 4 (45), Sueyoshi 1 (41), Nakijin 13 (80), Nakijin 14 (25)	50.3	20.19
Removed-gemmae	Chinen 7 (84), Chinen 6 (25), Sueyoshi 18 (42), Nakijin 7 (50)	50.3	21.47
Orphan	Chinen 16 (49), Sueyoshi 11 (25), Nakijin 6 (75)	49.7	20.42

rather than an individual worker, must be the independent unit, which resulted in small sample size. Thus to increase our sample, dissection data of other colonies which were not used for behavioural observation were added (10 control colonies and 3 orphaned colonies).

Quantification of the Intensity of Dominance Interactions

Aggressive interactions among mutilated workers are associated with competition for male production in this species (Peeters & Tsuji 1993; Nakata & Tsuji 1996). There are several possible ways to quantify the intensity of dominance interactions. In orphaned colonies, aggression often repeatedly occurs in some pairs of individuals, but not in other pairs during the same relatively short period, i.e. 1 d (Peeters & Tsuji 1993). This means that subsequent dominance interactions observed within a day are not independent. Moreover, previous observations suggest that dominance orders vary markedly among colonies (Peeters & Tsuji 1993). In some cases, only one individual performed most aggressions, while in other cases a few workers received most aggressions from many other individuals. Therefore, the following four complementary measurements were used.

- A. Number of aggressive interactions: the total number of 'bite and jerk' observed during a daily session. For example, if interactions occurred three times within the same pair of individuals in 1 d, it was counted as three events.
- B. Number of interacting pairs: the number of pairs of workers in which aggression was observed. Reversal of direction rarely occurred within a day.
- C. Number of attackers: the number of individuals that behaved dominantly, i.e. bite and jerk any other workers, at least once during a daily session.
- D. Number of victims: the number of individuals that were bitten and jerked by any other workers, at least once during a daily session.

Results

Dominance Interactions among Workers

When colonies were orphaned artificially, the intensity of dominance interactions increased dramatically for all A–D criteria (Fig. 1). For example, the number of aggressive interactions and the number of interacting pairs increased ≈ 10 and 8 times, respectively. The difference between before and after removing a gamergate was statistically significant in all three colonies (Mann–Whitney U-test, $p < 0.001$), and so was the Fisher's combined probability (A: $\chi^2 = 65.8$, df 6, $p < 0.001$; B: $\chi^2 = 65.7$, df 6, $p < 0.001$; C: $\chi^2 = 88.9$, df 6, $p < 0.001$; D: $\chi^2 = 69.7$, df 6, $p < 0.001$).

On the other hand, colonies of the coated-gemmae treatment (Fig. 1) showed no significant increase in the intensity of aggressive interactions (Mann–Whitney U-test, $p > 0.05$ in all four colonies for all A–D criteria), which was the same as in the control colonies (Fig. 1). Combined probabilities were also non-significant in all four measurements of interactions (coated gemmae group: A: $\chi^2 = 5.29$, df 8,

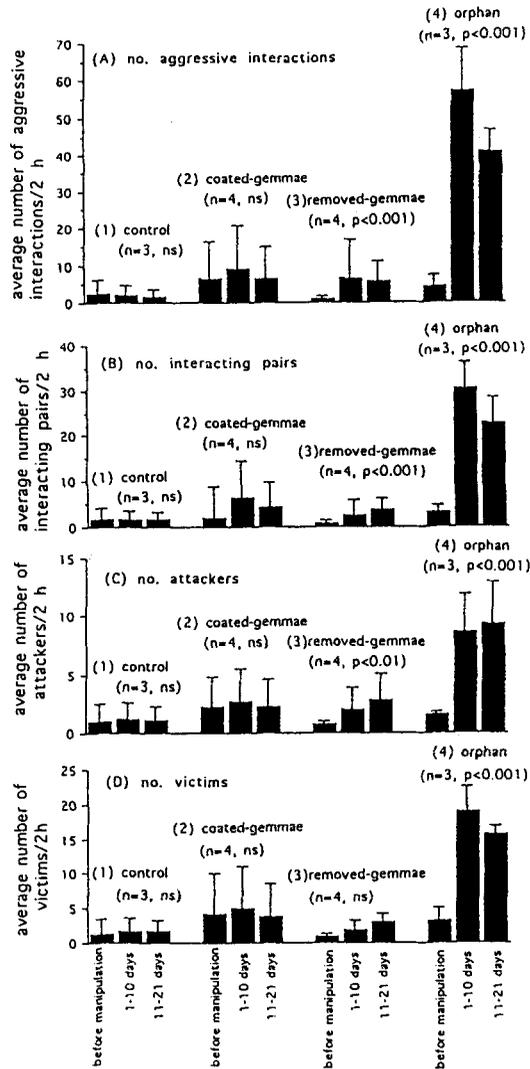


Fig. 1: Change in the frequency of aggressive interactions among mutilated workers following manipulation of gamergates. Four different measurements of the intensity of aggressive interactions (A)–(D) are shown. From left to right, results of different treatments (1)–(4) are presented (see text for details). Each bar indicates mean and SD over all colonies in the same treatment during the indicated period (an average score was calculated for each colony in each period then averaged again over all colonies). For convenience, we distinguished three periods (before manipulation, 1–10 d after manipulation and 11–21 d after manipulation). Fisher's combined probabilities over all colonies were compared before and after the same manipulation (based on Mann–Whitney U-tests applied to each colony)

$p > 0.7$; B: $\chi^2 = 3.87$, $df\ 8$, $p > 0.8$; C: $\chi^2 = 4.63$, $df\ 8$, $p > 0.7$; D: $\chi^2 = 5.82$, $df\ 8$, $p > 0.5$; control group: A: $\chi^2 = 4.48$, $df\ 6$, $p > 0.5$; B: $\chi^2 = 8.09$, $df\ 6$, $p > 0.2$; C: $\chi^2 = 6.24$, $df\ 6$, $p > 0.3$; D: $\chi^2 = 7.10$, $df\ 6$, $p > 0.3$). This indicates that the putative gemma pheromone is not involved in the regulation of worker behaviour.

Results of the removed-gemmae treatment differed between colonies. In colonies Chinen 6 and Nakijin 7, the intensity of dominance behaviour, as measured by three or four criteria (no. aggressive interactions, no. pairs and no. attackers), increased significantly (Mann–Whitney U-test, $p < 0.05$), while the number of victims increased significantly only in colony Nakijin 7. However, all these effects were quite small compared with the change occurring in orphaned colonies (Fig. 1). The other two colonies yielded negative results (U-test, $p > 0.05$), and we did not notice any qualitative change in the behaviour of mutilated workers. When we pooled results for all four colonies (Fig. 1 A–D), the intensity of aggressive interactions increased significantly (Fisher's combining probability) in three of the four measurements (A: $\chi^2 = 36.7$, $df\ 8$, $p < 0.001$; B: $\chi^2 = 35.3$, $df\ 8$, $p < 0.001$; C: $\chi^2 = 21.3$, $df\ 8$, $p < 0.01$; D: $\chi^2 = 13.6$, $df\ 8$, $0.05 < p < 0.1$).

Behaviour of Gamergates

Except in one colony, we did not observe any qualitative change in the behaviour of gamergates during the 3 w of observation. Manipulated gamergates (gemmae coated or removed) continued to lay eggs. Workers always behaved submissively (avoidance or crouching) when they encountered a gamergate, even after her gemmae had been artificially removed. Only in colony Chinen 7 was the mutilated gamergate aggressed by other workers (15 times in total, 12–20 d after gemmae removal). However, this aggression differed in its characteristics from the common dominance interactions (bite and jerk) among mutilated workers, because the dorsal gaster of the gamergate was bitten while her head was away from the attacking worker. Once the gamergate turned around, the worker stopped aggression and became timid. Interestingly, this is the only gamergate which aggressed mutilated workers (once on days 11, 20 and 21 after gemmae removal). In other colonies, the gamergate never participated in aggressive interactions.

Oviposition by Mutilated Workers

In the coated- and removed-gemmae treatments, mutilated workers never oviposited, indicating that the presence of a gamergate continued to be recognized. Only in control colony Chinen 8 was a mutilated worker observed to lay eggs. This egg was eaten by the gamergate immediately after oviposition, exactly as reported previously (Fukumoto et al. 1989; Nakata & Tsuji 1996). Nakata & Tsuji (1996) found that oviposition by mutilated workers becomes more frequent with increasing colony size, and it was not recorded when a colony contained fewer than 150 workers. However, colony Chinen 8 was not large (36 mutilated workers and 1 gamergate). Dissection revealed that the ovipositing mutilated worker had developed ovaries with yellow bodies and four fully mature oocytes. No other mutilated workers had a mature oocyte in the three control colonies.

In contrast, oviposition by mutilated workers was observed many times in orphaned colonies during the second and third weeks after orphaning, as already reported (Fukumoto et al. 1989; Peeters & Tsuji 1993).

Ovarian Conditions of Mutilated Workers

Fig. 2 shows the average proportion of workers with different categories of ovarian development in each treatment. In the removed-gemmae group, there is a slightly higher proportion of workers with mature ovaries than in the control group (no statistical significance). However, there was variability among the removed-gemmae colonies because mature oocytes were found only in Chinen 6 (two workers) and Chinen 7 (one worker). This variance parallels the observations of aggressive interactions (Fig. 1).

To compare treatments statistically, four parameters were used: (a) proportion (%) of workers with mature oocyte(s) in a colony, (b) average number of mature oocytes among the five most reproductively active workers in a colony, (c) average length of the longest oocyte among the top five workers in a colony, (d) absolute number of workers with mature oocyte(s) in a colony. For all (a)–(d) measurements, a significant difference was detected among treatments (Kruskal–Wallis test, $p < 0.01$). Then multiple comparisons were performed with the sequential Bonferroni method (Table 2). Results were consistent across all (a)–(d) parameters. There were significant differences between control and orphaned ($p < 0.01$), and between coated-gemmae and orphaned groups ($p < 0.01$), but the removed-gemmae group did not differ significantly from either the control group or the orphaned group.

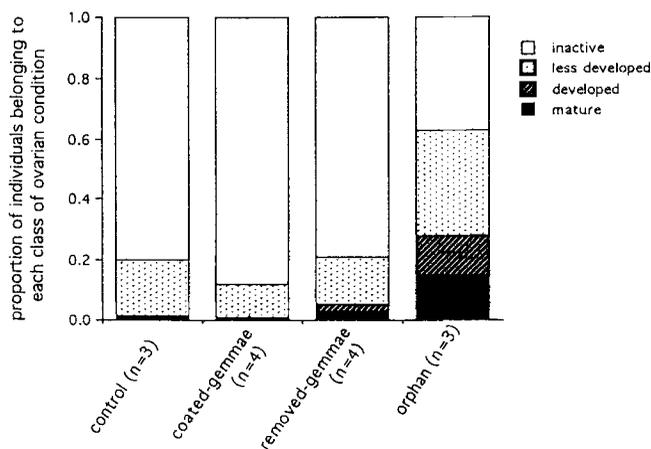


Fig. 2: Ovarian development of virgin mutilated workers under different treatments. Workers are classified into four types according to their ovarian condition (see text for details). Average proportion of each type of workers (over n colonies) is indicated.

Table 2: Comparison of ovarian development of mutilated workers among different treatments. \bar{X} and SD are shown. Different letters in the same row indicate a statistically significant difference in a sequential Bonferroni test (between a and b: $p < 0.01$)

Ovarian measurement	Treatment (n = number of colonies)			
	control (n = 13)	coated- gemmae (n = 4)	removed- gemmae (n = 4)	orphan (n = 6)
Proportion (%) of workers with mature oocyte(s)	0.26 ± 0.92 a	0.00 ± 0.00 a	2.75 ± 4.57 ab	28.63 ± 20.20 b
Average number of mature oocytes in top 5 workers	0.06 ± 0.22 a	0.00 ± 0.00 a	0.60 ± 1.07 ab	3.70 ± 0.83 b
Average length (mm) of longest oocyte in top 5 workers	0.13 ± 0.88 a	0.10 ± 0.34 a	0.42 ± 0.34 ab	1.26 ± 1.79 b
Number of workers with mature oocyte(s)	0.08 ± 0.28 a	0.00 ± 0.00 a	0.75 ± 0.96 ab	5.67 ± 1.86 b

Ovarian Condition of Gamergates

The numbers of mature oocytes in a gamergate's ovaries were compared between control, coated-gemmae, and removed-gemmae colonies (Table 3). To increase sample size, data from two other gamergates from unmanipulated colonies were pooled with the control. No statistically significant difference was found among treatments (Kruskal–Wallis test, $H = 0.561$, $p > 0.1$). This indicates that neither the coating nor the mutilation of gemmae affected markedly the reproductive physiology of gamergates, at least during the 3 w following manipulation.

Discussion

In *Diacamma* sp. from Japan, our manipulation of gamergates' gemmae (coating or removal) had only a slight effect on their reproductive supremacy. The behaviour and ovarian activity of experimentally mutilated gamergates did not change substantially during the 3 w of our study. In contrast, the behaviour of

Table 3: Number of mature oocytes (longer than 1 mm) found in a gamergate. $\bar{X} \pm SD$ are presented. There were no statistically significant differences among treatments (Kruskal–Wallis test, $J = 0.561$, ns)

Control (n = 5)	Coated-gemmae (n = 4)	Removed-gemmae (n = 4)
7.40 ± 3.05	8.50 ± 2.65	8.00 ± 0.82

young virgin workers changes from aggressive to timid within days of mutilation (K. Tsuji & C. Peeters, unpublished data), which reveals the distinct nature of a mutilated gamergate's response. Gronenberg & Peeters (1993) speculated that gemmae removal results either in a decrease in sensory input onto interneurons, or in neuromodulatory effects. In some colonies of the removed-gemmae group, however, there were occasional indications that mutilated gamergates were gradually losing their dominant status (i.e. frequency of dominance interactions increased slightly, various mutilated workers had mature oocytes in two out of four colonies, and in one colony the gamergate was attacked by mutilated workers).

Under natural conditions, workers are mutilated soon after emergence from the cocoon, which results in both their inability to mate and a decrease in aggressiveness. In contrast, their lack of ovarian activity is not a consequence of the absence of gemmae, because many mutilated workers can begin laying eggs soon after removal of the gamergate (Fukumoto et al. 1989; Peeters & Tsuji 1993). However, a dominance hierarchy is soon established, and only the top-ranked worker continues to produce eggs, while the ovaries of subordinates become resorbed (Peeters & Tsuji 1993). These various lines of evidence indicate that oogenesis is regulated by hormonal differentiation among individuals, itself influenced by social interactions. In our study, we mutilated gamergates which had already been reproductively active for a variable period of time (they had mated weeks or months earlier). We did not record an effect on ovarian activity, which again reveals the distinct physiology of gamergates. Because ovarian activity of mutilated workers reaches a maximum 2–3 w after orphaning (Peeters & Tsuji 1993), we observed for only 3 w after the manipulation and thereafter dissected workers. Apparently, however, observation longer than 3 w is needed to clarify the effect of mutilation on a gamergate's physiology.

Peeters & Billen (1991) distinguished between two separate physiological processes resulting from the absence of gemmae: a change in individual behaviour, and a halt in the emission of a pheromonal signal. To differentiate between experimental mutilation of gamergates and coating of their gemmae, our finding that ovarian activity remained suppressed implies that the putative gemma pheromone does not function to signal the presence of a gamergate. Three weeks is enough to study a releaser effect (because mutilated workers become aggressive within 1 d after orphaning). This raises two proximate questions. Firstly, what stimulus of a gamergate mediates the regulation of oogenesis in mutilated workers? Secondly, what is the function of exocrine glands inside the gemmae? Two points are worth noting. Firstly, Peeters & Higashi (1989) discussed that, once the unmutilated worker of *D. australe* mates, the inhibition of egg-laying by other workers becomes pheromonal. Experimental evidence indicates that the inhibitory substance in *D. sp.* is non-volatile and is perceived by direct contact between a gamergate and workers (K. Tsuji, unpubl. data). Secondly, it seems clear from a recent study (K. Nakata et al., unpubl. data) that gemmae do not release an attractant pheromone for males, as had initially been suggested by Peeters et al. (1992). Elsewhere, we will show the real function of gemmae pheromone (we have evidence indicating that the gemmae secretion is an orientation pheromone (K. Tsuji, unpubl. data), and present evolutionary dis-

cussions about the extraordinary regulatory system of the genus *Diacamma*, together with detailed experimental data and inclusive fitness calculations.

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