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Characterization of Queen Supergene Pheromone in the Red Imported Fire Ant Using Worker Discrimination Assays

Haolin Zeng[1] · **Jocelyn G. Millar**[2] · **Li Chen**[3] · **Laurent Keller**[4] · **Kenneth G. Ross**[1]

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Abstract

Ants use chemical signals to communicate for various purposes related to colony function. Social organization in the red imported fire ant, *Solenopsis invicta*, is determined by the *Sb* supergene, with colonies of the monogyne (single-queen) form lacking the element and colonies of the polygyne (multiple-queen) form possessing it. Polygyne workers accept new reproductive queens in their nest, but only those carrying *Sb*; young winged queens lacking this genetic element are executed as they mature sexually in their natal nest or as they attempt to enter a foreign nest to initiate reproduction after mating and shedding their wings. It has been suggested that queen supergene genotype status is signaled to workers by unsaturated cuticular hydrocarbons, while queen reproductive status is signaled by piperidines (venom alkaloids). We used high-throughput behavioral assays to study worker acceptance of paper dummies dosed with fractions of extracts of polygyne queens, or blends of synthetic counterparts of queen cuticular compounds. We show that the queen supergene pheromone comprises a blend of monoene and diene unsaturated hydrocarbons. Our assays also reveal that unsaturated hydrocarbons elicit discrimination by polygyne workers only when associated with additional compounds that signal queen fertility. This synergistic effect was obtained with a polar fraction of queen extracts, but not by the piperidine alkaloids, suggesting that the chemical(s) indicating queen reproductive status are compounds more polar than cuticular hydrocarbons but are not the piperidine alkaloids. Our results advance understanding of the role of chemical signaling that is central to the regulation of social organization in an important invasive pest and model ant species.

Keywords *Solenosis invicta* · Supergene · Pheromone · Cuticular hydrocarbons · Piperidines · Unsaturated hydrocarbons

Introduction

Ants (Hymenoptera: Formicidae) are enormously successful animals in a variety of terrestrial ecosystems, judging from both the proportion of biomass they represent and their ecological diversity (Hölldobler and Wilson 1990). The ecological success of ants is directly linked to their social habits, with division of reproduction and task specialization among members of different castes being a hallmark of most species. Their often complex and highly interdependent social habits can yield numerous ecological advantages over solitary animals under particular environmental conditions, most notably high efficiency in carrying out potentially challenging or risky tasks such as brood care, resource acquisition, and nest defense (Hölldobler and Wilson 1990).

To coordinate activities and maintain the cohesiveness of the colony social unit, ants have evolved communication systems that ordinarily involve the production and perception of diverse chemical compounds (Hölldobler 1995). For instance, colony members have unique cuticular semiochemical profiles advertising their life stage, caste, and sex (Kocher and Grozinger 2011), which allow adult workers to distinguish among these groups and engage in relevant interactions with their nestmates. Additionally, workers utilize various volatile and non-volatile pheromones to communicate in real time the necessity to perform complex tasks such as exploiting ephemeral food resources, expelling would-be

✉ Haolin Zeng
haolinzeng@uga.edu

[1] Department of Entomology, University of Georgia, Athens, GA, USA

[2] Department of Entomology, University of California, Riverside, CA, USA

[3] School of Life Sciences, Institute of Life Science and Green Development, Hebei University, Baoding, Hebei, China

[4] Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland

intruders, or quickly retrieving their queens and brood when the nest is disturbed (Vander Meer and Alonso 1998).

In most ant species, a distinct morphological queen caste is present, and these individuals are the sole or primary reproductive females in a colony (Crozier and Pamilo 1996). They communicate their presence to workers via pheromones. Queen pheromones also influence caste differentiation of the female brood, suppress the reproductive development of other adult queens, and modulate various behaviors of workers that are involved in regulating colony queen numbers (Fletcher and Blum 1981, 1983; Keller and Nonacs 1993; Vargo and Laurel 1994; Kocher and Grozinger 2011). Thus, understanding the composition and functions of queen pheromones can provide crucial insights into the regulation of colony ontogeny and can inform hypotheses on the evolution of social organization.

The red imported fire ant, *Solenopsis invicta*, has been the subject of many studies of the characteristics and functions of putative pheromones produced by queens. Since its introduction into the United States almost a century ago, *S. invicta* has become widespread and well established throughout the American South and in other Pacific-rim countries (Ascunce et al. 2011; Wylie et al. 2020). This invasive pest species causes substantial economic and ecological damage linked to its enormous colony populations, massive nest structures, aggressive nature, and potent venom (Jemal and Hugh-Jones 1993; Tschinkel 2013). However, it also has become a model organism for studies of behavior, ecology, and social evolution, due in part to its abundance, ease of collection, and amenability to rearing in the laboratory, facilitated by a vast literature on its basic natural history and social biology.

Perhaps the most important biological feature of *S. invicta* contributing to its utility as a model organism is its polymorphism in colony social organization. This polymorphism is manifested as the co-occurrence of two distinct social forms. A colony may house a single reproductive queen (monogyne form) or multiple reproductive queens (polygyne form) (Fletcher et al. 1980; Huang and Wang 2014). Polygyne behavior is directly correlated with the presence of an inversion-based complex of tightly linked genes spanning half of chromosome 16, termed the *Social b* (*Sb*) supergene (Wang et al. 2013), and which is marked by the *b* allele of the gene *Gp-9* in U.S. populations (Ross 1997; Keller and Ross 1998; Krieger and Ross 2002). While the *Sb* supergene is present in all reproductive queens and generally more than half of the workers in polygyne colonies, it is absent among the inhabitants of monogyne colonies (Ross 1997; Ross and Keller 1998, 2002). This region of reduced recombination includes several hundred genes (Wang et al. 2013), many of which are thought to have essential functions in the synthesis and/or transport of cuticular semiochemicals, or their perception (Wurm et al. 2011). At the behavioral level, colony queen number and identity are regulated by the adult workers. Temporarily queenless monogyne workers (which lack the *Sb* supergene) will accept only a single replacement queen that also lacks *Sb,* whereas polygyne colonies, all of which contain workers with the *Sb* supergene, accept multiple queens, but only those that bear *Sb* (Keller and Ross 1998; Ross and Keller 1998). Such “worker *Sb* discrimination” is mediated by chemical signals present on the queen’s cuticle that presumably communicate the supergene status of individual queens (Keller and Ross 1998; Trible and Ross 2016).

To aid in identifying pheromones underlying worker *Sb* discrimination, Eliyahu et al. (2011) conducted detailed chemical analyses of whole-body extracts of fire ant females. They found that cuticular semiochemical profiles of *S. invicta* queens vary according to the social form, age, degree of sexual maturity (reproductive development), and supergene genotype of individuals. Significantly, the concentration of a particular group of abundant, queen-specific piperidine venom alkaloids (Vander Meer et al. 1980; Vargo 1999; Vargo and Hulsey 2000) was positively associated with reproductive maturity of queens regardless of their supergene genotype. One of these piperidines, *cis*-2-methyl-6-*n*-undecylpiperidine (solenopsin), is the most abundant chemical in extracts of sexually mature queens, with the level increasing as a queen becomes fully reproductive (Eliyahu et al. 2011), leading these authors to hypothesize that the compound conveys information on queen fertility status.

Eliyahu et al. (2011) further determined that unsaturated cuticular hydrocarbons (CHCs), which along with myriad other CHCs form a protective wax layer on the surface of the insect cuticle (Blomquist and Ginzel 2021), differ in their abundance in queens with or without the *Sb* supergene. Young winged queens bearing the supergene produced increasing levels of unsaturated CHCs as they matured sexually in their natal nests. The highest amounts were produced by the fully reproductive queens carrying the *Sb* supergene while queens lacking the *Sb* supergene failed to produce appreciable amounts of unsaturated CHCs, no matter what their age or stage of reproductive development. Importantly, parallel time courses in the reproductive ontogeny of queens, in their dynamic CHC profiles, and in escalation of polygyne worker aggression toward queens lacking *Sb* as they mature sexually led Eliyahu et al. (2011) to hypothesize that unsaturated CHCs convey information as to whether or not a queen bears the supergene.

Trible and Ross (2016) subsequently took an important step in investigating the queen pheromone that elicits worker *Sb* discrimination after developing high-throughput worker behavioral assays. They confirmed the presence of a supergene-marking pheromone by showing that cuticular extracts of queens, applied to paper dummies, induced appropriate worker responses of acceptance or rejection as effectively as live queens or fresh queen corpses. We here extend the work of Trible and Ross (2016) by incorporating improved assay procedures and scoring metrics that capture the various behavioral elements that collectively emerge as the process of queen acceptance by workers, and by testing various constituents of queen cuticular extracts. Our goal was to better characterize the critical components of queen cuticular pheromones that elicit the worker responses central to regulating colony social organization in *S. invicta*.

Methods and Materials

Ant Collection and Rearing

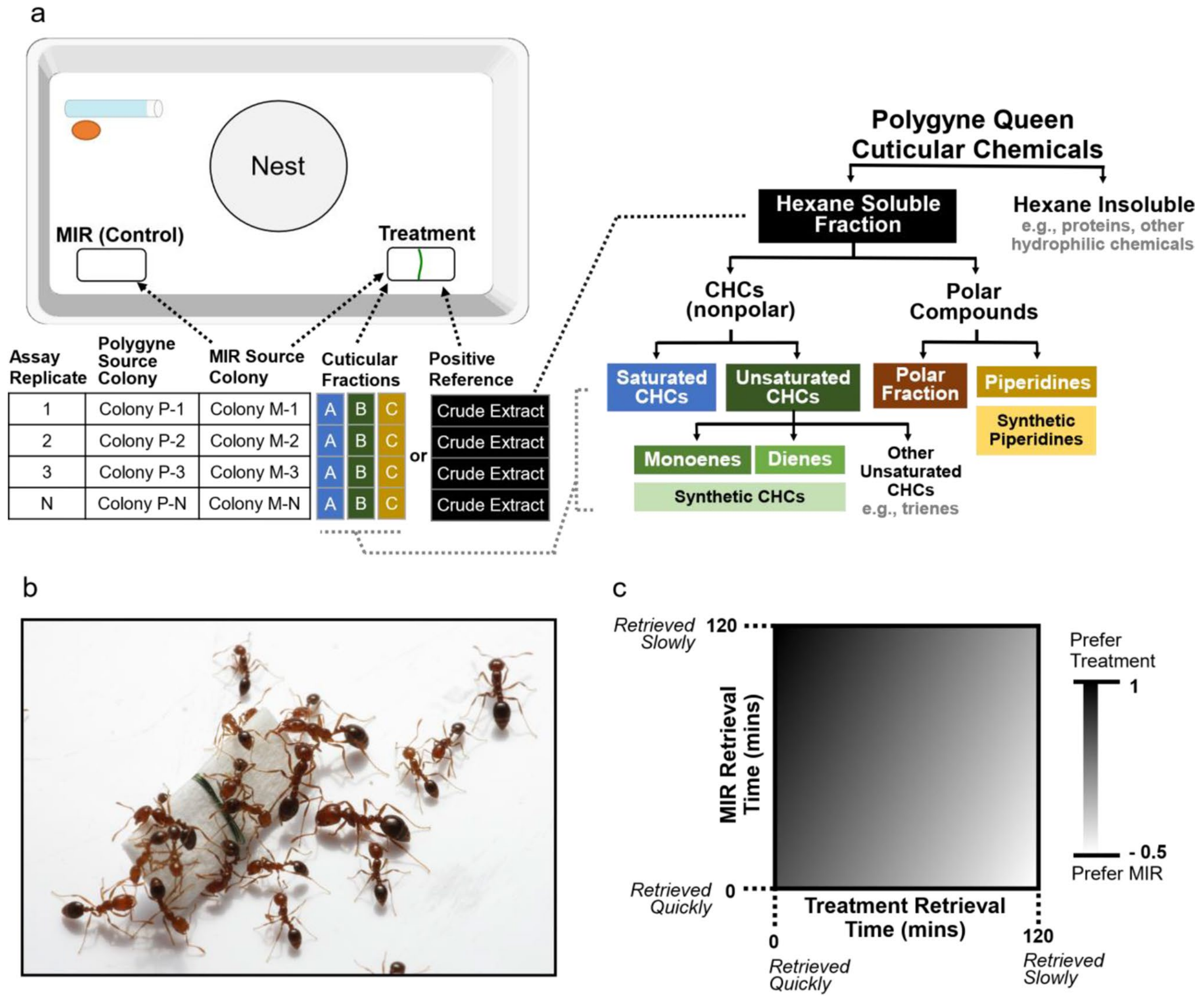
Colonies of *S. invicta* were collected in Athens-Clarke, Oconee, and Oglethorpe Counties, Georgia, U.S., from 2016 to 2020. Soil mounds separated by at least 10 m from each other were excavated into 5-gallon talced buckets, then returned to the laboratory (Banks et al. 1981). After removal from the soil by means of a water dripping method, colonies were maintained in large plastic trays, the sides of which were treated with Fluon$^{®}$ anti-traction compound, in a rearing room under standard conditions; all colonies and assay fragments derived from them were provided with nests (plastic Petri dishes with plaster bottoms; Ross 1988) and tubes of water, and they were fed every other day with a diet including freshly frozen or re-hydrated freeze-dried insects as well as high-sugar and high-protein artificial diets (Ross 1988; Ross and Keller 2002; Eliyahu et al. 2011). The social form of each colony was determined by carefully searching for reproductive queens. These determinations were confirmed by checking for the presence of *Sb* supergene genotypes diagnostic for the polygyne form; pooled DNA extracts of 10 – 20 workers per colony were used as template in a modified multiplex PCR procedure for scoring genotypes at the marker gene *Gp-9* (Valles and Porter 2003; Ross and Shoemaker, 2018).

Cuticular Extract from Monogyne Incipient-Reproductive Queens

To obtain the chemical extracts that provide workers with the information that the paper dummies (below) were reproductively active queens, we first removed the single reproductive queens (wingless queens with developed ovaries) from monogyne colonies also containing young pre-reproductive winged queens. Such removal induces the latter queens in the colony to shed their wings in a few days and initiate reproduction themselves by laying unfertilized eggs (Vargo 1992; Vargo and Laurel 1994; Mir et al. 2003). The cuticular extract from these wingless monogyne incipient-reproductive (MIR) queens contains a chemical signal of queen reproductive activity that is attractive to queenless workers of either social form. We examined the queenless monogyne colonies daily after removing the original queen and collected healthy MIR queens that were not being attacked by workers. The MIR queens were then stored immediately in a -80 °C freezer pending their use in an assay. MIR extract was obtained by placing three MIR queen corpses in 250 µl of hexane in 12 × 75 mm glass culture tubes. The tubes were then placed on an orbital shaker for 20 min. The queen corpses were removed, a paper dummy was placed in the MIR queen extract, and the dummy was dried down under a fume hood for ≥ 4 h, until there were no visible traces of hexane. The three-queen-equivalent (3QE) dosage in hexane extract added to each dummy corresponds to the dosage of treatment compounds used in all of the experiments described below. Our preliminary experiments suggested that 3QE was the optimal dosage for obtaining unambiguous, repeatable results across experiments.

Cuticular Extract and Extract Fractions from Polygyne Queens

Reproductive queens from polygyne colonies collected in Georgia were shipped on Dry Ice to The University of California, Riverside, where the chemical extraction and fractionation procedures were performed. Samples were thawed, then soaked in hexane for 10 min, yielding crude cuticular extract. Part of this crude extract was set aside to be used in Positive Reference assays (below), while the remainder was fractionated in multiple steps (see Figs. 1a and 2). The fractionation steps are described in detail in the Supplementary Online Information. Briefly, the crude extracts were first fractionated into nonpolar (saturated and unsaturated hydrocarbons) and polar compounds by liquid chromatography on silica gel and/or silica gel impregnated with 10% silver nitrate. The unsaturated CHC fraction was then further fractionated into monoenes, dienes, and trienes using a silver ion-loaded ion exchange column. The polar compounds were fractionated into piperidines and other polar compounds; henceforth, we refer to the latter fraction generically as the "polar fraction". Initial attempts to separate the piperidines as a class had variable outcomes; in some cases, elution of silica gel columns successively with ether and ethyl acetate removed the piperidines with other polar compounds, whereas in other cases, it was necessary to further elute the column with 4% Et_3N in ether to recover the piperidine fraction. Because of these inconsistencies, we developed an alternative fractionation method by first extracting the crude hexane extract with aqueous acid to remove the piperidines as their salts. The resulting aqueous solution was then made basic with NaOH, and the piperidines were back extracted into ether as their neutral forms. The remainder of the hexane extract was then fractionated



Assay Replicate	Polygyne Source Colony	MIR Source Colony	Cuticular Fractions	Positive Reference
1	Colony P-1	Colony M-1	A B C	Crude Extract
2	Colony P-2	Colony M-2	A B C	Crude Extract
3	Colony P-3	Colony M-3	A B C	Crude Extract
N	Colony P-N	Colony M-N	A B C	Crude Extract

Fig. 1 **a** Left half depicts the parallel assay design showing a cohort of dummy-assays comprising a single experimental series. Each polygyne colony was paired with a specific monogyne colony that was the source of the MIR queen extract. P = polygyne, M = monogyne, N = number of replicate assays. On the right is a diagram of queen cuticular chemical components. Colored text boxes indicate fractions or synthetic compounds used in behavioral assays. **b** Polygyne workers retrieving to the nest a paper dummy dosed with crude polygyne queen extract. The dummy typically would be retained in the nest for up to 8 h or longer. **c** Relationship of the times of retrieval of treatment and MIR dummies (or corpses) to the calculated "Initial Preference" measure for a single assay replicate. High Initial Preference measures result from rapid retrieval of the treatment dummy to the nest relative to the retrieval time for the MIR dummy (or corpses)

into saturated and unsaturated hydrocarbons and more polar compounds as described above. Each treatment chemical fraction was concentrated to 25 µl per 3 QE and stored in a -80 °C freezer before application to dummies or queen corpses.

Synthetic Cuticular Compounds

We synthesized eleven unsaturated CHCs present in reproductive polygyne queen cuticular profiles but effectively absent from those of reproductive monogyne queens, making them candidate components of a signal of supergene status (Eliyahu et al. 2011). Seven monoenes and four dienes were synthesized (Table 1). These CHCs were constituted into two solutions, in ratios matching those found in the queen crude extract cuticular profile. The first solution contained the five most abundant compounds, while the second contained all eleven. We also synthesized the most abundant piperidine, *cis*-2-methyl-6-undecylpiperidine (solenopsin A) which is a prominent component of the cuticular chemical profiles of all reproductive *S. invicta* queens but is effectively absent from pre-reproductive queens (and workers). According to Eliyahu et al. (2011), these piperidines may signal queen caste or fertility status.

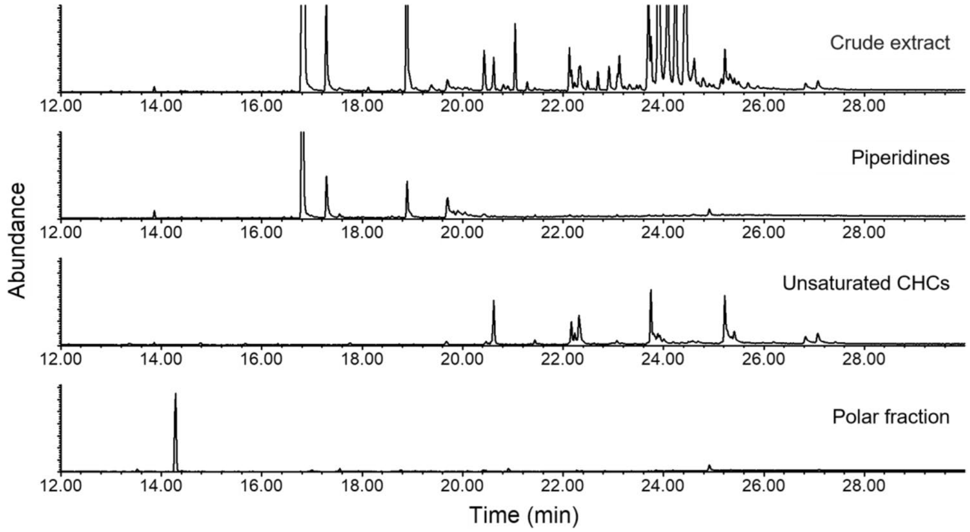


Fig. 2 Stacked plot of GC–MS traces of crude polygyne reproductive queen extract and of select fractions of this extract. All fractions were injected at the same concentration (in queen equivalents). The single large peak in the polar fraction is a contaminant from the solvent. The large peaks visible in the crude extract that are not present in any of the fractions are from the saturated hydrocarbons

Table 1 Synthetic unsaturated CHCs tested in dummy assays. The amount of a specific compound in each blend is in nanograms per three queen-equivalents, with the percentage of the total in parentheses

Compound Name	Compound Abbreviation	Compound Type	Amounts in Blend	
			Blend of Five CHCS	Blend of Eleven CHCS
(Z)-5-tricosene	5Z-C23	Monoene		25.5 (5%)
(Z)-5-pentacosene	5Z-C25	Monoene		19.8 (4%)
(Z)-9-pentacosene	9Z-C25	Monoene		18.3 (3%)
(Z)-9-heptacosene	9Z-C27	Monoene	73.2 (17%)	73.2 (14%)
(Z)-7-nonacosene	7Z-C29	Monoene		9.9 (2%)
(Z)-9-nonacosene	9Z-C29	Monoene	141.0 (32%)	141.0 (26%)
(Z)-9-hentriacontene	9Z-C31	Monoene	33.0 (8%)	33.0 (6%)
(6Z,9Z)-6,9-pentacosadiene	6Z,9Z-C25	Diene		16.2 (3%)
(6Z,9Z)-6,9-heptacosadiene	6Z,9Z-C27	Diene		11.7 (2%)
(6Z,9Z)-6,9-nonacosadiene	6Z,9Z-C29	Diene	42.5 (10%)	42.6 (8%)
(6Z,9Z)-6,9-hentriacontadiene	6Z,9Z-C31	Diene	147.0 (34%)	147.0 (27%)

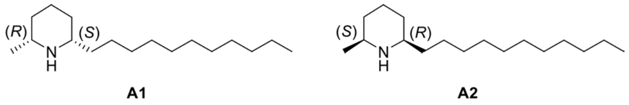


Fig. 3 Structures of the two enantiomers of 2-methyl-6-undecylpiperidine (solenopsin A). A1: (2*R*,6*S*)-2-methyl-6-undecylpiperidine; A2: (2*S*,6*R*)-2-methyl-6-undecylpiperidine

The (2*R*,6*S*)-2-methyl-6-undecylpiperidine and (2*S*,6*R*)-2-methyl-6-undecylpiperidine enantiomers (Fig. 3) were both synthesized. The two enantiomers may be required in a specific ratio to be functional, so we first tested solutions of the individual enantiomers, then a 1:1 racemic blend. The detailed methods used in estimating the relative natural abundance of each CHC on the queen cuticle, the approximate absolute amounts for dosage calculations, and the procedures for synthesizing the CHCs and piperidines are described in the online Supplementary Information.

Behavioral Assay Setup and Procedure

Our general assay methods were developed based on the findings of Trible and Ross (2016) that polygyne queen crude cuticular extracts contain chemicals comprising a putative pheromone (or blend of pheromones) that mediates worker *Sb* discrimination behavior. We tested specific fractions of crude hexane extracts of polygyne queens, with the aim of identifying the key fractions or even individual chemicals that are responsible for this specific pheromonal effect.

Each assay unit was set up by using a plastic spoon to transfer 3 g of adult polygyne workers (about 5000 ants) from the source colony into a clean plastic tray (40 × 25 x 5 cm) coated with Fluon® on the inside walls (Trible and Ross, 2016; Fig. 1a). A temporary nest, a water tube, and food (a mix of peanut butter and baby food) were provided to each unit. The workers were held in the tray for 24 h to make them more responsive to queen signals by virtue of having been queenless for a short period. The unit was then presented with two paper dummies placed equidistant from the nest on the floor of the tray. The paper dummies were 4 × 10 × 2 mm pieces of Ahlstrom Grade 470 chromatography blotting paper (Schleicher & Schuell CSS-470c) marked with a few loops of thin colored copper wire assigned randomly for identification. The size and weight of these paper dummies approximate those of a live *S. invicta* queen. For each replicate, one of the dummies was dosed with MIR reproductive queen extract, acting to control for the baseline attractiveness due to the queen caste (fertility) signal (Fig. 1a). The second, treatment dummy was dosed with a cuticular fraction or reconstructed blend, usually in addition to the MIR extract. Both dummies were dried down before being used in assays. If a treatment contains an appropriate mix of essential queen supergene pheromone components (presented at biologically appropriate relative abundances and dosages), assay workers are expected to display *Sb* discrimination by showing a significant preference for the treatment dummy over the MIR dummy. Such preference was evaluated utilizing a newly developed score metric, explained in detail in the next section.

For each experimental series, we first conducted "Positive Reference" assays for which dummies were dosed with crude hexane extract of polygyne reproductive queens (see movie in the Supplementary Online Information); this was done to obtain a baseline distribution of scores that reflected representative worker *Sb* discrimination behavior and to which treatment score values in the series were compared (Fig. 4, Ref). In this way, we accounted for variation in uncontrolled factors that might influence worker responses, including the colony of origin of MIR queens, the test colonies used, the time of year, and the length of time the colonies had been in culture.

We conducted the assays for each experimental series by dosing treatment dummies with designated cuticular fractions, solutions of synthetic compounds, or combinations thereof (Fig. 4, treatments A to O). We assessed the effectiveness of a treatment by comparing the scores for assays using that treatment with those of the matching Positive Reference assays. A treatment was considered effective if the treatment assay scores and the matched Positive Reference assay scores did not deviate significantly from one another, using paired Wilcoxon tests ($P > 0.05$). Particular treatment groups and their corresponding Positive Reference assay group were from the same parallel set of colonies—in other words, a specific source polygyne colony for the assay workers was always paired with a specific source colony for the MIR queen extract (Fig. 1a). This experimental design holds constant among-treatment variation arising due to interactions between specific polygyne source colony-MIR source colony pairs. All statistics were calculated using R 3.4.4 and RStudio 1.1.463 (RStudio Team, 2016; R Core Team, 2017).

Score Metric

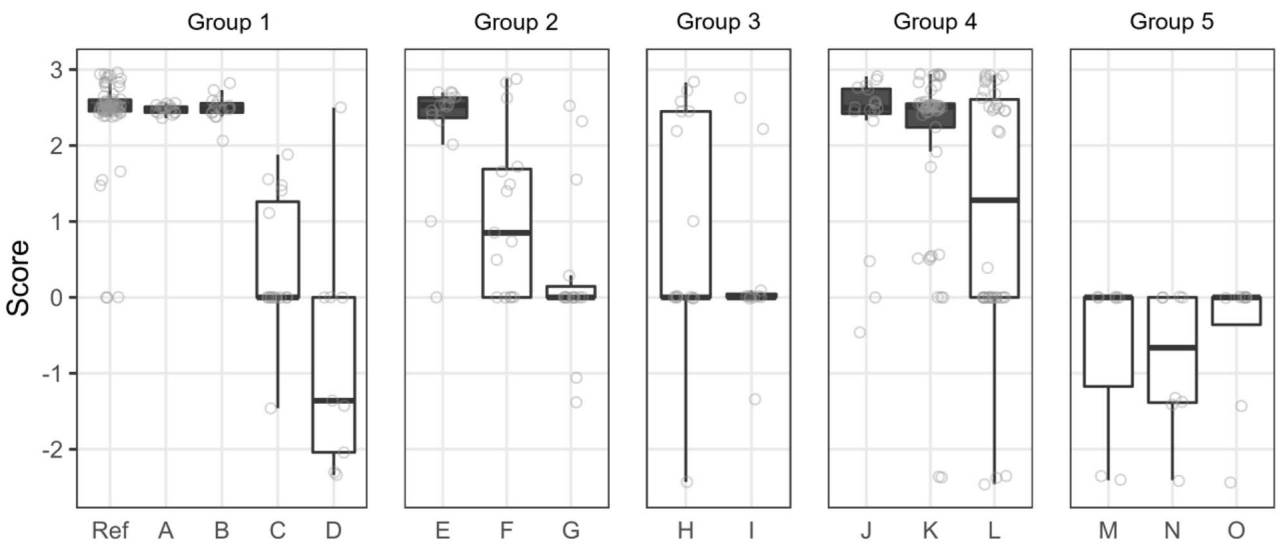
We developed a novel score metric to measure the degree of preference of workers towards the treatment dummy over the MIR dummy (or queen corpses used in another set of assays), if any, in each assay replicate. This metric was based on earlier experiments (Trible and Ross 2016) combined with extensive preliminary observations of how workers treated both fresh polygyne reproductive queen corpses and paper dummies dosed with crude queen cuticular extracts. The total score ranges from -2.5 to 3, with a higher positive value indicating a stronger preference for the treatment dummy and negative values indicating that the MIR dummy was preferred.

This total score is the sum of two measures, "Initial Preference" and "Retention." The Initial Preference measure assesses how quickly the dummies were retrieved into the nest (a behavior signaling acceptance of a queen or queen-like surrogate) (Fig. 1b; see Trible and Ross [2016]) as well as the relative difference in retrieval times between the treatment ($T_{treatment}$) and MIR (T_{MIR}) dummies (or queen corpses) over the initial 120 min of the assay period. The Initial Preference measure ranges from -0.5 to 1 and is calculated as:

$$[120 - (2 * \mathrm{T}_{treatment}) + \mathrm{T}_{MIR}]/240$$

If a dummy was not retrieved within the initial 120 min period, we set $T_X = 120$. The Initial Preference measure is highest if the treatment dummy is retrieved relatively quickly and the MIR dummy relatively slowly (Fig. 1c). As with the total score, a higher positive value indicates a stronger preference for the treatment dummy and a lower negative score indicates a stronger preference for the MIR dummy; scores around zero indicate no preference.

The Retention measure considers the positions of the two dummies in the assay unit (inside or outside the nest) at 2 h and at 8 h after their introduction. The Retention measure was + 1 if the treatment dummy was inside the nest. If neither dummy was inside the nest, the Retention measure was zero; if the MIR dummy was inside while the treatment dummy was outside, the Retention measure was -1. Therefore, the Retention measures, taken at 2 h as well as at 8 h, range totally from -2 to 2. After 8 h, the ants usually started to remove even highly preferred dummies from the nest, likely because the added chemicals degraded, volatilized fully, or were absorbed or adsorbed onto workers, so assays were terminated at this point. Similar to the Initial Preference measure, higher positive, zero, and lower negative Retention measures indicated a stronger tendency to



Group	Assay ID	Treatment Content	Sample Size	Mean Assay Score	*P* value – Wilcoxon	Effective
	Ref	Polygyne queen crude extract	66	2.42	NA	Yes
1	A	MIR ext. + Sat. CHCs + Unsat. CHCs	15	2.48	0.150	Yes
	B	MIR ext. + Unsat. CHCs	15	2.49	0.116	Yes
	C	MIR ext. + Sat. CHCs	15	0.40	<0.001	No
	D	Unsat. CHCs	15	-0.77	0.002	No
2	E	MIR ext. + Monoenes + Dienes	15	2.25	0.345	Yes
	F	MIR ext. + Monoenes	15	1.11	0.001	No
	G	MIR ext. +Dienes	15	0.28	<0.0001	No
3	H	MIR ext.+ 11 CHCs	15	0.92	0.003	No
	I	MIR ext. + 5 CHCs	11	0.33	0.003	No
4	J	Unsat. CHCs + Polar + Piperidines	16	2.12	0.589	Yes
	K	Unsat. CHCs + Polar	49	2.00	0.113	Yes
	L	Unsat. CHCs + Piperidines	38	1.14	0.003	No
5	M	Unsat. CHCs + Solenopsin racemic	7	-0.68	0.018	No
	N	Unsat. CHCs + Solenopsin A1	8	-0.82	0.011	No
	O	Unsat. CHCs + Solenopsin A2	8	-0.49	0.004	No

Fig. 4 Boxplots showing distributions of scores for experiments in five dummy-assay treatment groups. Whiskers show the upper and lower quartiles, horizontal lines the median values, and small circles the individual score values. Filled boxes indicate that a treatment was effective in eliciting worker *Sb* discrimination. Group 1 tests different fractions of CHCs; group 2 tests subfractions of unsaturated CHCs; group 3 tests synthetic CHCs; group 4 tests different polar molecule fractions (as queen fertility signals); group 5 tests synthetic piperidines. Sample size is the number of replicate assays conducted for each treatment

retain the treatment dummy, no preference, or a stronger tendency to retain the MIR dummy, respectively.

Assays of Queen Corpses Adulterated with CHC components

We hypothesized that if the unsaturated CHCs occurring on polygyne queens represent the *Sb* supergene signal, then the addition of these chemicals directly onto queens lacking *Sb* would convert their social phenotype, in the sense that workers would treat them as if they possessed the supergene. Applying unsaturated CHCs in hexane solution directly onto live queens might stress or even kill them, so we tested this hypothesis using queen corpses. We used the standard dummy assay procedures but replaced paper dummies with freshly frozen MIR queen corpses, distinguished by colored copper wires (randomly assigned) tied around their petioles. The procedures for these assays were identical to those for the assays with filter paper dummies (3 QE dosages of treatment chemical in 25 µl hexane), except that doped monogyne queen corpses rather than dummies constituted the test objects. The first two groups of corpse assays paralleled the "treatment vs control/MIR" design (Fig. 5, Assays Co-A and Co-B), while the third group directly compared unsaturated

Assay	Control	Treatment	N
■ Co-A	MIR corpse	MIR corpse + **unsaturated CHCs**	16
▨ Co-B	MIR corpse	MIR corpse + **saturated CHCs**	16
□ Co-C	MIR corpse + **saturated CHCs**	MIR corpse + **unsaturated CHCs**	18

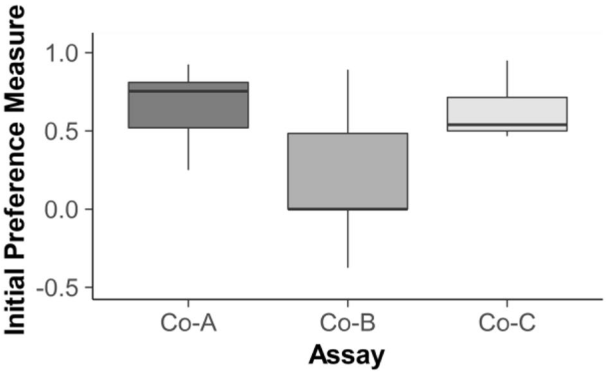


Fig. 5 Extracts applied to control and treatment queen corpses used in three different assays to test the effectiveness of unsaturated CHCs as signals conveying *Sb* supergene presence in queens (top). Boxplots (bottom) show the distributions of Initial Preference scores from these assays

CHCs (as treatment) and saturated CHCs (as control) to control for the effect of adding any hydrocarbons (Assay Co–C).

Results

Validation of Standard (Dummy) Assay and Score Metric

Our assay accurately recapitulated typical worker responses towards live polygyne queens and live MIR queens: scores for workers given dummies treated with queen extract (Positive Reference assays, mean score 2.42) were statistically indistinguishable from scores for workers given live queens (mean score 2.59, $N = 13$, *Mann–Whitney test*, $P = 0.912$).

Queen *Sb* Supergene Signaled by Unsaturated CHCs

Our first set of experiments (Fig. 4, group 1) aimed to test the role of cuticular hydrocarbons in queen *Sb* signaling. The first experiment (Fig. 4, treatment A) revealed that the combination of MIR extract and cuticular hydrocarbons (alkanes + alkenes) elicited responses from workers towards treated dummies that were as strong as the responses to dummies treated with crude hexane extract of polygyne reproductive queens (positive reference) (*Wilcoxon test*, $P = 0.15$).

We next separated saturated from unsaturated CHCs to test their relative efficacy. These experiments revealed that the combination of MIR extract and unsaturated CHCs from polygyne queens (Fig. 4, treatment B) elicited a full response, with no significant difference from the positive reference (*Wilcoxon test*, $P = 0.116$). In contrast, the MIR extract + saturated CHCs did not elicit retrieval of treated dummies, with a response significantly lower than the positive control (Fig. 4, treatment C, *Wilcoxon test*, $P < 0.001$).

When dummies were coated with unsaturated CHCs but no MIR extract there again was no discrimination response by workers (Fig. 4, treatment D, *Wilcoxon test*, $P < 0.005$), indicating that the MIR extract is necessary to elicit such responses. These data show that the MIR extract includes some signal of queen fertility or caste, which evidently fools workers into interacting with the dummies as if they were real queens; as such, it is required as a synergist of the unsaturated CHCs in signaling the presence of the *Sb* haplotype. Without it, workers simply disregard CHC-treated dummies.

Queen *Sb* Supergene Signal Requires both Monoenes and Dienes

To narrow the set of candidate chemicals comprising the queen supergene pheromone, we further fractionated the unsaturated CHCs into monoenes (one double bond) and dienes (two double bonds) for separate testing (Fig. 4, group 2). The combined application of MIR extract + monoenes + dienes from polygyne queens resulted in effective retrieval and retention behavior by the workers (Fig. 4, treatment E, *Wilcoxon test*, $P = 0.345$). In contrast, MIR extract + polygyne queen monoenes (Fig. 4, treatment F, *Wilcoxon test*, $P < 0.01$) or dienes (Fig. 4, treatment G, *Wilcoxon test*, $P < 0.001$) were not effective, indicating that both monoenes and dienes must be present to elicit full *Sb* discrimination behavior.

Queen *Sb* Supergene Signal Not Recreated Using Blends of Synthetic CHCs

We next generated synthetic blends of the most abundant unsaturated CHCs to test whether we could elicit worker *Sb* discrimination similar to that triggered by the natural fraction (Fig. 4, group 3). We constructed synthetic blends of unsaturated CHCs comprising either the five or the eleven most abundant unsaturated CHC compounds in proportions and concentrations matching those in the natural blend on a reproductive polygyne queen's cuticle (Table 1). Neither reconstructed blend succeeded in reproducing the effect of the dummies treated with crude hexane extract of polygyne reproductive queens when applied on an MIR background (Fig. 4, treatments H, I, *Wilcoxon tests*, all $P < 0.005$), suggesting that additional components may be required.

Queen Caste Identity/Fertility Signaled by Non-Piperidine Polar Compounds

Because previous work suggested that *cis*-dialkylpiperidines, which are abundant in extracts of reproductive queens, could be the substances signaling queen fecundity (Eliyahu et al., 2011), we investigated whether piperidines had a similar synergistic effect to the MIR extracts when used together with the unsaturated CHC compounds. In these experiments (Fig. 4, group 4) we also investigated the role of the non-piperidine polar fraction by conducting three separate experiments where unsaturated CHC compounds were combined either with the piperidines + polar fraction (treatment J), the polar fraction without the piperidines (treatment K), or the piperidines without the polar fraction (treatment L). These experiments revealed that the presence of the polar fraction with unsaturated CHCs elicited a response of workers towards dummies that was as strong as the response to dummies coated with crude hexane extract of polygyne reproductive queens (the positive reference) (treatments J and K, *Wilcoxon tests*, $P = 0.589$ and 0.113).

On the other hand, the piperidines apparently do not play a functional role in the pheromonal blend because the pairing of piperidines with unsaturated CHCs was much less effective than the Positive Reference (treatment L, *Wilcoxon tests*, $P = 0.003$). Moreover, there was no significant difference in scores when piperidines were added or not to extracts with the non-piperidine polar fraction + unsaturated CHCs (scores of treatment J vs. K, *Mann–Whitney test*, $P = 0.492$). Additionally, tests of the effects of synthetic versions of the racemic and enantiomeric forms of the major piperidine in extracts of reproductive queens (*cis*-2-methyl-6-undecylpiperidine, solenopsin A) validated that they were not effective at eliciting retrieval behaviors when combined with the natural unsaturated CHC fraction (Fig. 4, group 5, treatment M, N, O, *Wilcoxon tests*, all $P < 0.02$).

Queen Social Phenotype Converted by Applying Unsaturated CHCs

In a final experiment, we conducted tests with queen corpses to investigate whether we could also obtain full *Sb* discrimination responses from workers by the addition of the polygyne queen unsaturated CHC fraction to freeze-killed monogyne queens. Workers retrieved monogyne queen corpses treated with unsaturated CHCs significantly more often (94.1% of the time) than untreated corpses (41.9%, *Chi-square tests*, $P < 0.001$) or corpses treated with saturated CHCs (50%, Chi-square tests, $P < 0.001$). Also, there was no significant difference in retrieval proportions between untreated corpses and corpses treated with polygyne queen saturated CHCs (*Chi-square tests*, $P = 0.515$), further supporting the view that saturated CHCs play no role in worker *Sb* discrimination. Similarly, Initial Preference measures revealed that workers retrieved corpses treated with unsaturated CHCs significantly more quickly than untreated corpses (Fig. 5, Treatment Co-A; *Wilcoxon test*, $N = 16$, $P = 0.003$). In contrast, there was no significant difference between corpses treated with saturated CHCs and untreated corpses (Fig. 5, Treatment Co-B; *Wilcoxon test*, $N = 16$, $P > 0.05$). Accordingly, workers also retrieved corpses treated with unsaturated CHCs significantly more quickly than corpses treated with saturated CHCs (Fig. 5, Treatment Co–C; *Wilcoxon test*, $N = 18$, $P < 0.001$).

Discussion

The presence of the *Sb* supergene in mature/reproductive polygyne queens was previously hypothesized to be signaled by the presence of one or more of the unsaturated CHCs found on the cuticles of such queens but effectively absent from monogyne queens, which lack this genetic element (Eliyahu et al. 2011). We found that the *Sb* supergene signal indeed comprises unsaturated CHCs, based on a series of experiments progressively reducing the complexity of the CHC chemicals tested and, conversely, by combining relevant fractions to recover worker *Sb* discrimination. We further validated the effectiveness of unsaturated CHCs by applying them to monogyne queen corpses, which successfully converted their sociochemical phenotype from that of a queen lacking the supergene to that of one bearing it, as evidenced by the positive responses of polygyne workers to the treated corpses.

It is not unexpected that the *Sb* supergene signal resides in the CHCs; these molecules have the necessary properties to make them suitable to convey such information—they are collectively abundant and relatively stable on the cuticle, and are variable in their molecular structure and relative abundance (Blomquist and Ginzel 2021). Indeed, they are widely used as pheromones in ants and many other social insects (Gibbs and Pomonis 1995; Van Oystaeyen et al. 2014; Martin and Drijfhout 2009; Blomquist and Bagnères 2010). Additionally, we found that the unsaturated CHCs comprising the *Sb* signals likely consist of a complex blend that includes numerous different hydrocarbon molecules, because neither the monoene nor the diene fractions alone, nor our synthetic CHC blend composed of up to eleven CHC molecules, elicited the full effect of the natural pheromone.

Our findings help link previously reported genomic and gene expression data to fire ant social behaviors. Genes involved in both the production and, presumably, perception of CHCs differ in number and level of expression between the homologous regions of the alternate social chromosomes, Chr16 *Sb* and Chr16 *SB*. On the production side, Fontana et al. (2020) found duplications of genes likely involved in

CHC synthesis in the *Sb* supergene that do not occur in the homologous *SB* region of Chr16, including genes encoding fatty acid synthases, desaturases, elongases, reductases, and cytochrome P450s. The products of these genes are critically involved in various steps of the biosynthesis of unsaturated CHCs (Helmkampf et al. 2015; Holze et al. 2020). Some of the same genes likely involved in unsaturated CHC synthesis were found to be upregulated in *Sb* supergene-carrying (polygyne) queens compared to monogyne queens lacking the element (Nipitwattanaphon et al. 2013), as expected if unsaturated CHCs serve important roles as semiochemicals in the former.

On the perception side, many genes of the insect odorant-binding protein (OBP) family, some members of which have been shown to transport pheromones to odorant receptors in insect chemosensilla (Pelosi et al. 2018), have *Sb*-specific mutations or are duplicated in the *Sb* supergene, and some duplicates potentially have gained new functions via structural or regulatory diversification (Pracana et al. 2017; Dang et al. 2019). A classic example is the OBP gene *Gp-9*, commonly used to diagnose the presence of the *Sb* supergene because of the complete linkage disequilibrium between the two elements in the U.S. (Ross 1997). The *b* allele of *Gp-9*, which occurs in the *Sb* haplotype, has a considerable number of substitutional differences from the wild type *B* allele, which occurs in the *SB* haplotype, and several of these produce amino acid changes that may structurally change the ligand-binding cavity or C-terminal tail of the protein, possibly rendering a functional change to the protein that affects worker chemoperception (see also Crozier 2002; Krieger and Ross 2005; Gotzek and Ross 2007). Besides *Gp-9*, many other OBP genes are duplicated in the *Sb* supergene (Pracana et al. 2017). At the gene expression level, fourteen OBPs were reported to be differentially expressed between monogyne and polygyne workers (Pracana et al. 2017). Most recently, Dang et al. (2019) showed that an *Sb*-specific, duplicated OBP gene is expressed in the antennae of polygyne workers (as well as elsewhere in their bodies) and proposed that this unique OBP contributes to the detection of *Sb* signals. Arsenault et al. (2020) further showed that the expression of this OBP was 12-fold higher in *Sb*-carrying pre-reproductive queens than in other queens, pointing to the evolution of gene regulatory mechanisms acting specifically on OBP genes. Additionally, any differences in chemoreception function between *Sb* and non-*Sb* workers may be attributed to variation in their odorant receptors (OR); these are products of another large family of genes that interact with OBPs and their volatile ligands at the sensory periphery (Brand et al. 2018), and so are also critical elements in insect chemoreception (Yan et al. 2020). Cohanim et al. (2018) suggested that many OR genes in the *Sb* supergene were positively selected to evolve novel primary amino acid sequences compared to the wild-type variants and showed that two OR genes are completely deleted in the *Sb* supergene. Collectively, these genomic and transcriptomic data suggest that *Sb* supergene-carrying workers differ from non-*Sb* workers in their abilities to recognize the specific *Sb* signals we characterized, but neurophysiological and/or genetic editing/knockdown studies are needed to confirm these ideas (also Crozier 2002; Dang et al. 2019).

An important finding of our study was that, although unsaturated CHCs are necessary as *Sb* signals to induce worker *Sb* discrimination, they are not by themselves sufficient. Instead, they must be presented along with a reproductive queen caste (or fertility) signal, such as occurs in our crude MIR extract, in order to generate such worker discrimination (Fig. 4, treatment B vs D). Previously, a specific component of the piperidines, a class of compounds that are major components of fire ant queen venom, was hypothesized to constitute this queen signal, because it was absent from workers and its abundance in queens was associated with their reproductive state, regardless of supergene status (Eliyahu et al. 2011). However, our results do not support this hypothesis. When the piperidine fraction of polygyne queen extract was paired with unsaturated CHCs, the blend failed to produce full worker *Sb* discrimination behavior. Synthetic versions of the major queen piperidine also failed to produce such discrimination in several experiments (Fig. 4, treatments M, N, O), providing support for the initial finding, although factors such as inappropriate dosages or ratios may also have contributed to the failure of the synthetic versions. In contrast, our results indicate that these fertility signals reside in other non-hydrocarbon cuticular molecules in the polar fraction which, in combination with the unsaturated CHCs, elicited a full *Sb* discrimination response by workers. Potential queen caste signal candidates include fatty acids, esters, or other moderately polar compounds residing on the fire ant queen cuticle, although these molecules appear to be present at comparatively low levels (Fig. 2, bottom trace; Eliyahu et al. 2011).

This study helps lay the groundwork for future progress in identifying both the queen caste pheromone and the key molecules that signal queen *Sb* status in *S. invicta*, arguably two of the most fundamental semiochemical signals in the normal functioning of colonies of this ant. In particular, we have confirmed that compounds more polar than hydrocarbons comprise the former signal, and have confirmed that a blend of monoenes and dienes is involved in the latter signal. In so doing, we have advanced the goal of revealing the myriad complex links from genotype to collective social phenotype in an important social insect species. From an applied perspective, such progress may facilitate the future development of novel control strategies based on disruption of the chemical communication system that underpins worker regulation of colony queen number.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

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Code availability Not applicable.

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