SHORT COMMUNICATION

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Dysfunctional reproductive physiology, and not reproductive activation, triggers policing in experimental colonies of the clonal ant *Cerapachys biroi*

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We previously reported the existence of a unique policing system in the clonal ant *Cerapachys biroi*, where individuals that fail to synchronize to the colony reproductive dynamics and reproduce without control are recognized and executed by their nestmates. These executions help maintain the alternation of reproductive and foraging phases, a colony-level adaptive phenotype. In our previous study, we hypothesized that the specific chemical signature of non-synchronized individuals rather than some fertility-related cues serve as the proximate factor triggering their execution. We here examined this hypothesis by testing whether reproductively active individuals introduced in colonies in foraging phase are the target of aggression. We show that introduced fertile individuals display clear behavioral differences from sterile individuals of the foraging colonies, but are never targeted with aggressive behavior. Foraging workers, which usually perform aggressions, are able to discriminate the introduced individuals' subcaste but not their reproductive status. Our results therefore demonstrate that ovarian activation is not enough to trigger policing in experimental colonies, supporting our previous hypothesis that aggressed individuals are not just unsynchronized, but possibly non-responsive to colony-level regulation cues and thus dysfunctional in their reproductive physiology.

Colonies of the clonal ant *Cerapachys biroi* undergo stereotypic reproductive cycles made of two constantly alternating phases.[1-3] In the reproductive phase, eggs are collectively laid while pupae of the previous generation complete their development, whereas in the foraging phase, larvae feed on prey items provided by foraging workers and there is no reproductive activity until the onset of pupation. In a previous study, we showed that colonies of *C. biroi* are subject to a strict regulation of reproduction, with larval cues inhibiting ovarian activation in fertile adults.[4] Nonetheless, some individuals seem to lack sensitivity to these cues and fail to synchronize to the colony-level reproductive cycle. Those individuals are executed during their first foraging phase as reproductively mature individuals, when they exhibit for the first time their unusual physiology (i.e., reproductive activation during a non-reproductive phase). Interestingly, almost all the executed individuals belong to a worker subcaste specialized in reproduction (highly reproductive individuals, or HRIs, which have four to six ovarioles, lay up to eight eggs per cycle and do not engage in foraging; in contrast, low reproductive individuals or LRIs, which have two ovarioles, lay up to two eggs during few cycles and then become sterile foragers). Aggressed HRIs show significantly different cuticular hydrocarbons profiles compared with non-aggressed HRIs from both foraging and reproductive phases.[4] In our previous study, we hypothesized that the specific signatures of aggressed HRIs, rather than being fertility-related,[5] are the proximate cues revealing their peculiar reproductive physiology and trigger aggressive behavior. We concluded that this novel form of policing[6] is analogous to immunosurveillance on cancer cells in multicellular organisms,[7,8] where cells that do not respond anymore to the organism-level growth inhibition signals are killed by the immune system. These cells bear cancer-specific surface antigens,[9] which are the proximal cue triggering the action of immune cells. Accordingly, the profiles of *C. biroi's* aggressed HRIs exhibit a significant chemical difference from the profiles of reproductively active non-aggressed HRIs, differing for both relative proportions and absolute quantities of cuticular hydrocarbons. This suggests that they might be different from normal reproducers. According to our hypothesis, individuals' response threshold to the larval inhibition of reproduction might be distributed along a continuum. While most individuals in a colony refrain from reproducing in the presence of larvae, at the extremes of the distribution of these traits the

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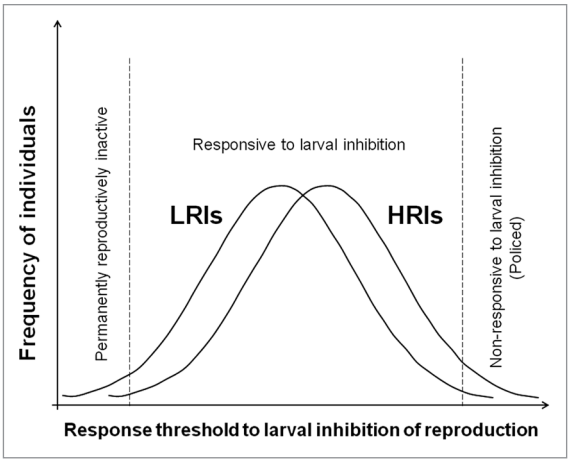

Figure 1. Distribution of responsiveness to the larval inhibition of reproduction for LRIs and HRIs in a *C. biroi* colony. We hypothesize that part of the HRIs do not respond to the larval inhibition of ovary activity and get executed by nest mates in order to maintain the alternation of reproductive and foraging phases. Permanently sterile LRI might exist having a so low threshold to the larval inhibition cues that they never activate ovaries. These individuals are however difficult to identify because contrary to non-synchronized reproducers, they are not a threat for the colony and do not get aggressed by nest mates.

individuals' thresholds to larval cues become extremely low or high (respectively, left and right side of the curve in **Fig. 1**), corresponding respectively to a permanently reproductively inactive or active phenotype. Aggressed HRIs are among those individuals with permanently activated ovaries and thus not simply non-synchronized in reproduction, but probably extreme in their reproductive physiology and dysfunctional from a colony-level perspective.

As it is possible to manipulate experimentally the reproductive status of individuals by exposing them to specific social contexts (i.e., if separated from larvae, fertile individuals produce fully developed eggs within roughly 5 d),[4] we tested whether individuals with artificially activated ovaries were targeted with aggressive behavior when introduced into foraging colonies. We conducted a first experiment in which we induced ovarian activation in some HRIs and LRIs, (details in the SI section) and observed the behaviors they performed and received during the days following their introduction in foraging recipient colonies made with splits of the same mother colony. Reproductively active individuals of both subcastes were less active than controls, spending more time in the nest chamber [LMM (linear mixed model), $F(1, 379) = 35, 037$, $p < 0.0001$, all detailed results are shown in the **Supplemental Materials**]. HRIs (both reproductively active and inactive) spent more time in the nest chamber than LRIs [LMM, $F(1, 379) = 261, 69$, $p < 0.0001$], which foraged more [LMM, $F(1, 379) = 309, 34$, $p < 0.0001$]. Interestingly, no differences were found between the behavior targeted at reproductively active or inactive individuals (LMM, all $p > 0.067$), including the only six observed episodes of biting (which indeed cannot be considered as true policing, in which individuals are normally immobilized and killed by nest mates) and the single observed immobilization (which was unexpectedly performed toward a LRI).

In a second experiment, we more precisely tested the reaction of foragers (which usually perform the aggressions) toward reproductively active and inactive HRIs and LRIs. We introduced experimentally treated ants in arenas positioned in the foraging areas of foraging colonies, which contained two foraging workers, and observed interactions during two minutes per test. No aggressions were observed when foraging workers faced reproductively active individuals. Moreover, although foraging workers showed more interest in HRIs than LRIs (they antennated them significantly more [LMM, experimental colony and reproductive status used as random factors, $F(1,88) = 11, 388$, $p = 0.001$], showing that they are possibly able to discriminate them from LRIs), reproductive status of introduced individuals alone had no effect on the behavior of foraging workers [LMM, experimental colony and subcaste used as random factors, $F(1, 89) = 0, 28308$]. The interaction between reproductive status and subcaste showed differences exclusively between subcastes [LMM, experimental colony as random factor $F(3, 87) = 3, 9388$; LSD post-hoc tests, all $p = 0.002$], confirming the previous results.

The results of our two experiments show that reproductive activation does not produce specific reactions in foraging individuals. Indeed, as we showed in our previous study, fertile *C. biroi* are able to regress rapidly their ovarian status by re-absorbing developing eggs in the presence of larvae. Reproductive de-synchronization is thus not enough to trigger aggressive behavior, and this supports our hypothesis that "naturally" aggressed individuals are indeed dysfunctional in their reproductive physiology and probably exhibit maladaptive extreme response thresholds to social colony-level cues. However, policing in *C. biroi* has been reported to occur in colonies of 500–5,000 individuals, and for this reason we cannot exclude that the small size of our experimental colonies (150 individuals) might have influenced the behavioral tests. Further work on the relation between policing and colony-level life history traits in *C. biroi* is needed to elucidate this issue.

Material and Methods

Experiment 1. A stock colony (T1, clonal line MLL4,[10] around 5,090 individuals, 9.3% HRIs) was split in two parts at the beginning of a foraging phase [larvae at first developmental stage (L1)]. 1,000 individuals were deprived of larvae and put in a separate nest; another 1,000 individuals were put in a separate nest with larvae. The goal of the procedure was to induce ovary activation in the larvae-less colony fragment, and to keep ovaries inactive in the other fragment. Twenty colonies were made with the remaining 3,000 individuals to be used as recipient colonies for the experimentally treated ants. The experimental procedure started 5 d after the fragmentation of the mother colony, which is the time needed by fertile ants to produce new eggs. The experiment consisted in introducing five reproductively inactive and active HRIs or LRIs (respectively, from the 1,000 individual fragments with and without larvae) in recipient colonies and observing their behavior during the following two days.

Experiment 2. The same procedure of de-synchronization was applied to colony T1 (20 months after experiment 1), in

order to focus on the individual interactions of foraging ants with reproductively active or inactive HRIs and LRIs. Experimentally treated individuals (n = 25 for each of four groups) were introduced in arenas placed in the foraging areas of recipient colonies, which contained two different foraging workers for each test. Interactions were observed during two minutes per test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/cib/aricle/24954

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