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Worker Reproduction in the Highly Polygynous Ant *Crematogaster pygmaea* Forel, 1904 (Hymenoptera: Formicidae)

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Abstract

In most ant species, workers have retained functional ovaries, allowing them to potentially lay viable unfertilized eggs that develop into males. Mechanisms (ex.: queen and/or worker policing) have nevertheless evolved to control worker reproduction when the queen is present. In many species with a high degree of polygyny, especially in tramp species, complete sterility of workers has evolved, presumably to "trap" them within their "worker phenotype". Our study showed for the first time that in the highly polygynous and polydomous ant *Crematogaster pygmaea*, workers retained the full capacity to produce reproductive eggs in queenless colonies, with at least some of them developing in adult males. We provide evidence that worker-produced males are reproductively functional. Although most queenless colonies produced eggs, few larvae developed into pupae and adult males. We conclude that workers strongly police the worker-produced offspring, even in the queen's absence. Probable high relatedness between queens of *C. pygmaea* colonies and strong genetic proximity between brood and workers could force the workers in their helper, non-reproductive function even if they keep the ability to reproduce. Our observations indicate that the production of adult males and gynes in *C. pygmaea* is controlled by seasonal factors related to the rainy season.

Introduction

Reproductive division of labor in which one or some females (queens) monopolize reproduction while all other females (workers) nurse the brood, build and maintain the nest, defend the colony, and forage for food is the hallmark of ant societies (and other eusocial Hymenoptera) (Wilson, 1971) and one of the main drivers of their ecological success (Hölldobler & Wilson, 1990). Such reproductive division of labor and its associated reproductive altruism is thought to have been evolutionarily promoted by high intracolony relatedness (of workers to the queen, among workers, and between workers and brood), thereby ensuring inclusive fitness of workers that give up their own reproduction (functional or total sterility) (Hamilton, 1964a, b; Crozier & Pamilo, 1996).

Except in some ant groups (ex: *Atta* Fabricius, 1804, *Carebara* Westwood, 1840, *Linepithema* Mayr, 1866, *Monomorium* Mayr, 1855, *Pheidole* Westwood, 1839, *Solenopsis* Westwood, 1840, *Tetramorium* Mayr, 1855) whose workers are completely sterile (reproductive organs lacking or vestigial), workers of most ant species have retained functional ovaries allowing them to potentially lay, at least under specific

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circumstances (death of the queen, for example), viable unfertilized eggs that can give rise to males (arrhenotoky) (and in some rare cases, to females, by thelytoky) (Bourke, 1988). Mechanisms to control worker reproduction have nevertheless evolved in ants since uncontrolled worker reproduction would be a source of internal conflicts over the male parentage (between queens and workers and even between workers) with potential costs for colony productivity (ex: loss of colony efficiency by disruption of the division of labor between queens and workers) (Bourke, 1988; Ratnieks and Wenseleers, 2005; Ratnieks et al., 2006). Generally, worker reproduction is controlled by the queen (through chemical and/or physical manipulation) (Holman et al., 2010) and/or, more commonly, by the workers themselves (self-policing or worker-policing), with worker-policing occurring at different levels: workers can prevent male production by congeners using aggression/killing towards workers that have ovarian activity (preovipositional policing) and/or by destroying/eating worker-laid male eggs (postovipositional policing) (Wenseleers et al., 2004; Kawabata & Tsuji, 2005; Ratnieks & Wenseleers, 2005; Ratnieks et al., 2006).

In some species, the presence of several reproductively active queens (true polygyny) can seriously reduce the within-colony relatedness values, more specifically the worker-brood relatedness, with a consequent rapid decrease of those values as the number of queens increases (Keller, 1995). Therefore, theoretically, in polygynous systems with workers keeping the ability to lay male-destined eggs, the presence of several breeders in the colony should promote workers' reproduction, with a consequent serious potential within-colony conflict over reproduction (Bourke et al., 1995). According to Keller (1995), one possible evolutive solution for such species with low within-colony relatedness could be the complete sterility of workers and the worker control over the reproductive brood since workers are "trapped" within their "worker phenotype" and have no other choice but to rear the royal brood. Indeed, workers are completely sterile in many ant species where a high degree of polygyny is observed, especially in tramp species (Passera, 1994; Keller, 1995; Bourke, 1988).

Although genetic databases and ovarian dissection have been commonly used to demonstrate workers' participation in male production, systematic direct observations of male production by workers are still lacking. Furthermore, no studies have been done to date to verify if males produced by workers were functionally reproductive (= able to mate with gynes and transfer viable and functional sperm to them), probably due partly to the fact that ants are typically less willing to copulate under laboratory conditions and that copulations are difficult to observe in the field (mating on the wing in many ant species) (Baer, 2011). Due to these technical problems, at least in non-poneromorph groups, this question of the reproductive functionality of the worker-produced males still needs to be investigated.

Crematogaster (Orthocrema) pygmaea Forel, 1904 is a highly polygynous and polydomous ground-dwelling ant that is a habitat specialist of generally anthropized and open areas in coastal and tabuleiro zones of the state of Ceará (Quinet et al., 2009; Martins Segundo et al., 2017), the only places where this species has been found so far, in addition to some specific areas of Caatinga (seasonally dry tropical forest) in the state of Piauí (northeastern Brazil) (Jory & Feitosa, 2020). Its colonies are formed by tens or even hundreds of underground and simple nests (each nest is formed by a single straight vertical tunnel several tens of centimeters long, with a mean of four horizontal chambers) interconnected by surface trails (Quinet et al., 2009; Carlos, 2015). The polydomous networks of *C. pygmaea* colonies have a seasonal dynamic since they rapidly expand at the beginning of the rainy season when the number of nests and queens increases (nearly 300 nests in the rainy season in one of the colonies studied by Carlos (2015)) while suffering a strong reduction in nests and queen number during the dry season (Quinet et al., 2009; Hamidi et al., 2012; Carlos, 2015). The number of queens per nest varies from 0 to 36 (mean ± SE, 4.27 ± 7.22) (Hamidi et al., 2012), each colony probably containing several hundred queens.

Preliminary and occasional observations (Quinet & de Biseau – unpublished) showed that *C. pygmaea* workers quickly lay viable eggs (i.e., non-trophic eggs) when isolated from queens. Based on these observations and the fact that *C. pygmaea* gynes easily mate with males in laboratory conditions (Martins Segundo et al., 2017), we conducted experimental studies to address several basic questions: Do workers' eggs develop into adult males? Is male offspring produced by workers viable, functional (able to mate), and fertile (producing viable and functional sperm)? Do workers' eggs are policed in queenright colonies?

Materials and Methods

Field colonies

Three large *C. pygmaea* polydomous colonies (Col-1, Col-2, Col-3) were used to collect the workers and queens used in the experiments described hereafter. All colonies were found on (or next to) the campus of the State University of Ceará (3°47' S – 38° 33" W) in Fortaleza (state of Ceará, Brazil), in anthropogenic and open areas with sparse herbaceous vegetation, the usual type of habitat of *C. pygmaea* (Quinet et al., 2009; Martins Segundo et al., 2017). The distance between the three colonies ranged from 0.7 km (between Col-1 and Col-3) to 1.5 km (between Col-2 and Col-3) (distance between Col-1 and Col-2: 0.8 km).

Monitoring of adult males' production in field colonies

From March 2004 to March 2005, at least ten nests were randomly excavated each month in the two colonies (Col-1 and Col-2). The number of males found in each excavated nest was registered.

Production of brood and adult males by workers in queenless experimental colonies

In April 2004, samples of workers were randomly collected by excavating several nests from the three colonies (Col-1, Col-2, Col-3). Those workers were used to form 25 queenless small experimental colonies with 200 workers each: six with workers from Col-1, seven with workers from Col-2, and 12 with workers from Col-3.

Colonies were kept in a laboratory room on the university campus and open to the outside under semi-natural light and temperature conditions. Each experimental colony was kept in a circular plastic box (12 cm in diameter, 15 cm high). A glass test tube (8 cm in length; 1 cm in diameter) with a water reservoir at the bottom, surrounded by a red plastic film, was used as a nesting site for the small experimental colony. The ants were fed *ad libitum* on a sucrose solution (0.1 M) (glass tube filled with sugar water) and a protein source (dry cat food). From May 2004 to February 2005, the number of eggs, larvae, pupae, males, and workers was registered each month in all experimental colonies.

To test the fertility of males produced in the queenless experimental colonies, five of them (all produced in queenless experiment colonies made with workers from Col-1) were kept with young gynes (N=5) (unmated winged queens collected in a *C. pygmaea* colony located ± 80 km from Fortaleza) in a Petri dish (8.5 cm in diameter), according to the method used by Martins Segundo et al. (2017) to study the mating and wings shedding behavior in *C. pygmaea*, in laboratory conditions. Males and gynes were observed continuously until mating occurred (Martins Segundo et al., 2017). After dealation (wings shedding), each young, mated queen was isolated in a glass test tube (10 cm in length; 1 cm in diameter) with a water reservoir, surrounded by a red plastic film and whose open end was closed by a cotton plug. Two months after the young queens were isolated, the presence of adult offspring (workers) was checked in each tube.

Policing of worker-produced brood in experimental queenright colonies

In April 2005, twenty nests containing at least one queen were excavated in Col-1 and Col-2 (ten nests in Col-1, ten in Col-2). The queen number per excavated nest ranged from one to 43 (mean ± SD 8.75 ± 10.20). Queens and workers from the same nest were kept together in an experimental colony (hereafter, "queenright experimental colony"). One hundred workers were then collected from each queenright colony and were used to form 20 experimental queenless colonies. The twenty queenright experimental colonies and the twenty corresponding queenless experimental colonies were each kept in a plastic box (23 cm × 18 cm and 4 cm high; sides coated with Fluon®). The conditions of colony maintenance (space for colony nesting, food, etc.) were the same as described above.

One month after the queenright and the queenless experimental colonies were formed, each queenright colony received a worker-produced egg from the corresponding queenless colony. It is assumed that all eggs used were non-trophic eggs (preliminary observations have shown that trophic and non-trophic eggs were morphologically different – Hamidi, 2010). To transfer an egg from a queenless colony to a corresponding queenright colony, a fine brush slightly humified was used to pick up the egg and to carefully place it into the foraging area of the queenright colony. In control experiments, an egg was collected from the royal brood of the queenright colony and was deposited in the foraging area of the same colony. To remove any chemical signal, the brush was cleaned using ethanol (90°) and dried between one egg transfer and the following egg transfer. Previous observations showed that the eggs were not conveyed between workers; they could therefore be easily followed using a simple magnifying glass. It was also observed in preliminary observations that a manipulated egg (in transfer or control experiment) could have two distinct fates in the queenright colony: destroyed (eaten or dropped on the waste deposit of the nest) or adopted (dropped by the transporting worker into the royal brood). After an egg from a queenless colony was introduced in a corresponding queenright recipient colony (or after an egg was picked up from the brood of a queenright colony and deposited in the foraging area of the same colony), it was observed for 20 min using a magnifying glass. Its fate (destroyed or adopted) was registered.

To test for a possible colonial odor drift after one month of isolation between the queenless and the corresponding queenright colonies, a worker collected from each one of the queenless colonies was introduced in the foraging area of the related queenright colony. After five minutes, responses (*i.e.*, adoption or aggression) of the resident workers toward the introduced worker were recorded.

Results

Production of adult males in field colonies

A total of 346 nests of *C. pygmaea* were excavated in Col-1 and Col-2 (165 in Col-1, 181 in Col-2) from March 2004 to March 2005. Adult males' production in the excavated nests began in September and October (Fig 1), at the end of the dry season. It peaked in December and February, at the very end of the dry season and the beginning of the rainy season, respectively (Fig 1).

Production of brood and adult males by workers in queenless experimental colonies

One month after they were formed, 84% (21/25) of the queenless experimental colonies had produced at least one egg (mean eggs number per colony ± SD: 18.3 ± 18.1; range: 1 to 79), while 68 and 28 % of them produced larvae and pupae,

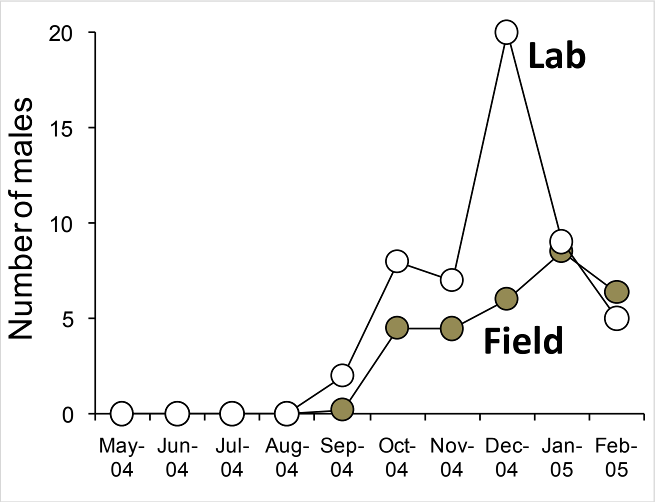

Fig 1. Time evolution of mean number of adult males per nest in *Crematogaster pygmaea* nests excavated in field colonies (Col-1 and Col-2) (Field), and of total number of adult males in experimental queenless colonies (Lab).

respectively (Fig 2). Only six colonies (24%) raised brood into adults (Fig 2). Furthermore, cumulated data showed that few larvae had turned into males: 51 males were produced out of a cumulated number of 2581 larvae over ten months (all colonies combined).

While six to seven weeks are needed to obtain adult workers from eggs (Martins Segundo et al., 2017), the first adult males were not observed before September and October, at the end of the dry season, i.e., not until five months after the queenless experimental colonies were formed, and their number reached a peak in December (Fig 1). Remains of crushed larvae, and pupae were regularly observed in the queenless colonies.

In the experiment where young gynes were exposed to males produced from eggs laid by workers of queenless colonies, mating behaviors were promptly observed, followed soon after by wings shedding behavior of all gynes, as observed by Martins Segundo et al. (2017) with gynes and males from queenright colonies. After two months, all young queens had produced nanitics workers, as Martins Segundo et al. (2017) observed in foundations with gynes fertilized by males from queenright colonies.

Policing of worker-produced brood in artificial queenright colonies

One month after they were formed from queenright colonies (N = 20), 80% (16/20) of the queenless colonies (7 of the ten queenless colonies formed from the nests excavated in Col-1, 9 of the ten queenless colonies formed from the nests excavated in Col-2) produced eggs. Each queenright colony then received an egg from the corresponding queenless colony. The behavioral observations showed that three types of situations could arise. The most common one (11/16 – 69%) was the egg being eaten by the worker who first found it. The second one (1/16 – 6,2%) was the egg being moved by the worker to the external waste deposit of the nest. In the third one (3/16 – 19%), the egg was moved to the nesting site by a worker, and deposited among the royal brood, in less than 1 minute. In this last case, the egg was considered as being adopted by the recipient colony. In one case (1/16), the egg was carried for more than 10 minutes by a worker and then was lost by the observer. In the control experiments (where an egg was picked up from the brood of a queenright colony and deposited in the foraging area of the same colony), a much higher rate of adoption (χ^2, df=1, $p = 0.0002$) was observed, with 94% (15/16) of eggs being adopted by the recipient queenright colonies.

Finally, all workers from queenless colonies introduced into the corresponding queenright recipient colonies were adopted. No aggressive behaviors were observed.

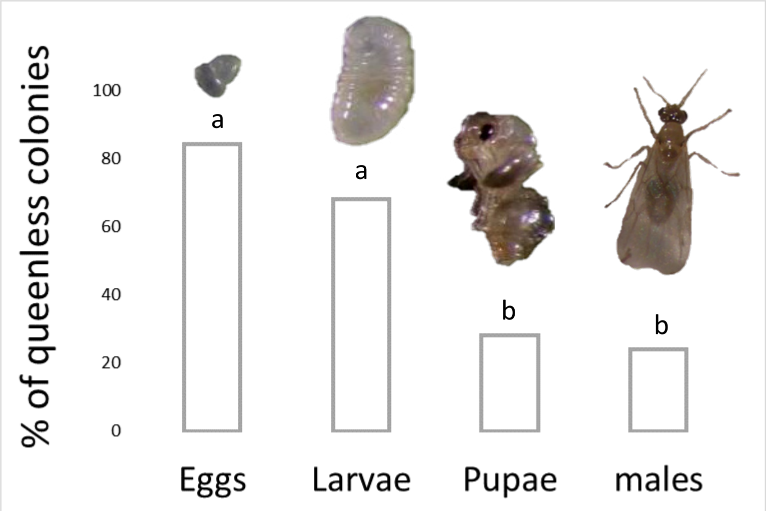

Fig 2. Percentage of *Crematogaster pygmaea* experimental queenless colonies (N = 25) that produced brood (eggs, larvae, or pupae) and adult males in a ten-month observation period (different letters on the bars indicate statistically significant differences at $p < 0.05$ (Fisher's Exact Test, two-sided).

Discussion

Although the potential reproduction of workers in ants and other hymenopteran societies is the subject of important issues in evolutive aspects of social organization (ex: social harmony and conflict over male parentage) (Hammond & Keller, 2004; Ratnieks et al., 2006) basic questions such as the fertility of males produced by workers remain remarkably uninvestigated. Furthermore, the potential reproduction of workers is of special interest in highly polygynous systems since a possible resulting low within-nest relatedness could lead theoretically to strong selection for workers to lay male eggs and, therefore, to important within-colony conflict over reproduction (Keller, 1995). According to Keller (1995), complete worker sterility, as observed in many highly polygynous tramp species (Passera, 1994), could represent an evolutive solution to preserve social harmony.

Our study demonstrates that in the highly polygynous ant *C. pygmaea*, workers retain their ability to lay eggs and to produce, at least in queenless conditions, males, contrary to what is observed in many other highly polygynous ants whose workers are completely sterile (Passera, 1994; Keller, 1995; Bourke, 1988). Furthermore, the worker-produced males of *C. pygmaea* were shown to be reproductively functional, being able to mate with gynes that, once inseminated, can initiate the foundation of new colonies, with an initial production of nanitic workers as observed by Martins Segundo et al. (2017) with gynes inseminated with males produced in queenright colonies.

However, our study also revealed that although most (84%) queenless colonies produced eggs one month after their formation and most eggs developed into larvae, few larvae developed into pupae and adult males. Although downsizing partly explains the reduction in brood size over time, frequent observations of crushed larvae and pupae lead to the conclusion that using brood's destruction and/or consumption, workers strongly police the worker-produced offspring in queenless colonies. In queenright colonies, worker policing of non-royal brood seems even stronger since almost any worker's egg introduced in queenright colonies was rapidly destroyed by workers (eaten by the workers or moved to the external waste deposit of the nest). Such selective elimination of worker-laid eggs is probably based on workers' abilities to discriminate between queen- and worker-laid eggs. Previous studies showed that in some ants (*Camponotus floridanus* (Buckley, 1866), for example), workers could distinguish between queen- and worker-laid eggs using surface hydrocarbons present on the eggs (Endler et al., 2004).

Despite the regular presence of eggs and larvae throughout the experiment with the queenless colonies, adult males were not produced until five months after those colonies were formed. Moreover, the period of production of males in the laboratory queenless colonies corresponded to that when the production of adult males was also observed in the field (see Fig 1). Similar results were observed in the polygynous and polydomous ant *Prolasius advenus* Smith, 1862 (Grangier et al., 2013). In *P. advenus*, queenless colonies produced males only seven months after they were formed and simultaneously in the field. Peak of adult males' production in *C. pygmaea* (December to February) also corresponds to the period when alate gynes are produced in *C. pygmaea* field colonies (peak in January and February) (Quinet et al. 2009). This strongly suggests that the production of adult males, and gynes, in *C. pygmaea* is under the control of seasonal factors and that such seasonal factors should be closely linked to a rainy season that extent mainly from January to May in the northeastern region of Brazil (Caatinga domain).

To conclude, our study demonstrates for the first time that *C. pygmaea* workers can produce reproductive eggs. It also shows that the workers from a highly polygynous ant can produce fertile males, unlike other highly polygynous ants whose workers are completely sterile (Passera, 1994; Bourke, 1988). Similar conclusions were drawn by Lee et al. (2017) with *Anoplolepis gracilipes* (Smith, 1857), an invasive polygynous and polydomous ant whose colonies contain 7 to 12% of physiogastric workers that can produce reproductive eggs in queenless conditions, some of them developing in adult males. However, their assumptions about the ability of those worker-produced males to copulate with gynes and to fertilize them are based on indirect evidence (functional genitalia, intact reproductive organs, and the presence of viable sperm). Our study is also the first systematic observation of worker reproduction in ants of the *Crematogaster* Lund, 1831 genus. However, some indirect evidence of worker reproduction was found in *Crematogaster impressa* Emery, 1899 (Delage-Darchen, 1974), and in *Crematogaster scutellaris* (Olivier, 1792) (Soulié, 1960), whose workers would be able to produce not only males but also females (through thelytoky).

As suggested by Hamidi et al. (2017), many of the gynes produced in *C. pygmaea* colonies probably stay in the natal colony where they mate with males of the colony, as part of a dual dispersal strategy where some gynes engage in long-range dispersal followed by independent colony foundation at the beginning of the rainy season, while others mate in the parental colony and are re-adopted leading to high polygyny. Thus, the consequent high relatedness between queens of colonies could lead to strong genetic proximity between brood and workers. Kinship selection should therefore force the workers to their helper, non-reproductive function even if they keep the ability to reproduce. Further investigations are nevertheless needed to clarify the kin structure of *C. pygmaea* colonie. Chemical analyses on royal and non-royal broods should also help to understand the fundamental mechanisms that regulate reproductive skew in *C. pygmaea.*

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Author's Contribution

RH: Conceptualization, methodology, investigation, formal analysis, writing (original draft/review and editing).
JCB: Conceptualization, methodology, formal analysis.
YQ: Conceptualization, methodology, formal analysis, writing (review and editing).

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