

# Kin-informative recognition cues in ants

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Although social groups are characterized by cooperation, they are also often the scene of conflict. In non-clonal systems, the reproductive interests of group members will differ and individuals may benefit by exploiting the cooperative efforts of other group members. However, such selfish behaviour is thought to be rare in one of the classic examples of cooperation—social insect colonies—because the colony-level costs of individual selfishness select against cues that would allow workers to recognize their closest relatives. In accord with this, previous studies of wasps and ants have found little or no kin information in recognition cues. Here, we test the hypothesis that social insects do not have kin-informative recognition cues by investigating the recognition cues and relatedness of workers from four colonies of the ant *Acromyrmex octospinosus*. Contrary to the theoretical prediction, we show that the cuticular hydrocarbons of ant workers in all four colonies are informative enough to allow full-sisters to be distinguished from half-sisters with a high accuracy. These results contradict the hypothesis of non-heritable recognition cues and suggest that there is more potential for within-colony conflicts in genetically diverse societies than previously thought.

**Keywords:** nepotism; social insect; cuticular hydrocarbons; social evolution; kin recognition

## 1. INTRODUCTION

Although the ancestral mating behaviour in social Hymenoptera (ants, bees and wasps) is monandry, multiple mating by queens with different males (polyandry) has evolved in approximately a third of the social hymenopteran species studied [1,2], leading to the presence of multiple genetic lineages (patrilines) within colonies. The evolution of polyandry increases substantially the potential for conflict within social insect colonies, because workers are three times more related to their full-sisters ( $r = 0.75$ ) than to their half-sisters ( $r = 0.25$ ). This skew in relatedness results in potential conflicts over reproduction and resource allocation, which seems likely to reduce the fitness of the colony. The potential for actual conflict between patrilines within social insect colonies is therefore expected to be selected against, at least as long as the colony-level costs of conflict exceed the benefit to individuals. Preferential treatment of full-sisters over half-sisters would require patriline-specific recognition cues. Although it is also possible that such cues may be present but ignored by workers to avoid the costs of conflict, it is generally assumed that between-patriline conflict is prevented by positive frequency-dependent selection removing genetically polymorphic cues from populations [3–7]. A homogenization of recognition cues within the colony would also improve the accuracy of nest-mate recognition among colonies, reducing the risk that colony resources are exploited by intruders [8,9].

Recognition systems in social insects have been extensively studied and are typically based on chemical cues [10,11]. Social insects must discriminate between nest-mates and non-nest-mates to guarantee that resources are directed to colony members only, and not to intruders. It has been shown repeatedly that cuticular hydrocarbons are the main cues used in social insect nest-mate recognition (e.g. [12–17]). They also play a role in processes of within-colony recognition, such as of queen fertility [18–22]. Therefore, we can assume that patriline-specific recognition cues, if they exist, should be found among the cuticular hydrocarbons.

The weight of empirical evidence appears to support the hypothesis that kin-informative recognition cues are eliminated from genetically diverse social insect colonies. Three studies have reported differences in recognition cues between patrilines in the honeybee *Apis mellifera*, but all had important limitations [23–25]. The study by Page *et al.* [23] involved colonies containing an artificially low number of patrilines (two, compared with 12 on average in natural colonies) and the results may also have been an artefact of patriline differences in tasks. The studies by Arnold *et al.* [24,25], which both used the same dataset, involved only a single colony and could only distinguish patrilines in bees that were very young (5 days old) or kept in isolation; cue differences between patrilines of older bees that were kept in their colony were an order of magnitude less. Although two studies have found that matriline within colonies of ants and wasps can differ in their cuticular hydrocarbon profiles [26,27], such differences can potentially be due to maternal differences (e.g. in queen health) or rearing conditions (e.g. owing to queens reproducing in different parts of the nest), rather

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than necessarily owing to genetic effects. Only two powerful studies of patriline-specific recognition cues have been conducted. The cuticular hydrocarbon profiles of neither *Vespa crabro* hornets nor the wood ant *Formica truncorum* were sufficiently informative to allow for a reliable recognition of patrilines [6,26] (see also [13]). The evidence for patriline variation in recognition cues under natural conditions in social insects is thus equivocal.

Here, we test the hypothesis that a lack of kin-informative recognition cues means there is no possibility for social insect workers to discriminate between full- and half-sisters [6]. We examined the genetic structure and cuticular chemistry of workers from four colonies of the leaf-cutting ant *Acromyrmex octospinosus* that were freshly collected from the field. If there is no expressed genetic polymorphism for recognition cues, as the hypothesis predicts, then the chemical profiles should vary significantly between colonies, but not between patrilines within colonies.

## 2. MATERIAL AND METHODS

Workers were collected from the fungus garden of four mature colonies of *A. octospinosus* in the field in Gamboa, Panama, in 2008 and 2009. To examine the genetic structure of the colonies the workers were sampled from, we used standard genotyping methodology. DNA was extracted from the legs of 96 workers per colony using 150  $\mu$ l Chelex, and amplified at five microsatellite loci: Ech1390, Ech3385, Ech4126, Ech4225 and Atco15 [28,29]. Reactions were performed in 10  $\mu$ l volumes with 1  $\mu$ l of DNA. The DNA was amplified in ABI 3700 thermal cyclers using an initial denaturation step of 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, an annealing step (Ech1390: 53°C; Ech3385: 54°C; Ech4126: 60°C; Ech4225: 54°C; Atco15: 62°C) for 45 s, 72°C for 2 min, with a final elongation step of 72°C for 7 min. Amplified products were multiplexed and run on an ABI 3130xl capillary sequencer. Allele sizes were scored by comparison with internal size markers using GENEMAPPER software. Multilocus offspring genotypes were used to infer the genotypes of colony queens and their multiple mates, and the workers were assigned to patrilines within their colony with negligible detection errors.

Before DNA extraction, workers were killed by freezing and the cuticular substances extracted by immersing individuals in 200  $\mu$ l of *n*-pentane (Sigma-Aldrich, Denmark) for 10 min under constant gentle agitation. The *n*-pentane was evaporated, and the cuticular substances then resuspended in 10  $\mu$ l of *n*-pentane immediately before analysis. Two microlitres per sample were injected into an Agilent 6890 N gas chromatograph equipped with an HP-5MS capillary column (30 m  $\times$  250  $\mu$ m, 0.25  $\mu$ m thickness) and a split-splitless injector, and coupled with a 5975 Agilent Mass Spectrometer with 70 eV electron impact ionization. The carrier gas was helium at 1 ml min<sup>-1</sup>. After an initial hold of 1 min at 70°C, the temperature rose to 150°C at a rate of 12°C min<sup>-1</sup>, then to 250°C at a rate of 8°C min<sup>-1</sup> and then to 320°C at 5°C min<sup>-1</sup>, where it was held for 25 min. The areas of 38 peaks common to all worker cuticular extracts were integrated for further analysis. The substances were identified on the basis of their mass spectra and their retention time as compared with standard linear hydrocarbons.

For each of the four colonies, the cuticular profiles of workers belonging to the five to six most abundant patrilines were analysed, with up to 11 workers per patriline (30–42

workers per colony). The peak areas of the cuticular profiles were normalized according to Aitchison [30] and used as variables in a principal component analysis (PCA), followed by a discriminant analysis (DA) based on as many principal components as were necessary to cover 90 per cent of the dataset's variance. Each sample was used in two analyses: one including all samples and comparing the profile of workers from different colonies, and another one that was conducted separately for each colony, to compare the profiles of workers from different patrilines within these colonies. To test whether the cuticular hydrocarbon profiles of workers differ between groups (colonies or patrilines), a Wilks's MANOVA was conducted for each analysis. Furthermore, a leaving-one-out cross-validation was computed, testing whether the group of a sample could reliably be predicted based on its PCA scores when compared with those of all other samples in the analysis. As a DA is able to use any variation in a dataset to predict groups, a permutation test with 1000 permutations was conducted. In each permutation, a DA was computed with randomly assigned groups. The maximum and 95 per cent percentile proportion of correctly assigned samples from the permutation test was compared with that for the DA using the real groups (i.e. the patrilines), to exclude overfitting.

As four colonies provide insufficient information about population-level variation, we did not calculate heritabilities. Instead, we carried out ANOVAs for each substance, using the log-transformed relative abundance of the substance as the response variable, with colony and patriline nested within the colony as explanatory factors. We then used the quotient of the *F*-values of the two factors to rank the substances according to how informative they were for patriline discrimination relative to colony discrimination. A high rank implied a substance relatively more informative about patriline identity than colony identity. The ranks were then compared between substance classes (linear alkanes, branched alkanes, unsaturated hydrocarbons) and correlated with the chain length (*z*-transformed number of carbon atoms) in a general linear model.

## 3. RESULTS

All four of the colonies we sampled were polyandrous, containing 7–11 patrilines. Generally, four to six patrilines made up 65 to 83 per cent of the sampled workers, with between 9 and 43 per cent of workers assigned to each individual patriline. Two of the colonies had a more even spread, with 9 to 19 per cent of workers assigned to each of five or six patrilines. However, the other two colonies were more skewed, with one patriline representing 30 to 43 per cent of workers and three others 9 to 25 per cent. The remaining patrilines were rare, containing only 1 to 5 per cent of workers (electronic supplementary material, table S1).

The four colonies had 32 peaks in common that contained one or multiple hydrocarbons each (electronic supplementary material, table S2). The chain length of the hydrocarbons ranged from 16 to 37 carbon atoms, with the shorter molecules being mainly linear alkanes while the longer molecules (more than 24 carbon atoms) were mostly branched alkanes and unsaturated hydrocarbons. The cuticular profiles differed between the colonies, allowing for efficient colony-specific recognition (table 1 and electronic supplementary material,

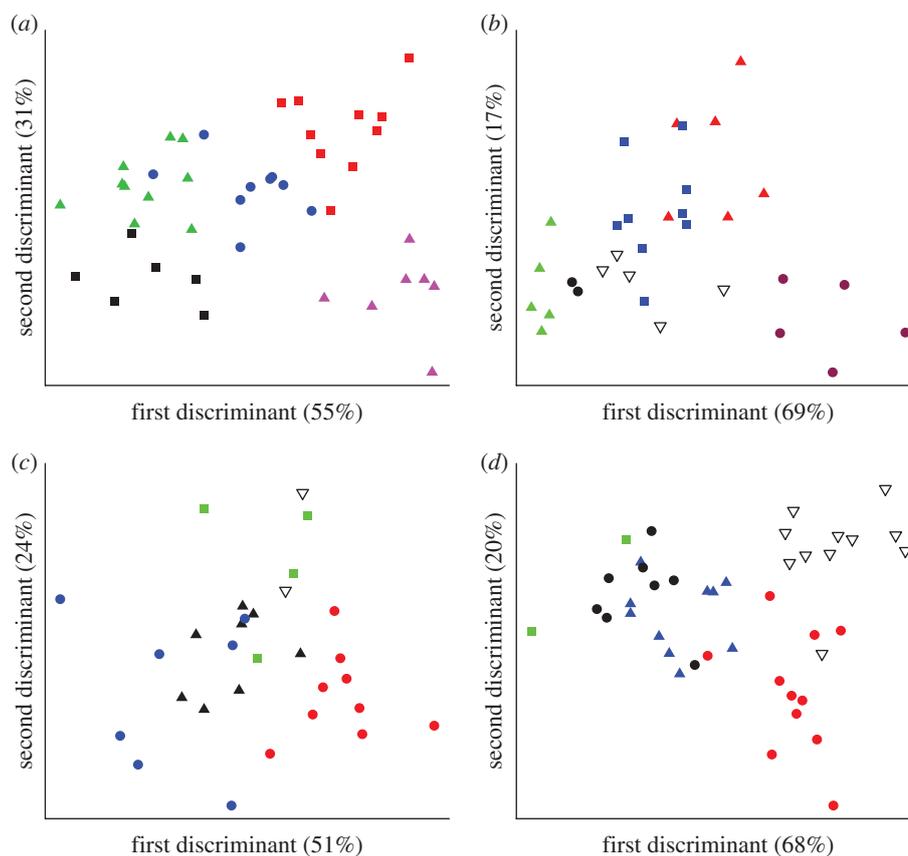


Figure 1. Patriline-level comparison of the first two functions of a discriminant analysis based on cuticular hydrocarbon profiles for the four colonies of *A. octospinosus* examined. Different patrilines within each colony are indicated by different symbols. (a) Colony Ao 081; (b) colony Ao 409; (c) colony Ao 412; (d) colony Ao 414.

Table 1. The success of predicting the colony of origin ('all') or the patriline within the four colonies (Ao 081–Ao 414) of *A. octospinosus* workers using the information encoded in the 32 cuticular hydrocarbon peaks all colonies had in common. Shown are the minimum number of principal components (PCs) that were needed to cover 90% of the variance; the significance of a Wilks's MANOVA between the groups based on these PCs; the percentage of workers that were assigned to the correct group (colony or patriline) in a leaving-one-out cross-validation; the 95% quantile (corresponding to a two-tailed  $p < 0.05$ ) and the maximum number of correctly assigned samples in a permutation test (1000 iterations) with randomly assigned groups; the minimum and maximum number of samples per group; and the number of groups in the analysis.

colony	PCs	Wilks's $p$	correctly assigned (%)	1000 permutation tests (95%/max%)	samples/group	$n$ groups
Ao 081	8	***	98	60/71	6–10	5
Ao 409	9	***	94	78/94	2–9	6
Ao 412	9	*	90	80/93	2–9	5
Ao 414	9	***	95	67/76	2–11	5
all	11	***	96	47/51	30–42	4

\* $p < 0.05$ .

\*\* $p < 0.01$ .

\*\*\* $p < 0.001$ .

figure S1). In all four colonies, the patrilines differed significantly in their CHC profiles, to the extent that the patriline of a worker could be predicted from the CHC profile with a very high accuracy (table 1). Most patrilines of all colonies can be clearly distinguished using only the first two discriminant functions (figure 1). Of the 32 substances, only two ( $C_{29:1}$  and  $C_{31:2}$ ) did not differ significantly between colonies, while six substances did not differ between patrilines (electronic supplementary material, table S2).

The information provided by substances for patriline discrimination relative to colony discrimination depended

on the substance class and chain length ( $F_{5,31} = 9.18$ ,  $p < 0.001$ ; table 2). Linear alkanes provided relatively little information to distinguish patrilines within colonies, but varied systematically between colonies (electronic supplementary material, table S2 and figure 2). Branched alkanes and unsaturated hydrocarbons provided more information about patriline identity and also discriminated between colonies. Of the six hydrocarbons most informative for patriline discrimination relative to colony discrimination, five were unsaturated hydrocarbons ( $C_{29:1}$ ,  $C_{31:2}$  in two different configurations,  $C_{31:1}$  and  $C_{37:2}$ ) and one was a methyl-branched alkane

Table 2. Estimates and significance statistics for a general linear model to predict the rank of a substance according to how informative it is for patriline discrimination relative to colony discrimination. The hydrocarbon class (hc) was used as a predictive factor and the chain length (number of carbon atoms in the main chain) as a covariate.

	estimate	s.e.	<i>t</i>	<i>p</i>
intercept	25.5	3.1	8.0	<0.001
class branched hc	-12.7	3.8	-3.3	<0.01
class unsaturated hc	-13.9	3.7	-3.8	<0.001
chain length	-2.1	2.2	-1.0	0.33
chain length × branched hc	9.5	3.8	2.5	<0.05
chain length × unsaturated hc	0.4	3.5	0.1	0.92

(11-, 13-MeC<sub>25</sub>). Five of these differed significantly between patrilines (the C<sub>29:1</sub> received a high rank because it differed neither between patriline nor colony and had a very low *F*-value for the between-colony effect). Two further substances (C<sub>27:1</sub> and 11-, 13-, 15-MeC<sub>31</sub>) were important for patriline discrimination, but did not rank highly because they also had a strong colony effect. The ANOVA results suggest that the unsaturated hydrocarbons are the most heritable substances. In spite of these differences between substances, however, discriminant analyses conducted separately for linear alkanes, branched alkanes and unsaturated hydrocarbons showed that each of these classes was informative enough to allow for a relatively good discrimination between patrilines (table 3).

Chain length also influenced how informative a substance was for patriline discrimination relative to colony discrimination. There was no overall pattern though, and the trend instead depended on the class of substance. While linear alkanes and unsaturated hydrocarbons were more informative for colony discrimination when short, and more informative for patriline discrimination when long, the opposite was the case for branched hydrocarbons (table 2 and figure 2).

#### 4. DISCUSSION

In all four colonies analysed, the cuticular hydrocarbon profiles were informative enough to allow the correct identification of the patriline of nearly all workers. As the permutation test shows, the information encoded in the cuticular profiles is a genetic polymorphism and not just random variation that could be used for any kind of discrimination. Our data demonstrate, therefore, that in colonies of *A. octospinosus*, workers have the information available to potentially discriminate full-sister from half-sister workers and thus to possibly act nepotistically towards them. Further behavioural experiments will be needed to establish whether workers utilize this information, but the fact that it is even present contradicts the generally accepted hypothesis of recognition cues having negligible heritability. It seems likely that the ants can at least detect the information. We estimate that *Acromyrmex* workers have at least 250 ng mm<sup>-2</sup> of hydrocarbons on their bodies (V. Nehring 2010, unpublished data), which is similar to other ants [31–33]. In our study, the hydrocarbon C<sub>31:2b</sub>, for example, varied in relative abundance by 2.1 per cent, equating to

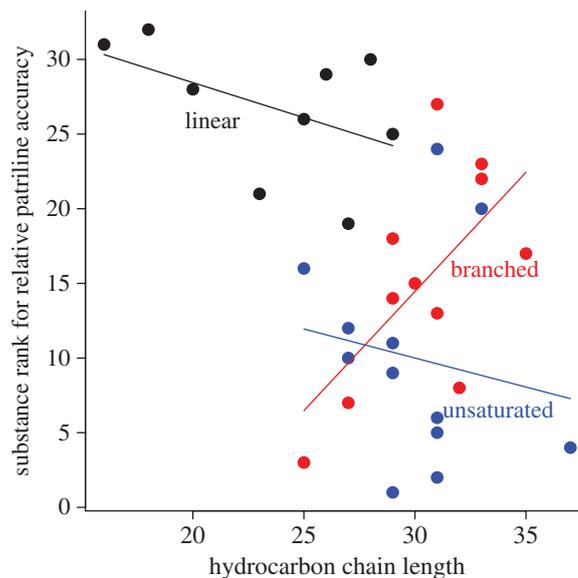


Figure 2. The effect of chain length and substance class (black, linear alkanes; red, branched alkanes; blue, unsaturated hydrocarbons) on the informative value of a substance for discriminating patrilines within colonies relative to its value for discriminating colonies.

Table 3. The success of predicting the colony ('all'), or the patriline within colony, of *A. octospinosus* workers, based on the different hydrocarbon classes.

colony	PCs	Wilks's <i>p</i>	correctly assigned (%)	1000 permutations (95%/max%)
<i>linear alkanes (9 substances)</i>				
Ao 081	5	***	69	55/64
Ao 409	4	**	50	56/69
Ao 412	3	*	57	53/63
Ao 414	5	***	71	57/64
all	3	***	67	39/44
<i>branched alkanes (12 substances)</i>				
Ao 081	5	***	83	55/64
Ao 409	6	***	75	66/78
Ao 412	6	0.15	53	67/77
Ao 414	5	**	64	55/67
all	6	***	88	42/47
<i>unsaturated hydrocarbons (12 substances)</i>				
Ao 081	4	***	60	48/60
Ao 409	4	**	69	56/72
Ao 412	6	0.16	70	70/80
Ao 414	5	**	55	55/67
all	6	***	65	42/46

\**p* < 0.05.

\*\**p* < 0.01.

\*\*\**p* < 0.001.

5.25 ng mm<sup>-2</sup>. It has recently been shown that ants can detect 0.05 ng mm<sup>-2</sup> of hydrocarbons [33] (i.e. at least two orders of magnitude less than the level of variation we found in individual compounds), and the overall profile will provide far more information. Although little is known about how ants process this information [34,35], it therefore seems very plausible that ants are capable of

at least detecting the levels of patriline variation in cues that we found. *Acromyrmex* workers are capable of producing viable eggs and occasionally do so even in queenright colonies [36], so workers could use the information about patriline identity to police half- rather than full-sisters that lay eggs. If similar patriline variation is present in the cuticular hydrocarbons of larvae or eggs, then workers may also be able to act nepotistically by selectively policing eggs laid by half-sister workers, or by preferentially nurturing full-sister larvae into queens. The less complex profiles of brood include some of the substances we found to distinguish patrilines [37], but further work will be needed to determine whether the level of information is sufficient to allow patriline discrimination of brood.

The substances that showed the greatest variance among patrilines were branched alkanes and unsaturated hydrocarbons, particularly long-chained alkenes and alka-dienes. Linear alkanes, in contrast, differed between colonies but were less informative of patriline, although this may in part be because they were generally shorter in chain length (and thus relatively more volatile). These three substance classes are increasingly thought to fulfil different functions for recognition, and they are each suitable for different recognition contexts owing to their specific physical properties. Branched hydrocarbons, for example, seem to be the major compound class that is used for nest-mate recognition in most systems [13,14,16,38] (but see [39]). It has been shown that branched hydrocarbons are heritable but easily transferred between nest-mate *Formica* ants, making them bad candidates for within-colony kin recognition, but good candidates for between-colony nest-mate recognition [40]. By sharing substances between nest-mates, differences in the abundance of substances are equalized between ants, and a gestalt odour, common to all nest-mates, is usually formed [8,9]. On the other hand, linear alkanes in *Formica* ants are much less easily transferred between nest-mates but have low heritability, making them not suitable for within-colony kin recognition either [40]. Our finding that the relative abundances of unsaturated hydrocarbons are very patriline-specific within colonies of *Acromyrmex* ants suggests that these substances are less prone to be transferred between nest-mates, and are thus ideal cues to recognize more genetically similar full-sisters. This variation may be enhanced in taxa such as *Acromyrmex* that engage in little or no trophallaxis. However, none of the substance classes individually allowed patriline discrimination as accurately as did the complete profiles. These findings indicate that small differences in many substances, as well as large differences in a few substances, may be the key to possible patriline discrimination.

Five previous studies have investigated patriline variation in the cuticular hydrocarbons of social insects. Three studies with *A. mellifera* honeybees found that patrilines were distinguishable, but were either limited to a single colony, involved colonies with artificially very low mating frequencies or were confounded by patriline variation in task [23–25]. The other two studies with *V. crabro* hornets and *F. truncorum* wood ants were far more powerful, but did not find enough variation between patrilines to allow for their discrimination [6,26]. In combination with both theory and the lack of empirical evidence for nepotism, it was therefore inferred that

kin-informative recognition cues are absent in social insects [5–7]. However, our results demonstrate conclusively that there is significant patriline variation in recognition cues in natural populations of *A. octospinosus*. The typical mating frequencies of the different species studied may explain these contrasting results. It is notable that queens of both *F. truncorum* and *V. crabro* exhibit low polyandry (40 and 20% of queens mating multiply, with effective mating frequencies of 1.4 and 1.1, respectively [41–43]). In contrast, queens of both *A. mellifera* and *A. octospinosus* are highly polyandrous (100% of queens mating multiply in both species, with effective mating frequencies of 11.6 and 4.3, respectively [44–47]). This contrast suggests that kin-informative recognition cues may be more likely to occur in highly polyandrous taxa than those in which colonies are most commonly headed by a single singly mated queen.

There are two theoretical arguments that kin-informative recognition cues should be removed in social insects [5–7]. First, because the nepotism they could allow for would result in reduced colony efficiency, and the resulting indirect fitness costs outweigh those gained by nepotism. Second, because the occurrence of recognition errors incurs a fitness cost owing to inappropriately directed behaviour. However, the indirect fitness cost from nepotism, which involves only a minority of individuals, owing to kin-informative recognition cues being expressed by only a subset of patrilines in a genotypically diverse colony, will be relatively low. In addition, while nepotism based on a negative action (such as killing less-related individuals) would carry a clear and significant cost in the case of recognition errors, nepotism based on positive action (such as preferential feeding of more-related individuals) may potentially carry relatively little cost from recognition errors.

Our finding that cuticular substances are heritable and vary between patrilines within colonies of leaf-cutting ants shows conclusively that there is the potential for patriline recognition in social insects. It remains to be determined whether the information is actually used for kin recognition in leaf-cutting ants. However, the variation we found contradicts the generally accepted hypothesis that kin-informative recognition cues are lacking in polyandrous social insect colonies [5–7]. In concert with the recent findings of ‘royal patrilines’ in *Acromyrmex echinator* leaf-cutting ants [48], our results thus provide momentum for a reconsideration of the ultimate and proximate mechanisms for conflict resolution in polyandrous social insect societies.

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