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Phenotypic plasticity in an ant with strong caste–genotype association

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Caste determination in social Hymenoptera (whether a female egg develops into a reproductive queen or a sterile worker) is a remarkable example of phenotypic plasticity where females with highly similar genomes exhibit striking differences in morphology and behaviour. This phenotypic dichotomy is typically influenced by environmental factors. However, recent studies have revealed a strong caste–genotype association in hybridogenetic ants: workers are all interlineage hybrids while queens are all purebred, suggesting that female caste fate is genetically determined. Using the hybridogenetic ant *Cataglyphis mauritanica*, we show that under laboratory conditions, purebred offspring develop into reproductive queens but occasionally give rise to workers. Moreover, while hybrids typically become workers, juvenile hormone treatment can switch their developmental pathway to the reproductive caste. These results indicate that phenotypic plasticity has been retained in an ant with a strong caste–genotype association, despite its lack of expression in natural conditions.

1. Introduction

Phenotypic plasticity is the ability of a genotype to produce different phenotypes in response to distinct environmental cues [1]. Social Hymenoptera provide spectacular examples of phenotypic plasticity in animals: females with similar genomes can develop into reproductive queens or sterile workers depending on environmental conditions. Recently, however, studies showed that genetic factors may also be involved in the female caste fate [2]. Evidence for genetic caste determination comes from studies on hybridogenetic ants, which are characterized by a strong caste–genotype association: workers are hybrids of two genetic lineages, whereas queens are purebreds [3]. This challenges the notion that all social insect castes are examples of phenotypic plasticity.

Three species of *Cataglyphis* ants have evolved a hybridogenetic reproductive system: *C. hispanica*, *C. mauritanica* and *C. velox* [4,5]. In each, two interdependent genetic lineages coexist. Sterile workers are all hybrids resulting from sexual reproduction between representatives of the two lineages, whereas reproductive queens are purebreds produced asexually by parthenogenesis. Not a single caste–genotype mismatch has been observed thus far [4–7]. This unerring association may be underlain by a genetic caste determination system, in which hybrid eggs and purebred eggs are fated to develop into workers and queens, respectively [2].

Here, we investigated whether phenotypic plasticity of the female castes was retained in the hybridogenetic species *C. mauritanica*. We took advantage of the fact that approximately 20% of queens mate with males from their own lineage to compare the development of sexually produced purebred eggs (a 'proxy' for eggs produced by parthenogenesis [6]) with that of hybrid eggs, under controlled laboratory conditions. We monitored the caste fate of female eggs under two sets of experimental conditions. First, we examined whether purebred eggs

could develop into workers outside the period of reproduction. Second, we tested whether an artificial increase in a juvenile hormone (JH) analogue could cause hybrid eggs to develop into new queens. JH is a key regulator of female caste determination in social insects [8]; an increase in JH stimulates the production of vitellogenin and promotes reproduction. This could be one mechanism involved in the strong caste–genotype association found in hybridogenetic *Cataglyphis* ants, if purebred genotypes have higher amounts of and/or are more sensitive to JH.

2. Material and methods

Colonies of *C. mauritanica* were excavated in Morocco and kept under standard laboratory conditions. As in other parthenogenetic species of *Cataglyphis* [9], queens and males develop from parthenogenetically produced eggs laid during a short period of time in early spring, whereas workers develop from sexually produced eggs laid later in the season. All our experiments were performed outside the period of sexual reproduction, when colonies raise *only* workers. *Cataglyphis mauritanica* colonies typically contain multiple queens (mean queen number per colony ± s.d.: 12.4 ± 11.8) [7]. We used queens that had mated with males from the same lineage (hereafter, intralineage queens) and queens that had mated with males from the alternative lineage (hereafter, interlineage queens).

To distinguish between interlineage and intralineage queens, individuals were isolated and their eggs were collected. The eggs' DNA was extracted and amplified at nine microsatellite loci that are diagnostic for the two lineages [6,7]. Interlineage queens lay hybrid eggs (i.e. with diagnostic alleles from both lineages), while intralineage queens lay purebred eggs (i.e. with diagnostic alleles from a single lineage). Queens laying eggs that belonged to more than one patriline were discarded.

To determine whether purebred eggs could develop into workers, ten field colonies were used to set up 14 experimental colonies, each headed by a single intralineage queen, and 16 control colonies, each headed by an interlineage queen. These colonies contained 150 workers but no brood. Larva number was assessed once a week over an eight-week period. Offspring caste and genotype were established post emergence.

To determine whether hormonal effects could cause worker-destined hybrid eggs to develop into queens, we exposed brood to increased level of methoprene (a JH analogue). Sixteen additional colonies were used to set up 13 treatment colonies and seven control colonies. Each colony contained a single interlineage queen, 500 workers and no brood. After two weeks, all larvae were removed, leaving only eggs, which were then treated topically with 2.5 $\mu g\ \mu l^{-1}$ methoprene dissolved in acetone in the treatment group or with acetone only in the control group [10]. Eggs were removed from each colony and 25 µl of methoprene solution was applied directly with a syringe onto batches of 20 ± 2 eggs. The acetone was allowed to evaporate before re-introducing the eggs into the colonies. Over the next five weeks, larva number as well as the caste of emerging adults were assessed weekly.

At the end of the study, all queens and their spermathecal contents were genotyped to confirm patriline inferences.

For statistical methods, see the electronic supplementary material.

3. Results

Intralineage and interlineage queens laid roughly the same amount of eggs. These eggs developed into larvae. However, purebred and hybrid larvae differed in their morphology: purebred larvae had a distinctive spheroidal shape, while hybrid larvae were more elongated (figure 1).

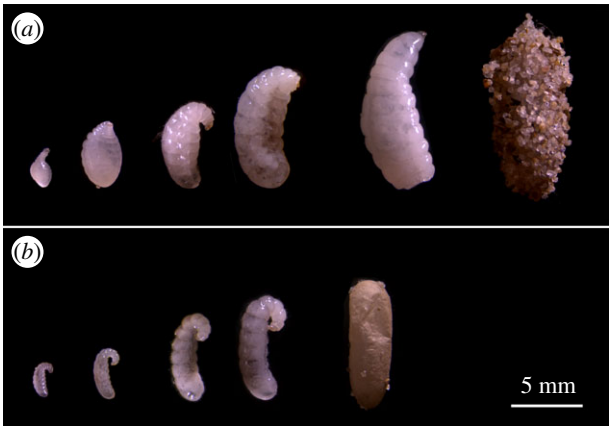

Figure 1. Morphology of *Cataglyphis mauritanica* larvae at different stages reared under laboratory conditions. In the first larval stages, purebred larvae (*a*) have a more spheroidal shape than hybrid larvae (*b*), which are more elongated and slender. Purebred larvae typically develop into queens and hybrid larvae into workers. (Online version in colour.)

Colonies headed by intralineage queens reared significantly fewer larvae (mean number of larvae produced per colony ± s.d. = 10.7 ± 6.43) than those headed by interlineage queens (52.9 ± 25) (two-way ANOVA, lineage effect: $F_{1,20} = 86.77$, $p < 0.001$; colony effect: $F_{11,20} = 1.75$, $p = 0.13$; electronic supplementary material, figure S1 and table S1). In several instances, purebred larvae suddenly disappeared, suggesting that their development had been aborted or that they had been culled by workers. The colonies headed by interlineage queens all produced hybrid workers over an eight-week period (mean number of workers produced per colony ± s.d. = 22.2 ± 18.5). Across all the colonies headed by intralineage queens, one purebred larva developed into a worker during the same period (figure 2*a*). The opposite trend was observed in queen production: twelve new queens were produced across four of the 14 colonies (28%) headed by intralineage queens, but no queens emerged in the 16 colonies headed by interlineage queens (Fisher's exact test: $p = 0.036$; figure 2*b*). All the new queens had purebred genotypes, which was consistent with their sexual origin (electronic supplementary material, table S2). Therefore, purebred larvae are targeted to the queen caste even outside the period of sexual production.

When hybrid eggs were exposed to increased levels of a JH analogue, their developmental patterns shifted towards those observed for purebred eggs. All 13 methoprene-treated colonies produced spheroidal larvae, whereas none of the seven acetone-treated colonies produced such larvae (Fisher's exact test: $p < 0.001$). However, most spheroidal larvae did not reach adulthood; only 11 larvae from three colonies developed into queens (figure 2*b*). These queens had hybrid genotypes, consistent with their production by interlineage sex. In contrast, only workers were produced in control colonies. The JH effect on the phenotype of the larvae and their tendency to develop into new queens (Fisher's exact test: $p = 0.25$, non-significant probably due to limited sample size) strongly supports that JH influences caste fate. Consequently, JH may cause hybrid eggs to develop into queens, indicating that both developmental pathways, reproductive and worker, are conserved in hybrids.

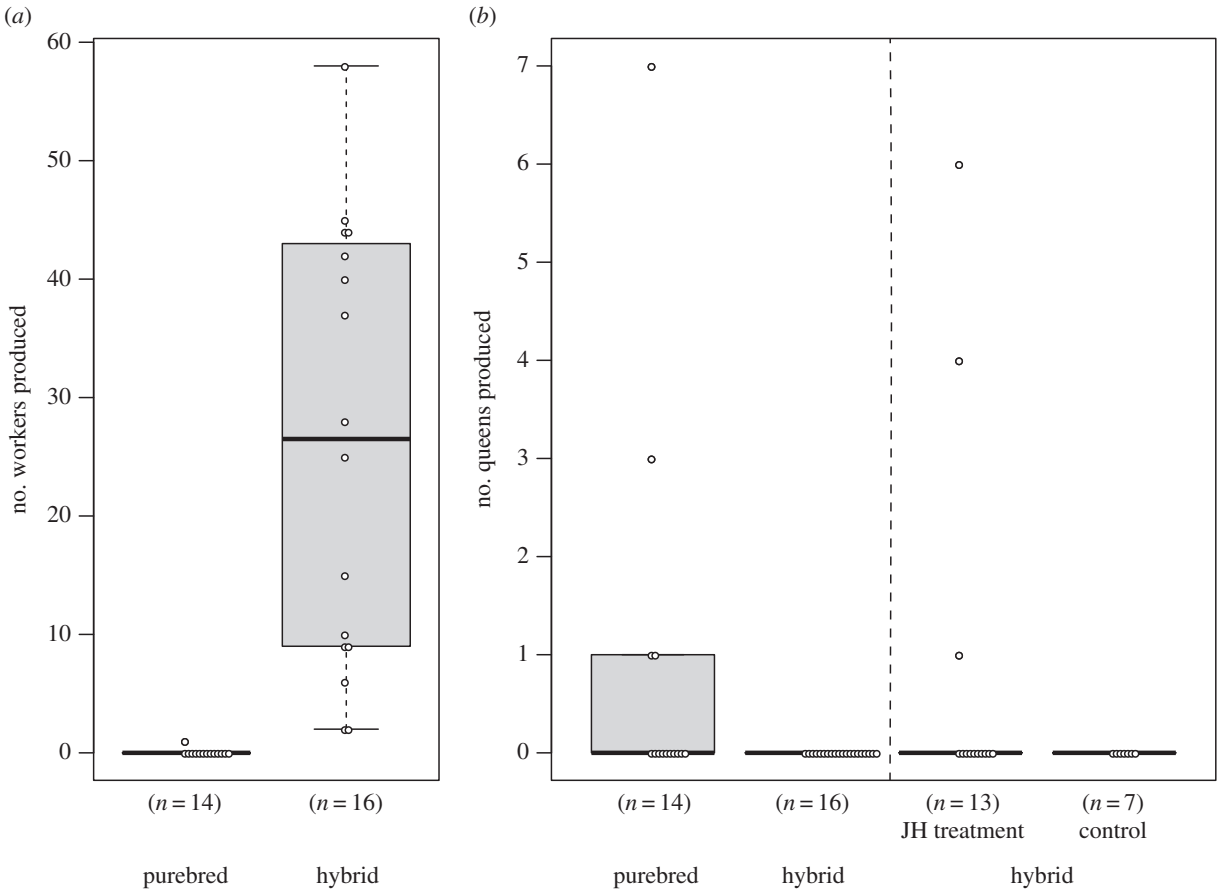

Figure 2. Boxplots of the number of (*a*) workers and (*b*) queens emerging from sexually produced purebred eggs and hybrid eggs in *C. mauritanica*. On the right in (*b*) are the numbers of queens produced from treatment-colony hybrid eggs (treated with JH analogue methoprene) and control-colony hybrid eggs (treated with acetone). Replicates are depicted as white dots.

4. Discussion

Strong caste–genotype associations are commonly interpreted as evidence for genetic caste determination in social Hymenoptera [3]. This study shows that in the hybridogenetic ant *C. mauritanica*, purebred eggs adopt a queen developmental pathway even outside the period of reproduction and hybrid eggs develop into workers, supporting a hardwired caste determination mechanism. However, our results provide the first empirical evidence that elements specific to both castes' developmental pathways are retained for purebred and hybrid larvae. One purebred larva indeed developed into a worker. Furthermore, treatment with a JH analogue dramatically shifted the fate of hybrid eggs, causing them to develop into spheroidal larvae and, ultimately, queens. This indicates that phenotypic plasticity is retained in *C. mauritanica*, despite strong caste–genotype association.

Remarkably, none of the 460 queens genotyped thus far in nature has displayed apparent hybrid ancestry [5,7]. Similarly, none of the 1928 workers had purebred genotypes. This loss of phenotypic plasticity in the wild might stem from individual constraints on offspring production or from responses to key factors initiating caste determination. For example, hybrid brood might have a limited ability to produce JH or a response threshold to JH too high for triggering the development of hybrid larvae into queens in naturally occurring hormonal conditions [2,3]. Likewise, whether JH antagonists favour development of purebred brood into workers awaits further studies. Another explanation for the absence of hybrid queens could be that both parthenogenetic and fertilized eggs are laid and raised simultaneously, and compete with each other to develop into queens. Assuming that the genomes of purebred larvae contain co-adapted genes that drive queen development, these adaptations may be broken up in hybrids, resulting in individuals that are less well equipped to developmentally compete and that thus develop into workers when purebreds are present [11]. Because no purebred larvae were present in the JH-treatment colonies, it would appear that no such competition constrains the development of hybrid larvae.

In *C. mauritanica*, reproductive queens readily develop from parthenogenetic eggs under natural conditions [5]. Our study shows that queens can also develop from purebred fertilized eggs in the laboratory. Whether this occurs in nature remains uncertain. Here, only a few purebred larvae reached adulthood; most may have been culled by workers. It is likely queen larvae were eliminated because their development was seasonally out of sync, a phenomenon documented in other ant species [3,12]. Our discovery that some purebred eggs developed into queens outside the period of sexual production contrasts with previous results obtained under similar conditions in a sister species, *C. hispanica*, where purebred eggs failed to hatch into larvae [6]. Taken together, these findings suggest that the association between genotype and caste destiny is underlain by different mechanisms in the two species. Similar results were reported in hybridogenetic *Pogonomyrmex* seed harvester ants: individuals with purebred genotypes were genetically constrained to develop into queens and either their development was aborted at the egg

stage when they were seasonally out of sync (as in *C. hispanica*) or they were culled by workers at the larval stage because of arrested development/seasonal asynchrony (as in *C. mauritanica*) [3].

Caste determination and sex determination share many similarities, with variation in environmental (e.g. temperature and nutrition) or genetic (e.g. sex chromosomes and genetic caste determination) factors leading to the development of alternative phenotypic states. In many species, the line between environmental and genotypic sex-determination is blurred, with certain environments altering the (otherwise genetically determined) sex of developing offspring [13]. Similarly, in species with a strong caste–genotype association, two regulatory developmental pathways, each associated with a genotype, could govern female caste fate. Under this scenario, the strength of the caste–genotype association would depend on the responsiveness of each pathway to environmental cues.

Our study shows that, in *C. mauritanica*, phenotypic plasticity has been retained despite its apparent lack of expression in natural conditions. Hybrid genotypes readily develop into sterile workers, but can grow into reproductive females under queen-favouring artificial conditions. The failure of purebred eggs to develop into workers forces queens to exploit sperm from an alternative lineage to successfully produce workers.

Ethics. This study was carried out in compliance with relevant guidelines related to research on insects.

Data accessibility. The numbers of workers and queens, and the microsatellite data are available in the electronic supplementary material, tables S1 and S2.

Authors' contributions. A.K. and H.D. conceived the study and performed the experiments. A.K., H.D. and S.A. analysed the data and wrote the article. All authors agree to be held accountable for the content of this paper and approved the final version of the manuscript.

Competing interests. The authors have no competing interests.

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