Reproductive differentiation and conflicts in *Diacamma*: A model system for integrative sociobiology

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ABSTRACT. The ponerine queenless ant genus *Diacamma* has attracted attention for its unique mode of reproductive differentiation through mutilation of the thoracic appendage, gemma, after emergence of monomorphic females. I will begin this review with a description of the scientific history of the discovery of this differentiation mechanism, followed by a review and discussion of the ontogenetic origins, physiological and behavioral effects, and adaptive significance of the gemma and mutilation. Next, I will discuss the adaptive significance of helper-worker reproduction and its regulatory mechanisms. The gene-eyes-view implications of prolonged mating, another unique characteristic of this genus, are also discussed. Finally, the physiology and molecular biology of reproductive differentiation and the territorial behavior and nestmate discrimination mechanisms will be briefly reviewed, along with a list of other topics.

INTRODUCTION

The ponerine ant genus, *Diacamma*, currently has around 44 taxonomically described species. Its geographical distribution ranges from India and south-eastern Asia east to Vanuatu and south to northern Australia (Shattuck & Barnett 2006). In many habitats, it stands out with its large body size (8-16 mm in body length). Colonies are small, containing 12 to 450 adult individuals. They often nest in rotten wood and under stones on the ground's surface. In some species, nests are also found in soil with a mound around the entrance, or occasionally in the cavities of live trees (Annagiri 2021). The systematics of this genus is underway (e.g., Shattuck & Barnett 2006; Laciny et al. 2015; Zettel et al. 2016), and we await a picture of the phylogenetic relationships among species and populations within the genus.
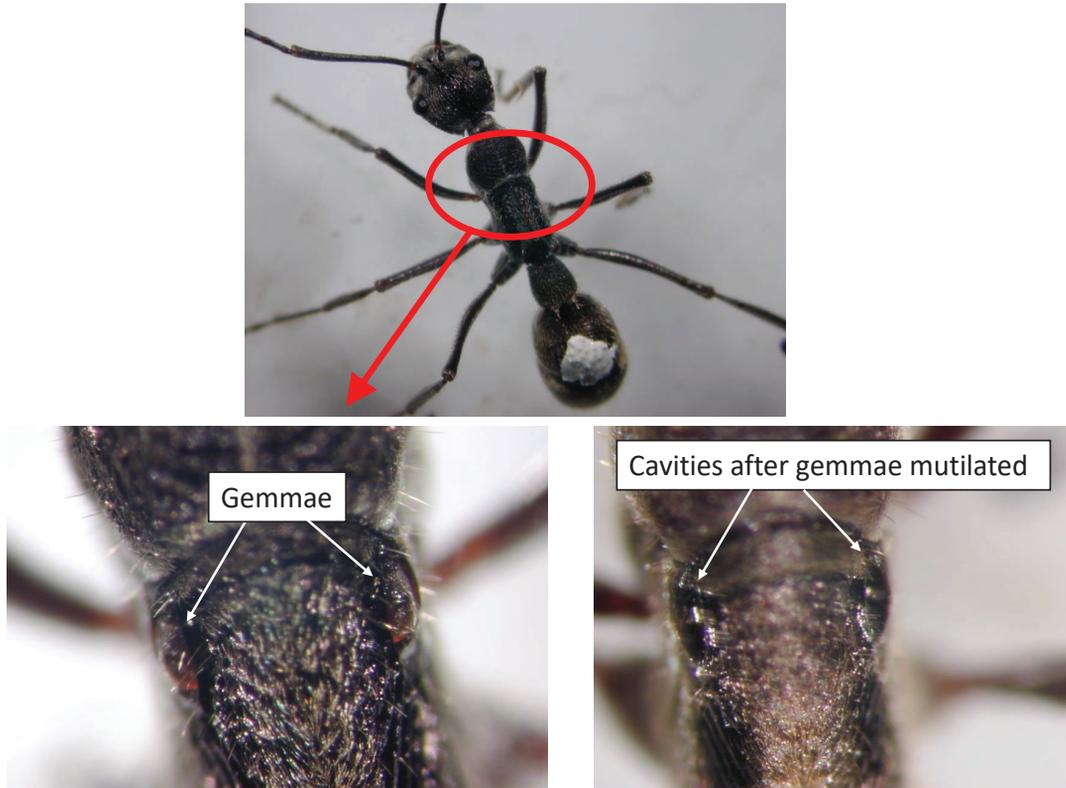
Fig. 1. Images of a *Diacamma* cf. *indicum* (from Japan) female (upper), gemmae (left) and gemmariums (right) which are the cavities remaining after the mutilation. The body size of a female is ca. 10 mm. Photographs were taken by Nao Fujiwara-Tsujii.

Gemma and its Mutilation
General

Diacamma has increasingly attracted attention due to its unique social structure, which has been studied by several groups worldwide using different approaches, such as behavioral, physiological and molecular methods as reviewed below. The genus lacks a winged queen and has no ergatoid queens (a morphological reproductive caste that is wingless but can be distinguished from workers by other traits such as body size) (Annagiri 2021). Females are monomorphic, with one mated female per colony assuming the reproductive role (functional queen) called gamergate (hereafter, GG: wingless females that reproduce sexually and are morphologically indistinguishable from other non-reproductive helper females). The other females are basically non-reproductive helpers. All species are monogynous (one GG per colony), and no species or population is known in which polygyny (multiple GGs per colony) is the norm (Annagiri 2021).

GGs are sporadically found in poneroid ant genera such as *Rhytidoponera, Pachcondyra, Harpegnathos, Diacamma, Dinoponera,* and *Streblognathus* (Peeters 1991; 2012). In these "queenless" poneroids, GGs are determined after adult emergence. The uniqueness of *Diacamma* lies in the differentiation mechanism of the female role between reproductive and non-reproductive individuals (Fukumoto et al.1989; Peeters & Higashi 1989). Although in many other cases, dominance behavior regulates reproductive division of labor, in *Diacamma* the roles are determined by specialized social manipulation (with the exception of the *Diacamma* sp. "nilgiri" population, as mentioned later). All females emerge with a pair of thoracic appendages called gemmae, which are however, mutilated in most females imme-

diately after the adult-eclosion (Figure 1). The mutilator is usually the GG, but in some species, helpers also take this role or help the GG mutilate (Baratte 2006a). Mutilated females do not mate and start to exhibit the behavioral developmental pathway as helpers. In an orphan colony that has lost the GG, the newly emerged callow females retain their gemmae, and aggress nestmates and attempt to cut each other's gemmae. The female that emerged earliest has an advantage in this fight (this is easy to imagine; she seems to be more mobile possibly owing to having a harder cuticle, etc.), and as a result the first emerged female usually takes over GG's role, and mutilates the gemmae of all her sisters that emerge later. This individual eventually mates and becomes the new GG for the colony. Thus, only the GG retains gemmae for life. This social biology is basically the same for all known species of the genus (with the obvious exception of "nilgiri", discussed below), with some quantitative differences. This remarkable social biology based on gemma mutilation was made widely known by the paper by Peeters & Higashi (1989). I am aware of the episode that led to this discovery and I would like to record it here because it is important for the history of myrmecology.

History

It has long been known that *Diacamma* colonies do not have queens, and that some of the workers play the queen role (Wheeler & Chapman 1922; Mofett 1986). However, unlike what has been circulated, the discovery of the control of reproductive role by gemmae mutilation was first described in the early 1980s by Yuji Fukumoto in his master's thesis (Fukumoto 1982; archived in the University of the Ryukyus Library) using a population in Okinawa, Japan. (At that time, it was described as *D. rigosum*, however, the taxonomic position of this Japanese population is yet undetermined. Fujioka et al. (2021) proposed the tentative name *Diacamma* cf. *indicum* (from Japan), since it appeared to be closely related to the Indian *D. indicum* (Viginier et al. 2004).) In the early 1980's Fukumoto was a graduate student of the late Takuya Abe, a well-known termite researcher. They, and especially Abe, did not fully recognize the importance of this discovery at the

time (Abe directly told me so), and instead were more interested in nest relocations and tandem behavior, a subject that has recently been actively studied in this genus. They published a pioneering paper on tandem running in *Diacamma* (Fukumoto & Abe 1983). The differentiation mechanism, however, remained unpublished except for Fukumoto's master dissertation in Japanese for a long time. During his fieldwork in Australia in the mid-1980s, Abe met Christian Peeters, who was a postdoctoral fellow with Ross Crozier at the University of New South Wales in Sydney. Abe honestly discussed their unpublished findings on Okinawa's *Diacamma*, and Peeters started working on an Australian species (*D. australe*) with Seigo Higashi, who was a member of Abe's field research in Australia. This led to the publication of Peeters & Higashi (1989). When Abe heard that this paper was about to be published, he hurriedly published Fukumoto et al. (1989) in a Bulletin of Kyoto University. From this start, *Diacamma* research projects were launched in many countries, and Peeters played the role of their facilitator. His comparative biological perspective became the driving force for the development of extensive research in several countries. I will review scientific knowledge of *Diacamma* to date, with references on its reproductive biology.

Ontogenetic origin

Although the Poneroid complex retains many ancestral traits such as small queen-worker dimorphisms, reproduction by GGs is clearly a derived phenomenon (Peeters 1991; 2012), attributed to the secondary replacement of the reproductive role by mated workers after the establishment of the morphological castes of queens and workers. The coexistence of winged queens and GGs in some species, such as *Harpegnathos* and *Rhytidoponera*, supports this view (Peeters 2012). However, it remains controversial as to whether the current adult female form (GG) of *Diacamma* is derived from the queen form or the worker form in the ancestral lineage. Previous studies on individual development suggest gemmae are homologous to forewings (Baratte et al. 2005; Gotoh et al. 2005), as many early authors described them as vestigial wings (e.g., Fukumoto et al. 1989). If they are homologous to the

wings, then females may have ontogenetic characteristics close to queens of other ants, or they may be an intermediate form between queens and workers. However, it has also been suggested that at least the developmental origin of the exocrine glands opening on the surface of gemmae may not be homologous to the wing, and therefore the gemma can be a developmentally novel form (Baratte et al. 2006b).

From what ancestral state did this unique mode of reproductive differentiation evolve? In the Australian wasp, *Ropalidia plebeiana*, a wing-biting struggle between potential queens is known (Saito & Kojima 2005). In termites, it has been argued that manipulation of nymphal wing buds by other individuals is a means of interfering with subsequent differentiation into alates (Zimmerman 1983), though this finding remains empirically questionable (Korb 2005). For ant gynes, wing damage prior to dispersal may severely limit the dispersal ability of gynes, so wing-damaged females may become helpers. Actually, in some ants it is occasionally observed that dealate virgin gynes act as helpers in their natal colony (e.g., Nehring et al. 2012). One can imagine that a wing-biting struggle between gynes over future queen status might have existed in the ancestral *Diacamma* that had a winged queen. Reproductive differentiation through such physical fights might have been followed by the evolution of other characteristics of *Diacamma* today, such as the development of gemmae instead of wings, and the practice of dependent colony founding.

A good comparison might be the Indian population known as the *D.* sp."nilgiri", which is closely related to *D. ceylonise*. These two are genetically isolated by allopatry, but mitochondrial data suggest some genetic introgression (Baudry et al. 2003), and there are interesting intraspecific karyotypic polymorphisms in both populations (Karnik et al. 2010). *Diacamma* sp."nilgiri" colonies are monogynous (one GG per colony), as in other *Diacamma*. Reproductive differentiation, however, is controlled by a dominance hierarchy among females rather than by mutilation (Peeters et al. 1992). (Note that in Peeters et al. (1992) *D.* sp. "nilgiri" was referred to as *D. vagans*). Nearly all females of "nilgiri" retain gemmae intact, and the highest ranked female is the GG of the colony. (I guess an accidental loss is likely responsible for the occasional absence of gemmae in "nilgiri"). When this GG is removed, aggressive dominance interactions occur among young females. The top-ranked female eventually engages in mating and reproduction (Peeters et al. 1992), similar to what is usually observed in the other queenless ants (Cournault & Peeters 2012). If this observed state of non-mutilation in *D.* sp. "nilgiri" is ancestral, the hypothesis that the wing-biting fight among gynes represents the origin of gemmae mutilation can be completely rejected.

The mode of reproductive differentiation in "nilgiri", however, is likely derived from the ancestral state of differentiation by mutilation. A clever interpopulation fostering experiment provided this conclusion. When GGs of "nilgiri" were presented with unmutilated callow females of *D. ceylonese*, the "nilgiri's" GGs, which naturally do not perform mutilation behavior, engaged in mutilation. In marked contrast, when callow "nilgiri" females were presented to GGs of *D. ceylonese*, which normally mutilate callows, the GGs failed to cut off the gemmae (although they showed interest in the callow females). Although "nilgiri" has gemmae, their morphology seems to have slightly altered with fewer exocrine glands (Cournault & Peeters 2012). This or an associated chemical change of the putative gemma gland secretions may caused GGs of *D. ceylonese* to fail to mutilate "nilgiri's" gemmae (Ramaswamy et al. 2004). The wing-biting hypothesis thus cannot be rejected. However, evidence supporting this hypothesis is admittedly also lacking.

Physiological and behavioural effects

What is lost by removal of gemme is not egg-laying ability but mating ability. This is because mutilated females, helpers, retain their haploid male egg-laying ability, and helper reproduction occurs frequently in orphan colonies (Fukumoto et al. 1989; Peeters & Tsuji 1993). A future gamergate (hereafter, FG: an unmutilated callow female that retains gemmae and is following the behavioral developmental pathway to be a GG but is still unmated) becomes sexually mature by about one week after emergence. A sexually mature FG performs the sexual calling behavior: she goes to the entrance of her nest and rubs the

dorsal surface of the abdomen with her hind legs, which are slightly raised, to attract males to mate. Males are alates in *Diacamma*. An early study that found the presence of exocrine glands in gemmae assumed that gemmae might secrete sex pheromones (Peeters & Billen 1991). However, Nakata et al. (1998b) suggested that sex pheromones are secreted from the metatibial glands (Hölldobler et al. 1996) of the hind legs and not from gemmae. The correct causality is that gemmae mutilation prevents females from expressing sexual calling behavior. Interestingly, Nakata et al. (1998b) reported that not mutilated but just gemmae-coated (with shellac) callows, ceased sexual calling behavior. Furthermore, after careful removal of this coating that avoided damaging the gemmae, the callow started (or resumed) sexual calling (although the sample size was small). The front surface of gemmae has many sensory hairs as well as gland openings, and nerves leading to the central nervous system extend from the gemmae (Gronenberg & Peeters 1993). These structures are destroyed by gemmae mutilation. This may suggest that random or specific external stimuli received from the sensory hairs of the gemmae may trigger (release) sexual calling behavior. Further neurobehavioral studies are needed. Again, a good comparative material is *D.* "nilgiri", in which nearly all helpers continue to possess intact gemmae, and the top ranked helper is known to perform sexual calling behavior (as a new FG) in the orphaned condition (Peeters et al. 1992; Baudry et al. 2003). It will be interesting to see if FGs of *D.* "nilgiri" of which gemmae are artificially removed perform sexual calling behavior or not.

It has been discussed that gemmae may be the queen pheromone secreting organs (Peeters & Billen 1991). However, this hypothesis is unsupported, because neither the artificial removal of gemmae of GGs nor their shellack coating changed the helper egg-laying inhibitory effect of GGs (Tsuji et al. 1998). Note that, as explained later, queen pheromone also exists in *Diacamma*.

Adaptive significance

The gemmae mutilation is performed by GGs in some species (*D. austral*, *D. ceylonese*) and in others also by helpers (*D. palldium, D.* cf. *indi-*

cum (from Japan)) (reviewed in p.308 of Baratte 2006a). Since callows undergo mutilation in colonies with a healthy GG or FG, this clearly has the effect of maintaining monogyny. However, to understand the adaptive significance of this behavioral interaction, we should focus on the inclusive fitness effect of both the mutilator (GG/helper) and the victim (callow). Callow females before being mutilated are potentially totipotent. However, presumably each callow needs to decide whether it will be an FG or a helper soon after adult-emergence. Perhaps this is because sexual reproduction requires the development of internal morphologies that helpers do not have, such as a thick spermatheca, well-developed spermathecal glands, a thick bursa copulatrix or genital chamber (Allard 2002; 2005) and developed ovaries (Okada et al. 2012). The robust bursa seems necessary to enable the female to withstand the longest known copulation in ants (hours to over a day in some cases) in which the male inflated penis is inserted tightly to grasp the female (Allard 2002). Before mutilation, these reproductive organs in callows quicky develop from a neutral state at their emergence (Allard et al. 2005). After mutilation, callows, now helpers, start to reduce the bursa copulatrix and spermathecae, and these are highly likely be irreversible changes (Allard et al. 2005). By one week after the emergence, FGs appear to be ready for mating and to start laying eggs (Allard et al. 2005).

As mentioned in the previous subsection, the gemmae secrete neither sex pheromones nor queen pheromones. Mutilation, therefore, does not appear to absolutely (physically) deprive females of their ability to become GGs but just causes physiological and behavioral changes. The mutilation of gemmae can be interpreted as a decision-making trigger for the victim callow females to differentiate into helpers and adopt indirect fitness-obtaining tactics, as mutilation is a reliable cue of the existence of a GG or FG. Otherwise, i.e., being unmutilated, the callow will adopt tactics to increase direct fitness as a GG in the future. The adaptive significance of this decision rule from the gene-eyes-view is understandable when we consider the costs of these decisions, especially those of choosing the FG pathway. The cost of choosing the FG pathway manifests when there is a rival nestmate in repro-

ductive competition. This may incur direct costs, such as death or injury due to fighting with the rival. Even if they coexist without fighting, if the limiting factor to the overall colony productivity is something other than GG's egg-laying ability, then direct fitness per GG will be reduced. Since GGs have to share limiting resources, such as food for example, reproductive success per GG will be diminished. Also, even if she wins this conflict, there will be an indirect inclusive fitness cost if the rival is a relative (with the largest cost if the rival is her mother). These situations have been discussed in skew models (see Reeve & Ratnieks (1993) for instance). When two sister GGs were experimentally put in a colony, the fight became violent and continued until one died (K. Tsuji, unpublished data). Because of these costs, the inclusive fitness model predicts that callows are more likely to choose helper strategies if their rivals are close relatives, are more physically competitive (Reeve & Ratnieks 1993), and are healthier (with relatively longer expected tenure as a reproductive) (Tsuji & Tsuji 2005).

The decision-making process of callows triggered by gemma mutilation is likely to save time and energetic resources compared to differentiation through dominance behavior (regardless whether based on ritual or aggression) observed in other queenless ants, and therefore may be adaptive (Baratte et al. 2006a). However, if this is an adaptation, why is the differentiation via dominance behavior that results in slower decision-making present in the majority of other queenless ants and in other social insects with undifferentiated queen-worker morphology? One reason could be the lack of key preadaptation such as development of special organs like gemmae that enable to trigger quick decision-making. In the eusocial wasp *Roparidia marginata* whose social structure resembles that of *Diacamma* (in the sense that all females are morphologically equal), females differentiate into reproductive or non-reproductive individuals before emergence, seemingly related to the demographic environment of the colony (Gadagkar et al. 1988). In this example, the timing of the developmental pathway determination is similar to that of *Diacamma* or even earlier. The wasp has no specialized structure like gemmae, nevertheless achieves this early role differentiation without involving dominance

behavior. This may provide counter evidence of the preadaptation hypothesis, and suggest that the environment has stronger effects on this issue. I believe that the apparent rarity of early developmental path fixation in eusocial insects that have no physical caste, may be due to a tradeoff with flexibility to environmental changes. An early determination seems to require certain irreversible changes in subsequent development. Such irreversible changes are more efficient when the environment is stable or predictable but may become costly when the environment unpredictably changes. If for example the GG dies when there is no brood in a *Diacamma* colony, the colony is destined to become extinct. Further comparisons between "nilgiri" and the other *Diacamma* will provide an opportunity to test these ideas.

Associated change in chemical/CHC profiles

In the case when the GG performs mutilation, callows may have, even partial, access to information on a reproductive rival. Baratte et al. (2006a) found that when a young immature FG attempted to mutilate, the target callow resisted or even attacked the FG and attempted to mutilate the FG's gemmae. In marked contrast, when callows encountered a mature GG (most likely the mother in natural situations) and were once captured by the GG, they did not resist and showed submissive behavior that facilitated mutilation. An unmutilated callow soon after adult eclosion is usually aggressive and frequently shows dominance behavior to her nestmates. However, within a few days, aggression ceases, and her reproductive dominance seems to switch from behavioral to pheromonal (Cuvillier-Hot et al. 2002; Barate et al. 2006a). This change is associated with change in the cuticular hydrocarbon (CHC, hereafter) profiles (Cuvillier-Hot et al. 2001; 2002), which are known to function as queen pheromones or signals for ovarian development in other social Hymenoptera (Holman et al. 2010; van Oystaeyen et al. 2014). An alternative hypothesis is that the information emitted changes depending on the mating experience. Counter evidence for this alternative is that FGs appear to be recognized by helpers in much the same way as GGs a week after hatching, even when unmated (Cuvillier-Hot et al. 2002). I believe, therefore, that mating does

not play an important role in the signal change, although rigorous experimental data are still needed. In any case, exposure to cuticular hydrocarbons of the GG during the mutilation process should reliably signal the presence of GG.

However, in species in which helpers also take on the role of mutilation in the presence of GG, the callow can only indirectly perceive the presence or absence of its rival (a GG or FG). The presence of information on the GG is transmitted to helpers through direct physical contact (Tsuji et al. 1999). Eggs laid by the GG also to some extent convey the same GG presence information (Shimoji et al. 2011), in which CHCs are the presumed semiochemical source (Shimoji et al. 2012). The use of a negative cue such as the absence of a pheromone would be costly, if there is a time lag between the disappearance of the GG and the effect of the helper's behavioral change (in this context, the cessation of gemmae mutilation by helpers) because of the increased uncertainty. In *Diacamma* after removal of the GG this response occurs in more than one day, but less than three days in *D. palldium* in Malaysia (Sommer et al. 1993) and in one day in *D.* cf. *indicum* (from Japan) (K. Tsuji unpublished data, see also Kikuchi et al. 2008, 2010). The cost of this time lag is likely to be small, since the pheromone durability in *Diacamma* is seemingly shorter than those of queen pheromones of other ants (e.g., Endler et al. 2004). Therefore, mutilation by helpers may also represent reliable information of the GG presence in *Diacamma*. I am tempted to infer that the durability of queen pheromone effect has been adaptively modified.

Gemmae mutilation by helpers in the presence of the GG can be regarded as genuine worker policing (interference with sexual reproduction opportunities by workers that do not reproduce on their own - not selfish policing; *sensu* Stroeymeyt et al. 2007).

Helper Reproduction
Regulatory mechanism

The question of how helper reproduction is regulated in *Diacamma* provides an excellent opportunity to test the theory of inclusive fitness, and I have focused most of my research on *Diacamma* on this question. The control of helper (worker)

egg-laying in social hymenoptera can be chemical (control by queen pheromones or refraining from egg-laying by pheromone perceived by helpers) or behavioral (dominance and policing behavior), all of which are known to be involved in *Diacamma*. Their relative importance varies with the growth stage of the colony, with queen pheromone transmission and self-restraint by helpers being important in small colonies (containing less than 100 helpers in the Japanese species). On the other hand, in larger colonies, aggression among helpers and destruction of helper-produced eggs via egg cannibalism by the GG and by other helpers can also control the reproductive opportunities of helpers (Nakata and Tsuji 1996; Tsuji et al. 1999; Kawabata and Tsuji 2005; Kikuchi et al. 2008; Shimoji et al. 2018).

In *Diacamma*, oviposition ability of a GG and that of a laying-helper are relatively small (usually are less than 10 eggs per day; K. Tsuji unpublished data). Nevertheless, because of the relatively small colony size (containing only 12-450 females), and the potential for oviposition ability of the helpers to be comparable to that of the GG, it appears that a single individual is able to monopolize the production of male eggs that the colony can raise into adulthood. Monopolization of egg-laying by a single helper actually happens in orphan colonies one to two months after the removal of the GG (Peeters & Tsuji 1993). Given this biology, potential conflicts over the rights to male production between the GG and helpers, and among helpers, are predictable. This is because mothers are related to their own sons (relatedness r=0.5) more than males produced by any other colony members (brothers produced by the mother GG have r=0.25, nephews produced by a full-sister are r=0.375, nephews produced by a half-sister have r=0.125). I will review empirical data on relatedness later.

Queen pheromones of GG are transmitted to workers by direct physical contact (Tsuji et al. 1999). The GG regularly patrols the nest (Kikuchi et al. 2008), transmitting pheromones and interfering with helper reproduction by eating eggs when she detects them (Kikuta & Tsuji 1999). A GG patrols more frequently in larger colonies, apparently in an attempt to maintain the efficacy of pheromone transmission (Kikuchi et al. 2008). This GG colony size "sensing" mecha-

nism has been mathematically modeled (Sugawara et al. 2009), but no empirical test has been performed as yet. The queen pheromone seems to be a CHC in *Diacamma* as well (Cuvielle-Hot 2002; Shimoji et al. 2012), but the rigorous bioassay data are yet to be published (Kikuchi, T. personal communication).

Aggressive dominance behavior involving biting and jerking a body part of the opponent occurs between helpers even in the presence of a GG, though usually much less frequently than in orphan colonies (Peetrs & Tsuji 1993; Tsuji et al. 1998). The GG does not participate in this aggressive dominance. Clearly the dominance behavior among helpers reflects a selfish struggle for the right to produce sons of their own. In small colonies with fewer than 20 helpers, queen pheromone transmission by the GG is highly efficient, and dominance behavior is rarely observed. However, the frequency of dominance behavior increases with colony size, and direct male production by some dominant helpers also occurs in larger colonies (Nakata & Tsuji 1996; Kikuchi et al. 2008). A network analysis revealed that the dominance hierarchy is almost perfectly linear with no loop structures (Shimoji et al. 2014) and always contains a single top-ranking (alpha) helper. Only limited members of the colony participate in this dominance interaction. It is not clear, however, whether the hierarchy length is related to colony size as predicted by the inclusive fitness theory (Monnin & Ratnieks 1999).

When a colony is orphaned, the frequency of dominance behavior (within three hours) and eventually helper oviposition (within one week) increase. Initially many helpers lay eggs, but after one to two months the alpha helper monopolizes oviposition (Peeters & Tsuji 1993). In *Diacmma* (except for "nilgiri"), aggressive dominance behavior among helpers remains frequent, even after the alpha helper establishes her status (Tsuji et al. 1998). Chemical inhibition by the alpha helper appears to be weaker than that of GGs, and she must continuously suppress rival helpers through aggression. In addition, regardless of the alpha status, when a new FG emerges in an orphan colony, the colony exits in the orphan state and the reproductive role is almost exclusively held by the FG (Baratte et al. 2006a). The differ-

ence in the "presence" or the "power" between a GG (FG) and the alpha helper can be clearly seen here.

Direct interference of helper oviposition by egg-cannibalism by the GG and other helpers rarely occurs in unmanipulated colonies with a GG. Under experimental conditions, in which eggs laid by orphan helpers are introduced into GG-present colonies, egg-destruction by the GG (Kikuta & Tsuji 1999 called this queen policing by GG) always occurs regardless of colony size (Shimoji et al. 2018; but see Nakata & Tsuji 1996). On the other hand, worker policing by helpers occurs in two ways: attacks on ovary-developing helpers (immobilization; Kawabata & Tsuji 2005) and egg-destruction (Kikuta & Tsuji 1999). Shimoji et al. (2018) found that ca. 90% of experimentally introduced helper-derived eggs were destroyed within the observation time in small colonies with less than 100 helpers, whereas the destruction rate plummeted down to about 10% in large colonies with more than 150 helpers.

In summary, in small colonies, the helpers' effort is mostly directed toward altruism and (potentially) policing, whereas in mature colonies the helpers' effort is partially directed toward self-reproduction and dominance behavior. Simultaneously, the policing efficiency is reduced in large colonies. As a result, alates (males) are produced predominantly in large colonies under GG-present conditions. In other words, a shift from the ergonomic stage to the reproductive stage, the well-known growth schedule in social insect colonies, emerges. Microsatellite DNA analyses estimated that an average of 65% of males are of helper origin in the Japanese species (Shimoji et al. 2018). These data are generally supportive of the predictions of inclusive fitness models (Ohtsuki & Tsuji 2009) as discussed later.

Policing and its adaptive significance

Worker policing by helpers occurs at least in the Japanese species (Kikuta & Tsuji 1999; Kawabata & Tsuji 2005). Behavioral observations suggest *Diacamma* are monogynous with a once-mated GG in each colony. This seems to contradict the theoretical prediction based on relatedness (Woycieckwaki & Lonminki 1987; Ratnieks

1988). However, the relatedness structure of a colony may not follow the strict Hamiltonian 3/4, because GG turnover can occur in the field. When a full sister of a helper becomes a new GG, from the helper's point of view relatedness of the GG's sons is 0.375, which is higher than the relatedness of males produced by a daughter helper of the new current GG (r=0.1875). Thus, in such "mixed" colonies worker policing is adaptive purely in terms of relatedness at least for some colony members. What then is the real intracolonial family structure estimated by molecular markers in *Dacamma*? In *D. cyaneiventre*, microsatellite analysis showed that such mixed conditions were observed in 15% (André et al. 2006) or 19% (André et al. 2001) of colonies. Nevertheless, the mean relatedness 0.73±0.015SE was not statistically significantly different from the monogyny-monandry expectation of 0.75 (André et al. 2006). GGs are estimated to live about three times longer than helpers (André et al. 2006; see also Tsuji et al. 1996). Such rapid turnover of helpers may be a factor for this high average relatedness. On the other hand, in the Japanese species, the degree of relatedness among helpers was slightly lower, r = 0.550.5SE, and some (1/5) GGs mated twice (Shimoji et al. 2018). However, neither of the two relatedness estimations fulfill the condition for worker policing - males produced by the GG have on average higher relatedness to the policer-heplers than males produced by other helpers. The average relatedness alone does not explain worker policing in *Diacamma*.

There are two possible explanations for the observation of worker policing in *Diacamma*. The first hypothesis is that what was observed was not worker policing, but rather a phenomenon similar to selfish dominance behavior. Policing has multiple definitions, and it is unclear how it differs from dominance behavior under some definitions, such as interference of direct reproduction of another individual (e.g., Ratnieks et al. 2006). However, I would like to distinguish it here with the following definition to clarify the issue. Dominance behavior between helpers, regardless of whether aggressive or ritualistic, is a demonstration of rivalry for male egg-laying rights toward other helpers, which has the effect of increasing the chance of male egg-laying for the dominant actor. This is a relatively simple selfish

reproductive competition that is related to direct fitness. The other hypothesis is that policing is a behavior that has the effect of increasing indirect fitness rather than the direct fitness of the individual performing the behavior (genuine worker policing: Stroeymeyt et al. 2007). To distinguish between the two hypotheses, we can first look at whether the actors themselves are attempting to lay eggs or develop ovaries; Kawabata & Tsuji (2005) found that most helpers that attacked other helpers after the colony split-and-fusion procedure did not develop ovaries, suggesting that this behavior was genuine worker policing. The second criterion of genuine worker policing is the involvement of a third party. Selfish dominance behavior is motivated by the need to deter any other helper from laying eggs in order to achieve the most favorable option for the actor (self-oviposition to produce r = 0.5 offspring). This is true for all helpers, so there is a conflict of interest for all. There is no adaptive significance for any helper to aid an attacker. Indeed, in *Diacamma*, the aggressive bite and jerk interaction always occurs as a one-on-one contest between two helpers, and this interaction has been correctly described as dominance interaction (Peeters & Tsuji 1993; Kawabata & Tsuji 2005). Worker policing, on the other hand, has a different story. If we look at the indirect benefits of inclusive fitness, there can be situations where the oviposition of one helper is not in the best interest of many or even all other helpers in the colony. In this situation a third party, another helper, can aid a helper's policing attempt. Kawabata & Tsuji (2005) reported that much of the aggression observed after colony reunification was "immobilization", in which multiple helpers simultaneously attacked a single individual. There seems to be no doubt that genuine worker policing exists in *Diacamma* cf. *indicum* (from Japan). To increase indirect fitness is likely the adaptive significance of worker policing behavior in *Diacamma*.

So, what is the indirect fitness benefit of worker policing if it is not explained by relatedness? The colony-level cost of worker reproduction is a possible theoretical explanation (Cole 1986, Ratnieks 1988). If the self-reproduction of helpers has the effect of significantly reducing colony productivity, it may reduce the inclusive fitness of helpers. This cost is a somewhat tricky

concept. It implies a proximate constraint or a trade-off, in which each female cannot simultaneously exhibit the best performance in both helping and self-reproduction. I think that to rigorously predict the cost size and its effect on the evolution of worker policing requires a theoretical model which specifically reflects the colonial life history of social Hymenoptera. Ohtsuki & Tsuji (2009) employed this approach. Their model assumed that at any given point in a growing colony each helper (worker) can make decisions about how it allocates its own effort to self-oviposition or helping (thus these two are in trade off). The sum of helping efforts over all helpers is assumed to determine colony productivity, i.e., the size of colony resources available at that time. The queen (GG) is assumed to be able to control the sex of eggs she lays. Also, each helper can quantitatively decide resource allocation they invest in offspring among the brood (egg) pool in the colony (GG-produced males, helper-produced males, females) using colony resources. Finally, at any time in her life, a helper can decide how strongly to engage in worker policing. Colony-level resource allocation and the colony-level strength of policing are assumed to be democratically determined, reflecting the colony-member average. No cost is assumed in egg production and in policing. Ohtsuki & Tsuji (2009) analyzed the output when each of all parties tried to maximize inclusive fitness and making decisions by using a dynamic game programing. The main predictions of the Ohtsuki & Tsuji (2009) model are as follows. Worker policing evolves even in monogynous and monandrous colonies. It occurs in a colony growth stage-dependent manner; worker policing is potentially strong and all helpers' efforts are directed to helping and new helper production in small colonies. This leads to the emergence of the ergonomic stage in which the colony produces only helper-workers. On the other hand, when the colony size exceeds a certain threshold, worker policing is relaxed and thereby helpers are able to direct a proportion their effort to self-reproduction. As a result, the colony enters into the reproductive stage producing reproductive offspring (helper-produced males). It also leads to a 14.3% drop in colony productivity. In this way, the cost of worker reproduction is expressed only in the reproductive stage. The basic logic underlying this switch is the adaptive bang-bang strategy in the life-history model of social insects (Oster & Wilson 1978). That is, when the colony is small, producing helper workers rather than to invest in reproductive offspring is a better strategy for all colony members in order to increase their own inclusive fitness, because investment in the workforce during the early colony growth stage results in growth of the colony. Worker reproduction means the production of males, i.e., reproductive offspring, that should be prevented in small colonies. Those predictions are largely consistent with what was observed in the Japanese *Diacamma* as mentioned earlier (Shimoji et al. 2018; Tsuji et al. 2012).

The only prediction that was not fully supported by empirical data in *Diacamma* was that at the reproductive stage, all males are helper-produced, whereas in real colonies, on average only 65% males are helper-derived (Shimoji et al. 2018). This is probably due to the unrealistic assumption of the model that helpers can perfectly recognize each egg's-mother and the sex of eggs and selectively raise their preferred eggs. Real ants may not be able to fully distinguish between male eggs and female eggs. Although *Diacamma* helpers can discriminate between GG-derived eggs and helper-derived eggs when eggs are presented one by one (Shimoji et al. 2012), the discrimination opportunity may be limited in natural conditions. According to Nakata (1998), when male eggs derived from orphan helpers are added to egg piles of GG-present colonies, the male emergence rate eventually increases. Male production by both the GG and helpers is not a major contradiction in the theory.

Another important prediction of the model is that the cost of helper reproduction is only observed at the reproductive stage, when helper reproduction occurs, representing a 14.3% reduction in colony efficiency. I consider that the exact value of the cost, 14.3%, can depend on the model's assumptions: thus, the exact cost size is not so important. The noteworthy point is that in monogynous-monandrous colonies, it is adaptive for individual helpers to raise their own sons rather than the GG's sons in terms of relatedness, so a selfish strategy of personal reproduction evolves at the reproductive stage, even at some colony-level cost. Then, a scientific mission is to

empirically determine the size of this cost. Many studies attempted to detect the cost of worker (helper) reproduction in various Hymenoptera, which were mostly qualitative. In *Diacamma*, a significant reduction in the immunocompetence of orphan helpers was shown in an *E. coli* injection experiment, which was considered evidence of the cost of helper reproduction (Bocher et al. 2007). Tsuji et al. (2012) attempted to quantitatively determine the cost. In a series of experiments, it was found that there was no difference in the brood production speed of orphan and GG-present colonies. However, the average lifespan of helpers that were orphaned or isolated from the GG while keeping in the same colony size was reduced by about 18%. This value was not statistically significantly different from Ohstuki & Tsuji's (2009) prediction of 14.3%. The significant reduction in lifespan was specifically observed in the lower-ranked helpers that did not lay eggs in orphan conditions, while dominant egg-laying helpers showed no change in lifespan. In orphan conditions, dominant individuals direct their effort to self-reproduction, abandoning their helper role and increasing dominance acts. Subordinate individuals, that are still the majority, then increase helping effort, such as brood care, which makes up for the cost and maintains the production rate of the offspring. However, it seems to lead to overworking and shortened lifespan of subordinate helpers (Tsuji et al. 2012). One may think that orphan colonies should differ from GG-present colonies, and therefore that the observed cost may not reflect the real cost in the presence of the GG. This criticism is not relevant for two reasons. First, the cost was observed also in GG-present colonies, when contact of some helpers with the GG was impeded (Tsuji et al. 2012). Second, and more importantly, I consider that individual helpers may not be able to distinguish the orphan condition and the reproductive stages in the presence of the GG, because both conditions share a lack of (frequent) contact to the GG and exposure to queen pheromone (Nakata & Tsuji 1996; Kikuchi et al. 2008). Therefore, I believe Tsuji et al. (2012) provides the best quantitative evidence for the cost of worker (helper) reproduction. What was empirically supported is the trade-off in performance between helping and reproduction at the individual level.

Other Reproduction-related Topics
Prolonged copulation

The copulation duration in *Daicamma* is the longest in ants, lasting from several hours up to a day. Histological studies show that sperm transfer is completed within two minutes after mating (Allard et al. 2002; 2007). Allard et al. (2002), therefore, considered that prolonged copulation may be a method of mate guarding to protect male paternity. *Dinoponera quadriceps*, which is a queenless ant, also shows relatively long copulation with an average of 30 minutes (Monnin & Peeters 1998). In this case, the female kills the male and the copulation finishes (Monnin & Peeters 1998). In *Diacamma* the female also tries to disengage from the male during copulation, but it usually seems less feasible. Instead, helpers remove the male (Fukumoto et al. 1989; Allard 2002). This process is of interest in the context of sexual conflict. After mating takes place at the entrance of the nest, the female moves into the nest with the male still attached. The male is then attacked by a number of helpers who cut off his head and wings. The male abdomen, however, usually remains attached for hours (Fukumoto et al. 1989). Sperm are transported as a spermatophore (Allard et al. 2002), and sperm seems to occupy only a small fraction of the volume of the spermatophore contents. The seminal fluid may contain a female remating inhibitor, as known in other insects (e.g., Radhakrishnan & Taylor 2007). The male's abdomen can act as a mating plug until the female loses her desire to remate owing to this putative substance or for other reasons (ant queens are generally sexually active only when they are young). If *Diacamma* females have a potential incentive to mate more than once (e.g., Boomsma 1996), the helpers' quick killing of the male to end copulation might be a rare example of a third party's commitment in a sexual conflict in ants. The reason why helpers side with the female is perhaps because the female is related and the male is unrelated to the helpers. The occasional twice-mated GG in Japanese *Diacamma* (Shimoji et al. 2018) may be cases in which the interest of the female and helpers was successfully reflected. An alternative and more likely interpretation of male killing behavior by helpers is that the male bears a chemical signature of a

foreign colony and is thus attacked. The helper's engagement in the sexual conflict is a byproduct. Recently, however, it was reported that in *Cardiocondyla elegans*, workers are engaged in the control of mating opportunities of sexual individuals (Vidal et al. 2021). These findings may suggest that mating interference by helper-workers can occur more easily in ants than previously thought, especially when mating takes place within the home range of the colony.

Physiology and molecular biology of reproductive differentiation

Recently, the involvement of biogenic amines with reproductive division of labor has been intensively studied in the Japanese species; Okada et al. (2015) examined amine dynamics after adult emergence in FGs and helpers. Brain dopamine (DA) level was higher in FGs, and there appears to be a positive relationship between DA levels and ovarian development; Shimoji et al. (2017) similarly found that in helpers, both queen pheromone exposure through contact with the GG and aggressive dominance behavior from other helpers led to reduced brain dopamine levels. Together with the fact that topical application of DA promoted ovarian development and increased transcription of DA receptors (*dorp1* and *dorp2*) in the abdominal fat bodies (the organs that synthesize yolk protein), the above results suggest that dopamine has a function as a hormone promoting ovarian development. Okada et al. (2018) further compared differences in brain and abdominal gene expression between FGs and helpers and showed that the expression levels of genes related to nutrient transport and storage, such as insulin signaling genes and hexamerins, differed significantly. Miyazaki et al. (2021) similarly examined the relationship between gene expression and the division of labor among helpers, and found that the abdominal expression of the *Vg* gene (only one copy of which is present in this species) and its receptor (*VgR*), which contribute to ovarian development, differed. That is, their expression level was higher in GGs, nurse helpers, and forager helpers, in that order. Furthermore, the expression levels of the *Vg* gene and *Vg*-like*A*, from the same gene family as *Vg*, in the brain were different between the nurse helpers and forager helpers. This result suggests the close involvement of the *Vg* gene family in the division of labor in this species. The above-discussed genes are thought to have changed from regulating reproduction according to nutritional conditions in solitary species to regulating their expression according to social contexts (such as social ranks). The rapid differential expression of those "toolkit" genes suggests their importance in regulating reproductive division of labor also in *Diacamma*, which lacks morphological castes and in which reproductive differentiation occurs at the adult stage. *Diacamma* and other ants, as well as honeybees, have derived gene networks for reproductive division of labor. In particular, the reproductive function of JH (juvenile hormone) is different from that of the more ancestral *Polistes* wasps. These results are important for comparative biology to test the ovarian ground plan hypothesis and the split-function hypothesis (Sasaki et al. 2021). Since crossbreeding in the laboratory is possible in *Diacamma*, an approach using transgenic organisms may be available.

Brood Abduction, Nestmate Discrimination and Territorial Behavior

Brood abductions (especially pupae) by other colonies of the same species has been observed in *D. indicum*, in which stolen brood are not consumed as food but become the work force as helpers after emergence (Bishwarup & Sumana 2016; 2018; 2019). It is non-adaptive for the abducted pupae to become helpers after emergence. However, counter-adaptations in which the abducted individual detects and modifies its behavior (e.g., becoming more selfish) upon abduction, do not generally evolve in ants. This is presumably simply because kidnapped individuals have no opportunity to leave their own offspring and thus contribute nothing to future gene pools.

Colonies on the receiving end of theft do however put up a defense line against nest invasion by the same species through nest mate recognition (Suwabe et al. 2007). Although the foraging distance in the field is about five meters from the nest (Eguchi et al., 2004; Uematsu et al., 2019), violent attacks on non-nestmates of the

same species occur specifically only within two meters of the nest, that is, only within the nest vicinity (Uematsu et al., 2019). It is reasonable to assume that this is a line of defense against brood abduction. However, the proximate mechanism of nest mate recognition in *Diacamma* remains a puzzle. The CHC profiles, which are considered to be a nest-mate recognition label in other ants, have less marked differences among colonies in *Diacamma* (Cuviller-Hot et al. 2001). Furthermore, since females are wingless and colonies are founded dependently, neighboring colonies can be genetically related. However, molecular markers suggest that males can fly and disperse over much longer distances than females (Doums et al. 2002), so outcrossing mediated by this male behavior could result in genetic differences between neighboring colonies. Further research is needed.

Other Subjects

Other research themes have been developed in a variety of ways. Among them nest relocations and mechanisms of tandem behavior are extensively being studied mainly in India (Fukumoto & Abe 1983; Maschwitz et al. 1986; Fujiwara-Tsujii et al. 2012; Anoop et al. 2021; Anoop & Sumana 2015; 2018; Kaur & Sumana 2014; 2015; Kaur et al. 2012; 2017; Swetashree & Sumana 2015; Parateek et al. 2019; Snigdha et al. 2019) and circadian and other activity rhythms are relatively well studied mainly in Japan (Hayashi et al. 2012; Fuchikawa et al. 2014; Fujioka et al. 2017; 2019; 2021; Win et al. 2018). Other studies have examined the age-polyethism and its plasticity (Nakata 1995; 1999; Shimoji et al. 2020), foraging behavior (Rasgoti et al. 1997), nest structures (Swetashree & Sumana 2015), population dynamics (Karpakakunjaram et al. 2003), symbiosis and interspecific relationships (Maschwitz et al. 2000; 2001; 2004; Kaufmann et al. 2003; Peeters et al. 2015), molecular mechanisms of sexual dimorphism (Okada et al. 2006; Dobata et al. 2012; Miyazaki et al. 2014), carcass removal behavior (Banik et al. 2011), exocrine glands (Morgan et al. 1999; 2003; Peeters & Billen 1991; Billen 2008), trophallaxis (Fujioka & Okada 2019) and mandibular morphology (Manting et al. 2013). I will not go into the details of these subjects here. Instead, to conclude this article I would like to mention that *Diacamma* is now a model organism for comprehensive and integrative research from molecules to communities.

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