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Report

Enforcement of Reproductive Synchrony via Policing in a Clonal Ant

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Summary

In insect societies, worker policing controls genetic conflicts between individuals and increases colony efficiency [1–6]. However, disentangling relatedness from colony-level effects is usually impossible [7–11]. We studied policing in the parthenogenetic ant *Cerapachys biroi*, where genetic conflicts are absent due to clonality [12, 13] and reproduction is synchronized through stereotyped colony cycles [14]. We show that larval cues regulate the cycles by suppressing ovarian activity and that individuals that fail to respond to these cues are policed and executed by their nestmates. These individuals are genetically identical to other colony members, confirming the absence of intracolonial genetic conflicts. At the same time, they bear distinct cuticular hydrocarbon profiles, which could serve as proximate recognition cues for policing. Policing in *C. biroi* keeps uncontrolled reproduction at bay and thereby maintains the colony-level phenotype. This study shows that policing can enforce adaptive colony-level phenotypes in societies with minimal or no potential genetic conflicts. In analogy to immunosurveillance on cancer cells in genetically homogeneous multicellular organisms [15–17], colony efficiency is improved via the control of individuals that do not respond properly to regulatory signals and compromise the functioning of the higher-level unit.

Results and Discussion

Worker policing in insect societies is often interpreted as a way to repress or reduce reproductive conflicts that arise between colony members because of intracolonial relatedness asymmetries [1–4]. Alternatively, it can serve as a regulatory mechanism to increase group-level efficiency [5, 6]. Although these two hypotheses are not mutually exclusive, their relative contribution has been much debated over the past decade, mostly because the two factors are hard to separate in any given species [7–11]. Parthenogenetic species with clonal societies provide important new insights, because conflicting individual interests can be excluded as an underlying selective factor.

In the parthenogenetic ant *Cerapachys biroi* [12], nestmates are genetically identical or very nearly so (average within-colony relatedness $R = 0.99$ [13]). Colonies consist only of workers, all of which reproduce during at least a period of their life. Dominance hierarchies, which can be the basis of aggressive behaviors in ants with totipotent workers [1, 9], are absent. Despite this, intracolonial aggressive behavior is regularly observed in laboratory colonies, where single ants are dragged out of the nest, immobilized, spread-eagled by multiple aggressors, and often killed through biting and stinging over the course of several hours or even days (Figure 1; see also Movie S1 available online). We conducted a series of experiments aimed at understanding the causes of this behavior.

Eleven colonies from three different clonal lineages (MLL1, MLL4, and MLL6 [18]; colony sizes were circa 500–5,000 individuals) were initially monitored for 13 months (see Supplemental Information section). The aggressed individuals and a subset of aggressing individuals were dissected to count the number of ovarioles. Of 201 aggressed individuals, 92.5% had four to six ovarioles (high-reproductive individuals, or HRIs, which constitute circa 5% of the individuals in normal colonies [19]), whereas 93.4% of 198 aggressing individuals had two ovarioles (low-reproductive individuals, or LRIs [19]). HRIs and LRIs were not randomly distributed among aggressive and aggressed individuals (general linear mixed model [GLMM], colony as random factor, chi-square = 139.42, df = 1, $p < 0.0001$). Colonies of *C. biroi* undergo reproductive cycles similar to phasic army ants, such as *Eciton burchellii* and *Neivamyrmex nigrescens* [14, 20]. During the course of each cycle, a cohort of larvae develops synchronously during a 16 day foraging phase that starts with larval hatching and ends with pupation. The adults then lay a new batch of eggs at the beginning of an 18 day reproductive phase that ends with larval hatching and the emergence of a new cohort of adults. In the foraging phase, workers do not reproduce, and they conduct raids on the brood of other ant species to feed the developing larvae. In the reproductive phase, the ants remain inside the nest chamber and lay eggs [14]. The stage of the colony cycle was noted for 167 of the observed aggressions to determine their chronological distribution. Of the aggressions, 85.45% occurred during the foraging phase, 4.84% during the reproductive phase, and 9.69% at the transition between the two phases (Figure 2). Aggressions were then recorded twice a week during one cycle for ten colonies. Twenty-seven instances of aggression were observed in six of the colonies (4.5 ± 2.9 SD per colony), 25 (92.6%) of which occurred during the foraging phase. The other two aggressions, although observed at the transition between foraging and reproductive phase, could have started during the foraging phase during the interval between two observations. Overall, the vast majority of aggressions were directed toward HRIs and occurred during the foraging phase.

Like other ants, *C. biroi* undergoes a process of melanization after emergence; i.e., workers darken as they age. To determine the age of aggressed individuals (n = 60, 10 from each of six colonies), we compared their cuticular melanization to individuals of known age (circa 2 weeks old, 1 month old, and 2 months old; n = 20 for each age group). Aggressed individuals were darker than 2-week-old individuals (linear mixed model [LMM], colony as random factor, F = 17, 58740; df = 4;

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Figure 1. Worker Policing in *Cerapachys biroi*

The focal individual is spread-eagled by several workers, sometimes over the course of several days.

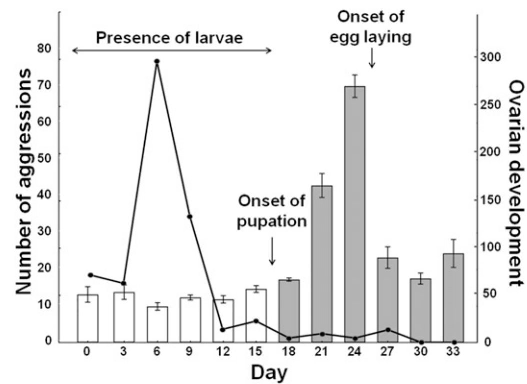

Figure 2. Course of Ovarian Development in HRIs and Worker Policing during the Colony Cycle

Ovarian development is measured as the square root of the picture's area of the biggest oocyte (mean ± SEM). For each column, n = 30, except for day 21 (n = 35) and day 30 (n = 40). Gray and white histogram columns represent the reproductive and foraging phase, respectively. The curve describes the number of observed aggressions in the corresponding parts of the colony cycle. Ovaries resume developing at the end of the reproductive phase, possibly because of the absence of larvae, and regress completely once the larvae have hatched.

$p < 0.0001$; least significant difference [LSD] post hoc test, $p < 0.001$) (Figure S1A), lighter than 2-month-old individuals (LSD post hoc test, $p < 0.01$), and not different from 1-month-old individuals (LSD post hoc test, $p = 0.157$). Aggressed HRIs therefore received aggression during the foraging phase following their first reproductive phase, when their ovaries were activated for the first time.

Because aggression was almost always directed toward HRIs during the foraging phase, we hypothesized that this behavior might have been linked to reproductive regulation in relation to the alternation of phases. We therefore determined the normal course of ovarian activity in HRIs throughout the colony cycle, in order to compare it to ovarian development in aggressed individuals. In normal HRIs, ovaries were activated only during the reproductive phase [LMM, colony as random factor, $F(11, 358) = 64, 574$; $p < 0.0001$] (Figure 2; details are given in the Supplemental Information section). Significant differences were found between the ovarian development of aggressed HRIs, aggressing LRIs, nonaggressed HRIs collected during the reproductive phase, and nonaggressed HRIs collected during the foraging phase [LMM, colony as random factor, $F(3, 659) = 71, 289$; $p < 0.0001$] (Figure 3 and Table S1). The ovarian development of aggressed HRIs was not different from that of nonaggressed HRIs collected during the reproductive phase (LSD post hoc test, $p = 0.4761$), but it was higher than that of nonaggressed HRIs collected during the foraging phase (LSD post hoc test, $p < 0.0001$).

Based on these results, we suspected that larvae inhibit ovarian development and thereby give rise to the colony cycles. We therefore monitored ovarian activity in experimental colonies with and without larvae. Individuals activated their ovaries in the absence of larvae, whereas larvae suppressed ovary development [LMM, colony as random factor, $F(31, 1144) = 30, 863$; $p < 0.0001$] (Figure 4; details in Supplemental Information section). This implies that aggressed individuals with active ovaries during the foraging phase did not respond to the larval inhibition of reproduction.

We then tested the hypothesis that aggressed HRIs act out of selfish genetic interest, i.e., that they constitute genetically distinct parasitic lineages. Across eight colonies (Table S2), aggressed HRIs, aggressing LRIs, and nonaggressed HRIs were genetically identical over six to eight polymorphic microsatellite loci. Two additional MLL1 colonies contained two multilocus genotypes (MLGs) differing by only one allele, and there was no skew in the distribution of the two MLGs among the three groups in either colony (Fisher's exact tests $p = 1.0$ and $p = 0.81$). According to these results, aggressions were not related to genetic conflicts of interest or directed toward unrelated parasitic lineages.

Given that cuticular hydrocarbons (CHCs) signal reproductive and dominance status in ants [21], we hypothesized that the CHC profile was the proximate cue eliciting aggression; i.e., we expected aggressed individuals to exhibit a reproductive phase-like profile during the foraging phase. The profiles of aggressed HRIs, aggressing LRIs, nonaggressed HRIs collected during the reproductive phase, and nonaggressed HRIs collected during the foraging phase showed significant differences (discriminant analysis [DA] across 16 CHC peaks; Wilks' lambda test: 0.16829; F approximately $(36, 2051) = 47, 170$; $p < 0.0001$) (Figure S1B; compounds are listed in Table S3). Aggressed HRIs were different from the other three groups ($p < 0.0001$). No difference was found between reproductive phase nonaggressed HRIs and foraging phase nonaggressed HRIs [$F(1, 35225)$; df = 12, 694; $p = 0.1842$]. These results suggest that the unique CHC signature of aggressed HRIs, rather than specific fertility-related compounds, might serve as the proximate cue that elicits aggression. However, this requires additional confirmation. Moreover, compared to the other groups, aggressed HRIs had significantly lower amounts of all compounds (Table S3). Because CHC quantities usually increase with age in social Hymenoptera [22, 23], this is in accordance with our result based on melanization level that aggressed individuals were young.

The results of our study show that larvae of *C. biroi* restrict the colony's reproductive investment to coordinated cohorts of brood by regulating reproduction directly via oogenesis inhibition. By limiting egg-laying to a short time window after pupation, larvae act as pacemakers of the alternating phases. Individuals that are not reproductively inhibited by the presence of larvae are costly because they threaten to disrupt

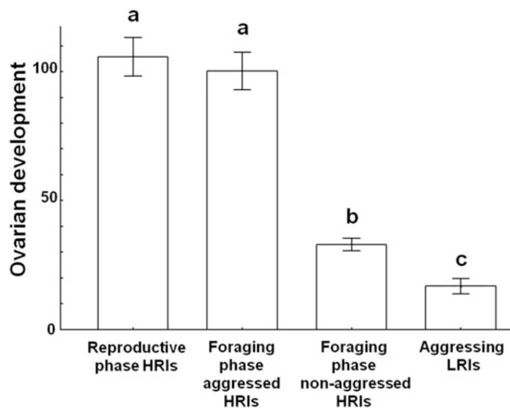

Figure 3. Ovarian Development in Different Groups of Individuals, Measured as in Figure 2

Letters indicate statistically significant differences (linear mixed model [LMM] with colony as random factor; least significant difference [LSD] post hoc test). The reproductive status of aggressed individuals was the same as that of reproductively active egg-layers during the reproductive phase. Aggressing LRIs are older foragers and show the lowest level of ovarian development. The reported statistics include only the six colonies for which all four groups were available (four colonies from MLL1 and two from MLL4) (Table S4). However, results do not change qualitatively when including all aggressed and aggressing individuals (Table S1). Additional information on the four groups is given in Figure S1.

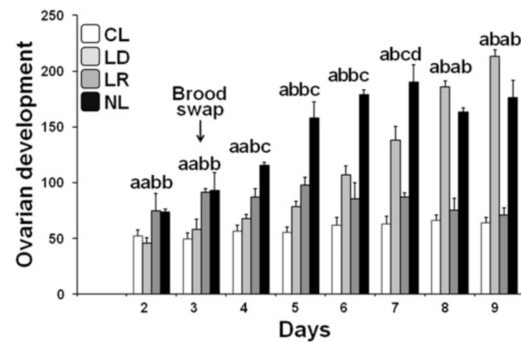

Figure 4. Larvae Inhibit Ovarian Development, Measured as in Figure 2

CL, control group with larvae; LD, larvae donor group; LR, larvae receiver group; NL, control group without larvae. Letters indicate statistically significant differences (LMM with colony as random factor, LSD post hoc test).

the colony cycle: in the absence of policing, the alternation of phases would disappear. Eliminating those nonphasic individuals is therefore adaptive even if, as our results show, they are not abundant in normal colonies. As is the case for ovariole number [19], individual response thresholds to larval cues that inhibit oogenesis might vary along a continuum in *C. biroi*. Although the presence of larvae prevents most colony members from reproducing, some HRIs might have such a high response threshold that their ovaries remain active irrespective of the social environment. Less fertile LRIs have too low a threshold to be nonphasic, and this is probably why they hardly ever get aggressed. Given that aggressions occur regularly (we estimate that 0.09%–0.9% of all individuals are aggressed) and it seems improbable that allelic mutations occur at a similarly high rate, we suggest that the nonphasic phenotype is, at least in most cases, due to epigenetic differences. Although we cannot exclude the possibility that mutations could in some cases account for the occurrence of desynchronized HRIs, such mutant cheater lineages are expected to be unstable and therefore rare in clonal groups [24].

In insect societies, policing rarely results in the death of the focal individual (but see [25] and, in a different context, [26]). Contrarily, the death of policed individuals is the norm in *C. biroi* and serves to permanently eliminate dysfunctional individuals immediately after they have become reproductively active. According to our results, *C. biroi* can develop ovaries and lay eggs within 5–9 days in the absence of larvae. This means that, whereas "normal" egg-layers lay once per cycle, noninhibited egg-layers could lay more in the same time-lapse, increasing the reproductive output of a hypothetical nonphasic colony. These superproductive colonies should outcompete phasic colonies and spread in populations, but this is not what we observe. Selective pressures have likely favored the conservation of the reproductive cycle, and an effective policing system has evolved to enforce the alternation of phases (the adaptive value of the phasic cycle is discussed in the Supplemental Information section). Although we cannot exclude the possibility that worker policing has originated in a sexual ancestor of *C. biroi* as an adaptation to genetic conflicts (see, e.g., [27]) its main current function is clearly to increase colony efficiency.

Earlier studies on another parthenogenetic ant, *Platythyrea punctata*, suggested that policing occurs in clonal societies to establish dominance hierarchies and maximize the reproductive output of colonies [9]. However, it has since become clear that despite parthenogenetic reproduction, colonies of *P. punctata* are often genetically heterogeneous due to colony fusions [10] and that high levels of policing are correlated with genetic heterogeneity [11]. Similarly, in the clonal ant *Pristomyrmex punctatus*, genetic heterogeneity within colonies negatively correlates with assembling behavior [28]. Even though we cannot exclude the possibility that chimeric colonies occur in some populations of *C. biroi*, genetic conflict would still seem an unlikely explanation for worker policing during the foraging phase. The reason is that desynchronized individuals that reproduce during the foraging phase are easily detected and removed from the colony. Instead, a social cheater lineage should show disproportionate reproduction during the reproductive phase.

Due to clonality, individuals in *C. biroi* colonies act as genetically identical replicators. In this context, interindividual reproductive conflicts are largely absent, and cooperation is promoted as it enhances the fitness of the common unique genotype. The individuals disrupting this organismal-like harmony are adaptively eliminated. However, in other parthenogenetic social Hymenoptera, social parasitism by unrelated genetic lineages has been reported. In the ant *P. punctatus*, for example, parasitic lineages spread by horizontal transmission across host colonies of the same species [29, 30]; the Cape honeybee, *A.m. capensis*, parasitizes another honeybee subspecies [31]. Because of uncontrolled reproduction, selfishness, and transmissibility, these social parasites have been compared to specific types of transmissible cancer found in mammals such as the Tasmanian devil *Sarcophilus harrisii* [32–34]. The example of nonphasic HRIs in *C. biroi* allows us to develop the analogy to cancer much more generally. Cancer is a disease where cellular proliferation is no longer under normal growth control, and the unrestrained division of cells interferes with the normal functioning of the organism [35]. The cell cycle is regulated through a series of transductional systems at the transitions between phases,

and if this regulation is lost, cells may undergo uncontrolled proliferation [36]. There are several ways in which this phenomenon can occur, e.g., DNA mutations or epigenetic changes can constitutively activate oncogenes or inactivate tumor-suppressor genes, resulting, for example, in the deactivation or underexpression of membrane receptors of extracellular growth-suppressing factors [37–39]. Insensitivity to the larval inhibition of reproduction, which is in most cases probably mediated via epigenetic effects, produces an analogous phenotype in nonphasic HRIs. Remarkably, these individuals exhibit specific chemical signatures and are detected and killed through the coordinate action of their colony-mates. This is analogous to immunosurveillance in multicellular organisms [15, 16], where cancer cells are detected and killed because they bear tumor-specific surface antigens [17]. These processes occur at different levels of organization (societies and multicellular organisms), involve selfish entities at the lower level (single ants or single cells), and are adaptive at the higher level. Policing in *C. biroi* is an example of how the regulation of individual reproduction is necessary in organismal associations to maintain group-level coherence, even in the absence of genetic conflicts. This selective pressure has produced analogous regulation systems at different levels of biological organization.

Experimental Procedures

Colonies

Twelve colonies of *C. biroi* were used in this study (details are given in Table S4). Colonies were housed in plastic boxes with a plaster of Paris floor containing a single nest chamber covered with red Plexiglas.

Cuticular Melanization Measurements

A picture of each individual was taken under standardized settings (see Supplemental Information section). Pictures were transformed to 32-bit grayscale, and melanization was measured as the average gray level value of a standard area in the center of the abdomen.

Reproductive Status

A picture of the ovaries was taken for each dissected individual. The status of ovarian development was assessed by measuring the picture surface area of the biggest egg, using the software ImageJ.

Larval Inhibition of Ovarian Development

Four experimental colonies were established from each of four stock colonies (16 in total), and each of those received a different treatment. On day 0, two experimental colonies were deprived of larvae (no larvae [NL] and larvae receiver [LR] treatments); the other two received equal amounts of larvae (control larvae [CL] and larvae donor [LD] treatments). On day 3, larvae were removed from LD colonies and placed in LR colonies. Individuals from each experimental colony (five fertile LRIs and three to five HRIs, depending on their availability in the different colonies; total n = 1,335) were collected daily from day 2 to day 9 in order to follow ovarian development in the different treatments (details in the Supplemental Information section).

Genetic Analyses

Genotyping procedures and marker loci have been described in Kronauer et al. [18], and details are given in Table S3. The software GenClone 2.0 [40] was used to assign individuals to recurrent MLGs.

Chemical Analyses

An Agilent Technologies 7890A gas chromatography system connected to an Agilent Technologies 5975C mass spectrometer was used for chemical analyses.

Supplemental Information

Supplemental Information includes one figure, four tables, Supplemental Experimental Procedures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.01.011.

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