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BEHAVIORAL REGULATION OF GENETIC CASTE DETERMINATION IN A *POGONOMYRMEX* POPULATION WITH DEPENDENT LINEAGES

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Abstract. The fate of a social insect colony is partially determined by its ability to allocate individuals to the caste most appropriate for the requirements for growth, maintenance, and reproduction. In pairs of dependent lineages of *Pogonomyrmex barbatus,* the allocation of individuals to the queen or worker caste is constrained by genotype, a system known as genetic caste determination (GCD). In mature GCD colonies, interlineage female eggs develop into sterile workers, while intralineage eggs become reproductively capable queens. Although the population-level consequences of this system have been intensively studied, the proximate mechanisms for GCD remain unknown. To elucidate these mechanisms, we brought newly mated queens into the laboratory and allowed them to establish colonies, nearly half of which unexpectedly produced virgin queens only seven months after colony founding.

We genotyped eggs, workers, and the virgin queens from these colonies. Our results showed that queens in young colonies produce both interlineage and intralineage eggs, demonstrating that queens of GCD colonies indiscriminately use sperm of at least two lineages to fertilize their eggs. Intralineage eggs were more frequent in colonies producing virgin queens. These findings suggest that intralineage eggs are predetermined to become queens and that workers may cull these eggs when colonies are not producing queens. Virgin queens produced by young GCD colonies were smaller than field-caught virgin queens, and often had developmental problems. Hence, they are probably nonfunctional and represent an intense resource drain for developing colonies, not a contribution to colony fitness.

Key words: *genetic caste determination;* Pogonomyrmex barbatus; *reproduction; social insects.*

Introduction

The discovery of a genetic system of caste determination (GCD) in pairs of dependent lineages of the seed-harvester ants *Pogonomyrmex barbatus* and *P. rugosus* has led researchers to question GCD's implications for colony development and reproduction relative to non-GCD populations (Julian et al. 2002, Volny and Gordon 2002*a*, Helms Cahan et al. 2004). In GCD colonies, queens develop from eggs fertilized by males of the same lineage (intralineage matings), while workers develop from eggs fertilized by males of the opposite lineage (interlineage matings; Volny and Gordon 2002*a*). Queens are therefore consistently homozygous at several diagnostic loci while workers are heterozygous at the same loci (Volny and Gordon 2002*a*). This contrasts greatly with most systems of caste determination in social insects, which are independent of an individual's genotype and depend instead on differences in environmental factors during development (Wheeler 1986, 1991).

Several other known types of genetic constraints on caste in ants involve allelic differences between individuals either within reproductive castes (Fersch et al. 2000) or within sterile worker castes (Fraser et al. 2000, Hughes et al. 2003, Goodisman and Crozier 2003). Allelic differences within the worker caste are typically thought to result from the tradeoff between the need for high genetic variability for increased colony productivity and the need for high inclusive fitness within a colony. In such cases, it is believed that optimal levels of genetic variability are achieved through multiple mating or the presence of multiple queens within a single colony. These are not the same as GCD in the present system. Genetic differences between reproductive and sterile castes have different implications. This form of GCD places severe genetic constraints on colonies because it limits a colony's ability to optimize caste production during different phases of the colony life cycle (Oster and Wilson 1978). Under a similar type of genetic constraint between reproductive and sterile castes, *Solenopsis invicta* queens in certain populations produce diploid males and workers in a 1:1 ratio, which diverts substantial resources from worker production and hinders colony growth (Ross and Fletcher 1985). Similarly, GCD *P. barbatus* queens founding new colonies produce both intralineage and interlineage eggs, but the proportion of intralineage individuals decreases across developmental stages, and only interlineage workers survive (Volny et al. 2006). Therefore, during the colony-founding stage, *P. barbatus* queens

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devote substantial resources towards the production of non-viable offspring, representing a considerable drain on colony resources.

To maximize fitness, *P. barbatus* GCD colonies must be able to efficiently produce interlineage females (workers) during the growth phase of colony development, but switch to additionally producing intralineage virgin queens (gynes) during the subsequent reproductive phase. For this switch to occur, workers or queens must be able to discriminate between eggs of differing genotypes and allow intralineage individuals to mature only during the reproductive phase.

There are three ways that a colony could control the production of intralineage virgin queens. First, the switch to queen production may be governed by differences in the nutritional thresholds of intralineage and interlineage larvae, such that intralineage individuals only survive and develop if the amount of available food is adequate (nutritional hypothesis; Wheeler 1986, 1991). Helms Cahan et al. (2004) show that GCD *P. rugosus* queens that have mated only with males of the intralineage genotype can lay eggs, but virtually no intralineage workers fully develop during colony founding. This suggests that, during colony founding, GCD queens cannot provide intralineage larvae with enough nutritional resources to allow them to develop into adults. A nutrition mechanism would not require direct recognition of larval genotypes; workers could control queen production by generally altering larval feeding levels. However, the system would still represent an overall drain on colony resources with no additional recognition or culling mechanism, because under some conditions interlineage larvae would be overfed, while under others nonfunctional intralineage larvae would be tended but would not fully develop.

Second, the development of interlineage and intralineage individuals could be controlled by the workers that feed and tend them (worker screening hypothesis). Workers could control brood development by culling early in development or by discriminative feeding. Both mechanisms would likely be in place, and have been shown to occur in non-GCD ant species (Aron et al. 2001, Rissing 1987). Either behavior would require that workers recognize brood genotypes. Culling would be an efficient mechanism to ensure interlineage worker production without the costs of directing colony resources to production of nonfunctional intralineage brood during colony growth.

Third, queens may directly regulate the production of intralineage and interlineage eggs during oviposition (queen control hypothesis), laying only interlineage eggs in young colonies and during non-reproductive phases, and then laying intralineage eggs to initiate reproduction, or by altering the hormonal resources provided to eggs of different genotypes. This is unlikely, however; *P. barbatus* queens are known to indiscriminately lay both intralineage and interlineage eggs during colony founding, when virgin queens are not being produced and the cost of devoting resources to intralineage eggs is the highest (Volny et al. 2006).

These mechanisms are not exclusive; more than one could regulate GCD. They do, however, generate different expectations. Under the queen control hypothesis, one should find intralineage brood only during discrete reproductive periods. Under the nutritional hypothesis, one would expect to observe no differences in the percentage of intralineage eggs in queen producing and non-queen producing colonies. However, queen-producing colonies would contain more intralineage larvae, pupae, and adults than non-queen producing colonies. There should also be an overall size difference between reproducing and non-reproducing colonies, reflecting colony differences in resource availability. Under the worker control hypothesis, one would expect to find a difference in the proportion of intralineage eggs in queen-producing and non-queen-producing colonies.

Materials and Methods

Colony establishment and discovery of queens

Newly mated foundress queens ($n = 50$) were collected in July 2004 from mating aggregations found north of Lordsburg, New Mexico, USA. Queens from this population are members of GCD *Pogonomyrmex barbatus* dependent lineages, and this population corresponds to population 10 in Anderson et al. (2006). Queens were individually sealed in tubes with water-soaked cotton until the first workers emerged, when test tubes containing surviving colonies were transferred to $12.5 \times 17.5 \times 5.5$ cm plastic boxes. Colonies were fed an ad libidum diet of Kentucky bluegrass seed and crickets once a week and were maintained at a constant temperature (34°C) and light : dark cycle (12 h : 12 h). Colonies were monitored weekly for worker number and for the presence of daughter queens or males.

In February 2005, seven months after colony establishment, virgin queens were observed in four out of nine surviving colonies (queen-producing colonies; overall survival, 18%). The virgin queens, as well as samples of workers and egg masses, were collected from eight of the nine colonies and frozen at −20°C to determine the colonies' genetic structure. At least half ($n = 8$) of all the eggs present in each colony at the time of sampling were used in genetic analyses. One colony was deemed too small to permit removal of eggs and workers at the time, and was not included in the study. Virgin queen larvae (distinguishable by their larger size), pupae, and adults continued to be collected from colonies through April 2005.

At the end of the study, all remaining live workers in all colonies were counted and weighed to determine whether a size threshold could explain the onset of queen production. Since virgin queen mass may change greatly between eclosion and mating, we used thorax width measurements to compare laboratory-reared queen size to the size of virgin queens collected from mature field colonies ($n = 28$ from a total of eight different colonies).

Genetic analysis of colony structure and confirmation of GCD

We genotyped individuals at multiple life stages (eggs, pupae, and adults) to confirm that colonies exhibited characteristics of GCD, and to assess differences in genetic structure between queen- and non-queen-producing colonies. Eggs, workers, and virgin queen pupae and adults were each genotyped by amplifying two microsatellite loci previously identified as diagnostic of the colony-level GCD phenotype (*Pb8* and *Myrt3*; Volny and Gordon 2002*a*). Intralineage individuals are consistently homozygous at these loci, while interlineage individuals are consistently heterozygous at the same loci. DNA was extracted and amplified from eggs, workers, and queens using protocols that were similar to those outlined by Volny and Gordon (2002*b*). Details of modifications to the original protocols are provided in Appendix A. PCR products were separated, sized, and genotyped on denaturing polyacrylamide gels (4%) run with an ABI 377 automated sequencer.

The appropriateness of the *Myrt3* and *Pb8* loci as diagnostic markers for caste was assessed by comparing worker and queen genotypes to their allozyme signatures for PGI, which is known to be diagnostic of caste in our source population (Anderson et al. 2006). Worker and queen gasters were analyzed for PGI following standard extraction and staining protocols (Richardson 1986). In preliminary surveys of eggs, we found that PGI appears consistently homozygous, reflecting maternal genotypes; therefore egg genotypes could not be verified with PGI.

The colony-level GCD phenotype was also confirmed by using methods outlined by Anderson et al. (2006). To determine whether each colony clustered with a mitotype clade associated with GCD lineages (Helms Cahan and Keller 2003, Anderson et al. 2006), we compared worker sequences for cytochrome oxidase I of two workers from each colony to the sequences that Anderson et al. used to construct the phylogenetic tree for GCD.

The relative frequencies of the genotyped alleles within this population are unknown, so it is not possible to directly predict the level of homozygosity within colonies. Therefore, we used the highest possible predicted level of homozygosity based on Hardy-Weinberg expectations (0.5) to test for homogeneity between individual colonies to determine if data could be pooled (Zar 1999). All tests failed to reject homogeneity (see Appendix B for summary statistics), and therefore data were pooled based on colony type for further analysis. All remaining statistical tests were conducted on data that met the assumptions required for parametric statistical analysis.

Results

Confirmation of GCD colony status and genetic structure

All mitochondrial sequences clustered with two mitotype clades that are associated with GCD (J1 and J2 clades; see Anderson et al. 2006). Queen-producing colonies belonged exclusively to the J1 clade. Two of the colonies that did not produce queens also clustered with the J1 clade; the other two colonies clustered with the J2 clade.

Table 1. Summary of worker and queen genotypes determined with the allozyme marker PGI.

Colony type and number	Workers		Queens	
	X^iX^i	X^iX^j	X^iX^i	X^iX^j
Queen-producing				
3	0	8	2	0
6	0	8	3	0
7	0	8	2	0
10	2	6	6	0
Total	2	30	13	0
Non-queen-producing				
4	0	8		
12	0	8		
16	0	8		
28	0	8		
Total	0	32		

Notes: As expected, all newly produced queens were homozygous (X^iX^i), whereas all but two workers were heterozygous (X^iX^j). The two homozygous workers indicate that homozygous individuals are still capable of developing into workers and that PGI is not completely linked to the genes generating GCD.

Out of the three markers that were used to classify caste, PGI associated most consistently with caste; all daughter queens ($n = 13$) produced by our colonies were homozygous for this marker, while only two out of 64 workers (3.1%) were homozygous (Table 1). In contrast, three of the daughter queens (20%) were heterozygous for *Pb8*, and three (27.3%) were heterozygous for *Myrt3* (Tables 2 and 3). Whereas the number and size of alleles for *Pb8* corresponded with those described by Volny and Gordon (2002*a*), all alleles for *Myrt3* were at least 66 base pairs shorter than previously reported (present allele sizes were 104, 106, 112, and 114 base pairs). However, all three markers conformed to the typical pattern seen in GCD colonies, in which almost all workers are heterozygous while almost all virgin queens are homozygous ($\chi^2 = 36.6$, df = 1, $P < 0.001$ for PGI; $\chi^2 = 22$, df = 1, $P < 0.001$ for *Pb8*; and $\chi^2 = 19.7$, df = 1, $P < 0.001$ for *Myrt3*; Tables 1, 2, and 3; see Appendix C for detailed genetic information).

Queen-producing colonies contained significantly more homozygous eggs than non-queen-producing colonies ($\chi^2 = 5.48$, df = 1, $P = 0.0192$, $n = 58$ for *Pb8*; $\chi^2 = 5.53$, df = 1, $P = 0.019$, $n = 45$ for *Myrt3*; Tables 2 and 3). In contrast, the number of homozygous and heterozygous workers did not differ in queen- and non-queen-producing colonies for PGI, *Pb8*, or *Myrt3*, because all colonies produced primarily heterozygous workers ($\chi^2 = 2.06$, df = 1, $P = 0.151$, $n = 64$; $\chi^2 = 1.87$, df = 1, $P = 0.171$, $n = 57$; and $\chi^2 = 2.43$, df = 1, $P = 0.144$, $n = 59$, respectively).

Other studies have found differences in the success of DNA extractions and amplifications of eggs depending on

TABLE 2. Summary of egg, worker, and queen genotypes determined with the microsatellite marker *Pb8*.

Colony type and number	Eggs		Workers		Queens	
	X^iX^i	X^iX^j	X^iX^i	X^iX^j	X^iX^i	X^iX^j
Queen-producing						
3	1	5	0	8	1	1
6	0	8	0	8	1	1
7	1	5	0	8	2	0
10	5	4	2	6	5	1
Total	7	21	2	30	9	3
Non-queen-producing						
4	1	5	0	6		
12	0	8	0	6		
16	0	4	0	6		
28	0	11	0	7		
Total	1	28	0	25		

Note: As expected, the majority of all newly produced queens were homozygous (X^iX^i), whereas most workers were heterozygous (X^iX^j), suggesting that *Pb8* is at least partially linked to genes causing GCD.

the microsatellite locus (see Volny et al. 2006). In the present study, there was no difference in amplification success at the locus *Pb8* between queen-producing and non-queen-producing colonies ($\chi^2 = 2.24$, df = 1, $P > 0.05$, overall success of 73.4%). However, when the same eggs were genotyped with *Myrt3*, fewer eggs from non-queen-producing eggs were successfully amplified ($\chi^2 = 10.2$, df = 1, $P < 0.05$, 40.9% success in non-queen-producing colonies vs. 77.1% success in queen-producing colonies).

Worker and colony size differences

Mean worker masses differed between queen-producing colonies and non-queen-producing colonies (nested, unbalanced analysis of variance; $F_{1,6} = 7.649$, $P < 0.0001$; Fig. 1). Specifically, workers from queen-producing colonies were typically larger than workers from non-queen-producing colonies (unequal *n* Tukey's hsd used for post hoc comparisons; see Appendix D for exact *P* values for pairwise comparisons). Workers in the laboratory-reared colonies were still substantially smaller than workers collected from mature field colonies (mean masses [±SE] are 6.7 ± 0.7, 7.7 ± 0.4, and 16.4 ± 0.9 mg for non-queen-producing, queen-producing, and field colonies, respectively). Interestingly, the mean number of workers present in each colony was similar for the laboratory queen-producing and non-queen colonies (41.8 ± 7.8 individuals; $t_6 = 0.961$, $P = 0.374$). All lab-reared queens that successfully eclosed were substantially smaller than typical field-caught virgin queens, but had typical queen morphology, including, for example, wings (mean thorax widths were 1.807 ± 0.054 and 2.215 ± 0.019 mm for lab-reared and field-caught queens, respectively; $t_{26} = 9.016$, $P < 0.0001$). However, we also observed workers culling four queen pupae.

DISCUSSION

Until recently, it was not possible to differentiate between the alternate mechanisms for colony control of GCD, because daughter queens had not been produced in any laboratory-reared colonies. GCD colonies of *P. barbatus* were generally assumed to follow a life cycle typical of seed-harvester ants, wherein the production of daughter queens and males does not begin until colonies have grown to a size of 10 000–12 000 workers, a process that requires approximately five years in the field (Gordon 1995). However, almost 50% of our GCD laboratory colonies of *P. barbatus* produced daughter queens within seven months after colony founding. At the time, these colonies had fewer than 100 workers.

Our data suggest that in young, GCD, *P. barbatus* colonies, the production of workers vs. daughter queens is regulated by worker and/or queen culling and nutrition. For the nuclear markers tested here, queen-producing colonies contained more homozygous (intralineage) eggs than non-queen-producing colonies (Tables 1–3). This trend suggests that intralineage brood may be culled by workers very early during the egg stage of development. Alternatively, queens in non-queen-producing colonies may not have mated with males with the complementary genotype needed to produce intralineage brood. Although we know that *P. barbatus* queens mate multiply (Volny and Gordon 2002*a*), the exact likelihood of the second possibility cannot be determined for this population because the allele frequencies of the markers used and the average effective mate number are unknown.

The larger worker masses in queen-producing colonies suggest that all individuals in these colonies received more nutritional resources during development, thus supporting the nutritional threshold hypothesis for queen development. However, nutritional separation would first become apparent at the larval stage when feeding occurs, and would not explain the different genotypic ratios observed at the egg stage. Our data also suggest that intralineage eggs in GCD lineages of *P. barbatus* are limited with respect to their phenotypic plasticity and

TABLE 3. Summary of egg, worker, and queen genotypes determined with the microsatellite marker *Myrt3*.

Colony type and number	Eggs		Workers		Queens	
	X^iX^i	X^iX^j	X^iX^i	X^iX^j	X^iX^i	X^iX^j
Queen-producing						
3	2	4	0	7	0	1
6	0	8	0	8	2	0
7	0	4	0	8	0	2
10	5	4	2	6	6	0
Total	7	20	2	29	8	3
Non-queen-producing						
4	0	5	0	8		
12	0	2	0	6		
16	0	1	0	6		
28	0	10	0	8		
Total	0	18	0	28		

Note: As expected, the majority of all newly produced queens were homozygous (X*i*X*i*), whereas most workers were heterozygous (X^iX^j), suggesting that *Myrt3* is at least partially linked to genes causing GCD.

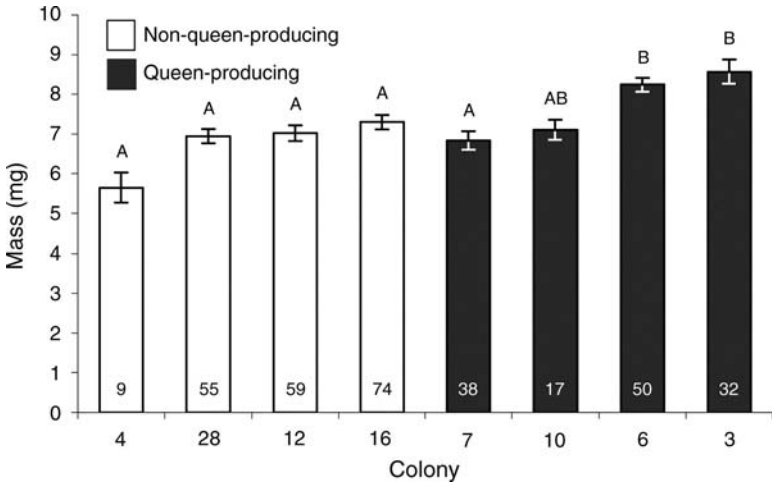

FIG. 1. Worker masses (mean ± SE) from queen-producing and non-queen-producing colonies of *Pogonomyrmex barbatus*. A nested unbalanced ANOVA revealed that workers from queen-producing colonies were significantly heavier than workers from non-queen-producing colonies ($F_{6,326} = 7.649$, $P < 0.0001$). Differences in mass among individual colonies are indicated by different letters above the columns (Tukey's unequal *n* hsd; $P < 0.05$). Sample sizes are indicated inside columns.

consistently develop into queens rather than workers, so a nutritional threshold alone would not suffice to explain the observed pattern. Thus, we suggest that both nutrition and culling may contribute to the differential production of intralineage queens and interlineage workers.

The virgin queens produced by these colonies are probably not functional or competitive as foundresses of new daughter colonies because they are significantly smaller than field-caught virgin queens. They also make only limited contributions to their parent colony through some brood care (K. Anderson, *personal observation*). Thus, we suggest that the shift to reproduction in the present study may be due to an imperfect worker screening mechanism in young colonies, in which poor detection and/or culling of queen-fated eggs allowed some intralineage individuals to mature into nonfunctional queens despite a cost to the colony. We also observed workers killing and eating several pupae that were clearly developing into queens, supporting this hypothesis and suggesting that culling can happen at any time during development, from the egg through the pupal stage, depending on workers' ability to detect differences between intralineage and interlineage individuals. Production of nonfunctional queens may be common in young colonies with small worker pools, as indicated by the fact that half of our laboratory colonies produced intralineage virgin queens, but we still do not know at what point fully functional queens are produced. The early production of queens could indicate that GCD colonies may become capable of reproducing earlier relative to non-GCD colonies, thus giving GCD colonies a potential advantage over non-GCD colonies. However, the putative benefits of early reproductive success must still be weighed against the longer-term ergonomic costs of allocating resources to reproduction.

Given the costs imposed on colonies by imperfect screening, one would expect that GCD colonies would develop compensatory mechanisms to minimize the negative consequences of GCD. The development of intralineage females in colonies outside of the mating season represents a genetic load analogous to the production of homozygous diploid males (which are nonfunctional) in honey bee colonies (Tarpy and Page 2002). Honey bee workers recognize and cull diploid male brood early in development (Drescher and Rothenbuhler 1964). Similarly, Aron et al. (2001) found that *Linepithema* workers that are forced into reproduction will selectively cull reproductives but not workers when available protein is limited. Some equivalent mechanism may occur in *P. barbatus* colonies in which workers recognize and cull intralineage offspring under colony conditions that favor growth over reproduction. GCD colonies that could detect differences between intralineage and interlineage individuals early in development could more effectively allocate resources towards the development of the individuals most beneficial to the colony at a given time.

We did observe two workers that were homozygous for PGI, the nuclear marker most closely associated with GCD (Table 1), which suggests that either the markers used to discriminate between homozygotes and heterozygotes are not completely linked to genes causing GCD (supported by the findings of Anderson et al. 2006), or that GCD colonies of the B clade still retain a low level of phenotypic plasticity in caste fate. Overall differences in the number of homozygotes and heterozygotes detected with *Pb8*, *Myrt3*, and PGI suggest that these markers are not equally linked to genetic regions determining caste. The most consistent indicators of GCD on the colony level are the mtDNA markers for dependent lineages; however, these do not directly demonstrate heterozygosity and thus cannot be used to determine the caste of individuals (see Anderson et al. 2006).

In conclusion, our current findings suggest several possible mechanisms for the early onset of reproduction in GCD *P. barbatus* colonies. Although early reproduction does not currently appear to be adaptive in this population, future studies including factors such as nutrition and colony clade membership should more clearly elucidate the proximate causes of GCD and factors triggering the switch to reproduction. Additionally, the geographically widespread nature of GCD in lineages of both *P. barbatus* and *P. rugosus* suggests that comparative studies of different populations may reveal alternative solutions to the genetic constraints imposed by GCD.

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APPENDIX A

DNA extraction and amplification protocol for microsatellite markers *Pb8* and *Myrt3* (*Ecological Archives* E087-134-A1).

APPENDIX B

Summary statistics for tests for heterogeneity to assess validity of pooling data across colonies (*Ecological Archives* E087-134-A2).

APPENDIX C

Mitochondrial clade assignments and genotypes for workers, eggs, and queens at microsatellite loci *Pb8* and *Myrt3* and allozyme marker PGI (*Ecological Archives* E087-134-A3).

APPENDIX D

P values for post hoc pairwise comparisons of worker masses (Tukey's unequal *N* hsd) (*Ecological Archives* E087-134-A4).