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Report

Cuticular Hydrocarbons Reliably Identify Cheaters and Allow Enforcement of Altruism in a Social Insect

Adrian A. Smith,[1,3] Bert Hölldober,[1,2] and Jürgen Liebig[1,3,*]

[1]School of Life Sciences and Center for Social Dynamics and Complexity
Arizona State University
Tempe, AZ 85287-4501
USA

[2]Biozentrum
Zoologie II
Universität Würzburg
D-97074 Würzburg
Germany

Summary

Cheaters are a threat to every society and therefore societies have established rules to punish these individuals in order to stabilize their social system [1–3]. Recent models and observations suggest that enforcement of reproductive altruism (policing) in hymenopteran insect societies is a major force in maintaining high levels of cooperation [4–6]. In order to be able to enforce altruism, reproductive cheaters need to be reliably identified. Strong correlational evidence indicates that cuticular hydrocarbons are the means of identifying cheaters [7–14], but direct proof is still missing. In the ant *Aphaenogaster cockerelli,* we mimicked reproductive cheaters by applying a synthetic compound typical of fertile individuals on nonreproductive workers. This treatment induced nestmate aggression in colonies where a queen was present. As expected, it failed to do so in colonies without a queen where workers had begun to reproduce. This provides the first direct evidence that cuticular hydrocarbons are the informational basis of policing behaviors, serving a major function in the regulation of reproduction in social insects. We suggest that even though cheaters would gain from suppressing these profiles, they are prevented from doing so through the mechanisms of hydrocarbon biosynthesis and its relation to reproductive physiology. Cheaters are identified through information that is inherently reliable.

Results and Discussion

Induction of Worker Policing in Queenright Colonies by Application of a Synthetic Compound Indicating Fertility

In the ant *Aphaenogaster cockerelli*, we find a strong correlation between reproduction, cuticular profiles, and policing. On the cuticle of reproductive queens, there are straight alkanes (tri-, tetra-, penta-, and hexacosane) in high quantities that do not appear in the profiles of nonreproductive workers [15] (Figure 1). However, workers that are separated from the queen and allowed to reproduce develop profiles with these compounds [15] (Figure 1). Previous experiments showed that such workers with active ovaries receive aggression upon reintroduction to their colony [16]. In these experiments, when groups of workers are experimentally separated and reunited with their nestmates and queen, individuals capable of producing viable eggs are immediately singled out and attacked. This observation in *A. cockerelli* is strongly indicative of policing, because studies in other ant species have shown that such aggression will lead to inhibition of any ovarian activity [7, 17]. The correlation of worker policing with fertility-related changes in the cuticular hydrocarbon profile of *A. cockerelli* workers suggests the involvement of reproductive-specific alkanes in cheater identification. If such alkanes are indeed responsible for worker policing, a transfer of such compounds on nestmates in similar concentrations should trigger worker aggression toward the manipulated workers.

We transferred the most pronounced reproductive-specific alkane, pentacosane, onto the gaster of nonreproductive workers in a concentration matching that of queenless workers that had fully developed ovaries, capable of producing viable eggs (Figure 1). (Pentacosane on workers after addition treatment [median relative abundance: 5.3%, range: 3.3%, 15.1%, n = 11] was not different than pentacosane abundance on workers with reproductively active ovaries [median relative abundance: 6.3%, range: 0.4%, 30.5%, n = 10; Mann-Whitney U-test: $z = -0.493$, 2-sided $p = 0.654$].) After reintroduction of the treated ants, we recorded directed aggression of nestmate workers that encountered the manipulated individual. For each trial, we used a different colony to exclude any colony-level effects (n = 11). To exclude any manipulation effect, we also transferred similar amounts of nonacosane, an alkane that occurs on the cuticle of all workers and queens, on a second group of workers in addition to a solvent control (Figure 1). (The increase in relative abundance of nonacosane on treated workers [median increase: 5.9%, range: 3.3%, 17.5%, n = 12] was not different from the relative increase of pentacosane on pentacosane-treated workers [median relative abundance: 5.3%, range: 3.3%, 15.1%, n = 11; Mann-Whitney U-test: $z = 0.246$, 2-sided $p = 0.833$].)

If simply manipulating the cuticular hydrocarbon profile induces aggression, we should find the same level of biting in this control group. However, aggression was significantly more frequent toward the pentacosane group than in the nonacosane group (Figure 2A, Movies S1–S4 available online). In addition, antennal inspection, indicative of treatment perception, was significantly raised in the pentacosane-treated group in comparison to the hexane control. Nonacosane-treated workers received intermediate levels of antennation (Figure 2B). By applying the major hydrocarbon compound of fertile workers, we were able to induce aggression toward these manipulated workers. This strongly suggests that cuticular hydrocarbons reveal reproductive status and are used to identify reproductive cheaters.

Lack of Worker Policing toward Pentacosane-Treated but Nonreproductive Workers in Queenless Colonies

When *A. cockerelli* workers are kept in queenless groups, some of them will activate their ovaries and eventually lay eggs [15]. This change in ovarian activity is accompanied by a shift in the cuticular hydrocarbon profile in *A. cockerelli* as

*Correspondence: juergen.liebig@asu.edu
[3]These authors contributed equally to this work

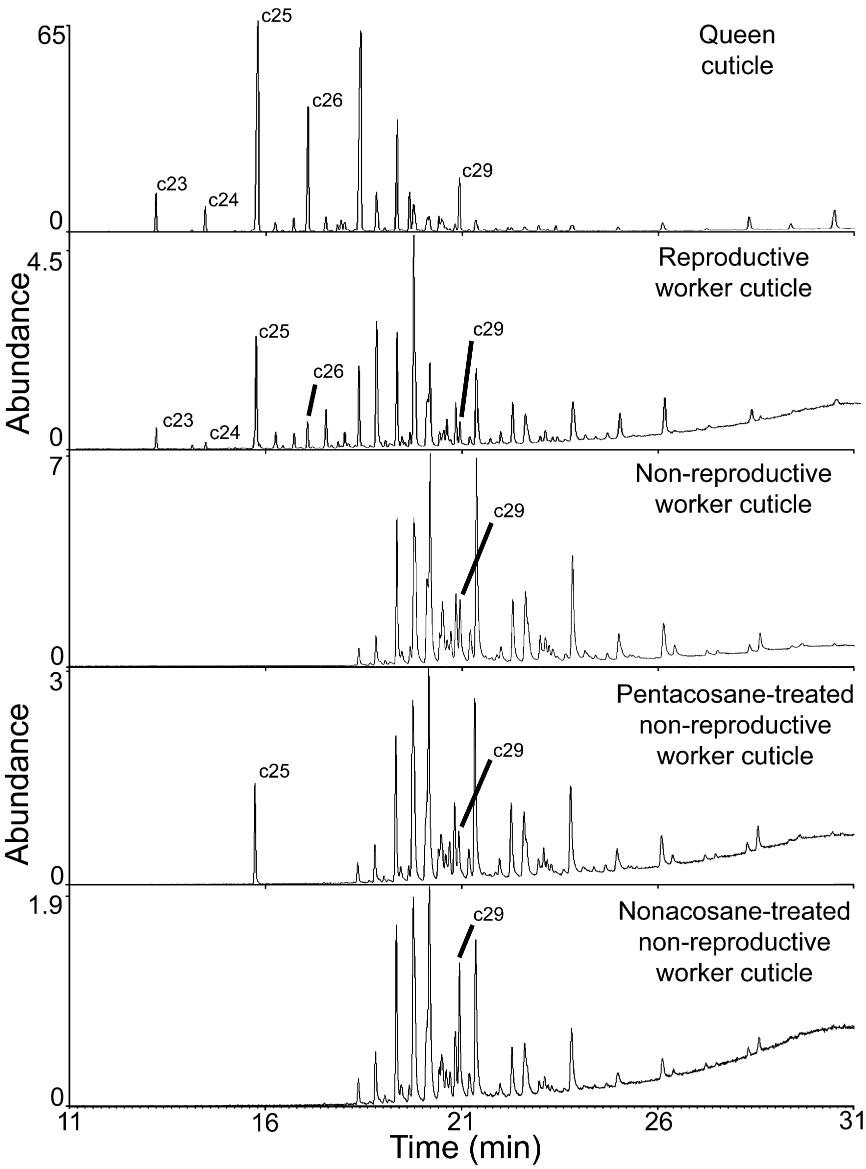

Figure 1. Chromatograms Showing Differences in Cuticular Hydrocarbon Profiles

Natural profiles of a queen, reproductive worker, and nonreproductive worker are shown. The profiles of pentacosane- (c25) and nonacosane- (c29) treated nonreproductive workers are provided for comparison. Straight chained alkanes tricosane (c23), tetracosane (c24), pentacosane (c25), hexacosane (c26), and nonacosane (c29) are labeled in the profiles where they are present.

well as in other ant species [11, 14]. Although these workers were policed in queenright colonies upon reintroduction, we did not observe aggression directed to them when they became reproductively active in queenless worker groups. We predict that nonreproductive workers treated with pentacosane should not be policed in queenless worker groups in contrast to the outcome in queenright colonies, because such workers are already present in the queenless group.

We repeated the same experiment in queenless worker groups already containing egg-laying workers. As predicted, pentacosane-treated workers were not policed but received the same low level of aggression as the nonacosane group and the controls (treated only with hexane), whereas foreign workers were attacked at significantly higher rates (Figure 3A). Workers in the queenless groups antennated pentacosane-treated workers more frequently than the hexane control group, indicating that workers still detected the profile manipulation (Figure 3B). The acceptance of the pentacosane-treated nonreproductive workers in queenless worker groups indicates that they were accepted as fertile workers even though they would have been policed in queenright colonies. This again is in line with our hypothesis that pentacosane indicates the reproductive status of a worker. It also confirms that pentacosane-treated workers are still considered as nestmates and that the aggression received in queenright colonies is not a consequence of mistaking them as foreign workers.

The Mechanism of Hydrocarbon Synthesis and Distribution and Its Unique Advantages as a Reliable Information System of Reproductive Status

It is counterintuitive that workers express changes in their cuticular hydrocarbon profile that consistently reveal themselves as reproductive cheaters. Such cheating in ant colonies is represented by egg-laying in the presence of the queen to gain direct fitness benefits [18, 19]. However, the linkage of cuticular hydrocarbon profiles with the surface profiles of their eggs may prevent a successful cheater strategy. Reproductive cheaters would need to escape physical policing as an adult and ensure that their eggs escape policing. In several species, nestmate workers are able to recognize worker-laid eggs with a surface hydrocarbon profile differing from the established queen's eggs, leading to the destruction of worker-laid eggs [9, 20–24]. In fact, in the egg-policing species *Camponotus floridanus*, the policing of viable worker eggs is strongly decreased when these eggs are treated with an artificial queen egg hydrocarbon profile [9]. Egg profiles, though not identical, are correlated to the cuticular hydrocarbon profile of the egg layer resulting from the mechanism of their production and distribution. Oenocytes within the fat body synthesize new hydrocarbons, which are transported through the hemolymph to target tissues, including the cuticle and the ovaries [25]. *A. cockerelli* workers produce viable eggs that are indistinct in surface hydrocarbon profile to queen-laid eggs and are not policed by workers [15]. Although egg policing has not been demonstrated in *A. cockerelli*, we would expect to see policing of eggs with a hydrocarbon profile that does not have the reproductive-specific compounds. This, however, cannot be tested, because all our analyzed viable eggs showed the reproductive profile. Because the presence of the reproductive-specific pentacosane on the cuticle of workers identifies these individuals as

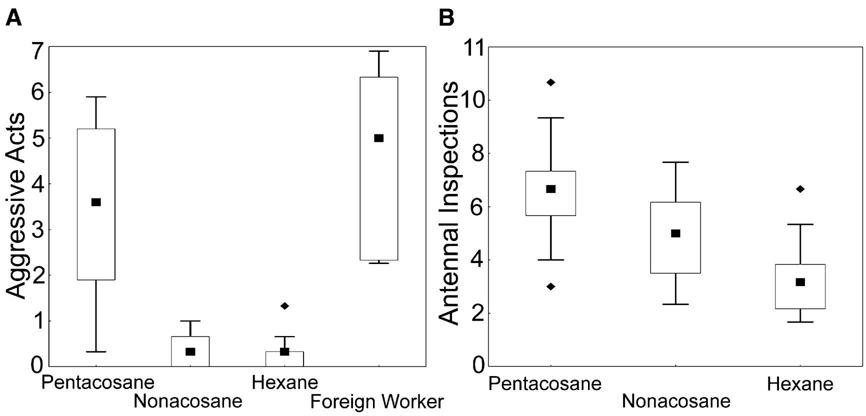

Figure 2. Response to the Introduction of Treated Workers into Queenright Colonies

Medians, 25%–75%, nonoutlier range, and outlying data points. Sample size (n = 11 all groups) represents number of independent colonies.

(A) Acts of aggression were more frequently directed toward pentacosane-treated workers and foreign workers. Friedman's ANOVA, $p < 0.001$; Wilcoxon-Wilcox multiple comparisons, $p < 0.001$ (foreign worker versus nonacosane and hexane), $0.01 > p > 0.005$ (pentacosane versus nonacosane and hexane), and $p > 0.05$ (pentacosane versus foreign worker and hexane versus nonacosane).

(B) Antennal inspections toward treated individuals. Friedman's ANOVA, $p = 0.003$; Wilcoxon-Wilcox multiple comparisons, $0.005 > p > 0.001$ (pentacosane versus hexane), $p > 0.05$ (nonacosane versus pentacosane and hexane).

reproductive cheaters [15], a successful cheater strategy of reproductive *A. cockerelli* workers would require two conditions: (1) to completely suppress the reproductive profile on the cuticle and (2) to continue to express it on the eggs. Only with these two requirements fulfilled would they be capable of gaining direct fitness benefits. However, this would require physiological changes leading to a complete uncoupling of the profile expression on eggs and the cuticle in workers, which has never been observed. Furthermore, this is opposed by strong selection on maintaining this physiological connection in queens that benefit from indicating their reproductive status on the cuticle and on the surface of their eggs to their workers [26, 27].

Hydrocarbon synthesis, transport, and uptake in target tissues are most likely under the regulation of epistatically interacting genes, so that single mutations in any involved genes are unlikely to remove the reproductive compounds on the cuticle and leave them on the eggs. On the other hand, a stepwise uncoupling of these profiles would lead to a transitionary phase that is prevented by efficient policing. In addition, pleiotropic constraints may be involved, as indicated by the fact that changes in one profile (cuticular or egg surface) result in changes in the profile expression of the other. For a general understanding of the absence of cheating, it is important to uncover the specific genetics and physiology of these underlying mechanisms. The inability to cheat may not be based on costs associated with producing or not producing a fertility signal, but rather on the conservation of basic biochemical mechanisms that are difficult to change. Epistatic and pleiotropic gene interactions are likely to play an important role in reliable cheater identification [29].

Conclusions

Correlational evidence has indicated that hydrocarbons contain sufficient information necessary to identify cheating egg-layers and their eggs [30–33]. Herein we provide the first direct evidence that reproductive cheaters are identified by nestmates through changes in their cuticular compounds. Cuticular hydrocarbons represent the proximate mechanism for the regulation of reproduction in insect societies for which only indirect evidence has been presented so far [10, 11, 34]. Cheater resistance in this system is crucial in ensuring social stability. The mechanism of hydrocarbon synthesis and transport provides a system that is uniquely resistant to cheating, which may explain its widespread occurrence among social insects [28, 30, 31, 33]. Similar to means of cheater prevention in slime molds [29], pleiotropic and epistatic effects may be constraining cheating. Such communicative mechanisms should be expected in other cooperative groups.

Experimental Procedures

Colonies of *A. cockerelli* were collected and cultured as described [15]. Solutions of 1 μg hydrocarbon per 40 μl of hexane were made for pentacosane and nonacosane (Sigma Aldrich). 0.5 μl of the solution was applied to the dorsal anterior portion of the gaster. The treated individual was marked with a single dot of flat blue Testors enamel paint on the dorsal section of the thorax and introduced into the appropriate colony 20 min after treatment. Hexane controls were treated with 0.5 μl of hexane and also marked with

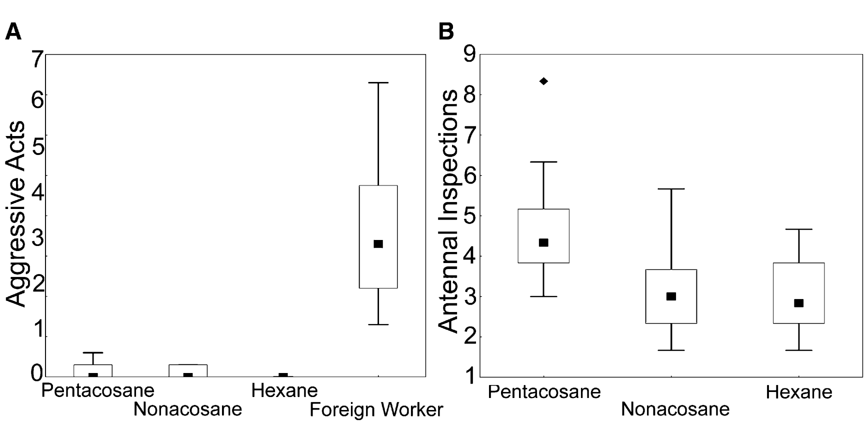

Figure 3. Response to the Introduction of Treated Workers into Groups of Queenless Reproductive Workers

Medians, 25%–75%, nonoutlier range, and outlying data points. Sample size (n = 12 all groups) reflects number of independent colonies.

(A) Acts of aggression were more frequently directed toward foreign workers. Friedman's ANOVA, $p < 0.001$; Wilcoxon-Wilcox multiple comparisons, $p < 0.001$ (foreign worker versus hexane), $0.005 > p > 0.001$ (foreign worker versus nonacose and pentacosane), and $p > 0.05$ (pentacosane versus nonacosane and hexane).

(B) Antennal inspections of treated individuals. Friedman's ANOVA, $p = 0.002$; Wilcoxon-Wilcox multiple comparisons, $0.005 > p > 0.001$ (pentacosane versus hexane), $p > 0.05$ (nonacosane versus pentacosane and hexane).

a color dot. To verify the effect of treatment, in-between each replicate colony, cuticular profiles were gathered [15] from pentacosane- and nonacosane-treated workers before and after treatment. After introduction into either queenless reproductive-nestmate groups or queenright colonies, any interactions that involved prolonged antennal inspection of the treated area were recorded. Aggressive actions (open mandible contact, clearly distinguishable from allogrooming, holding, and pulling) were recorded only when they followed antennal contact with the treated area. Ants introduced into queenright colonies (300–1000 workers) were observed for 5 min directly after reintroduction, whereas, because of smaller colony size, individuals placed in queenless colonies (50–150 workers) were observed for 10 min. A paint-marked foreign worker was introduced into each colony (queenright and queenless), and aggressive acts were recorded. Each data point for antennal inspection and aggression is the average of three trials with separate treated workers and foreign workers. Reactions to all four groups of individuals were tested on the same day, under the same laboratory conditions. A single queenright colony that did not respond aggressively to foreign workers in all three trials was excluded from analysis.

Supplemental Data

Supplemental Data include four movies and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01619-9.

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