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Chemical communication of queen supergene status in an ant

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Abstract

Traits of interest to evolutionary biologists often have complex genetic architectures, the nature of which can confound traditional experimental study at single levels of analysis. In the fire ant *Solenopsis invicta*, the presence of a Mendelian 'supergene' is both necessary and sufficient to induce a shift in a fundamental property of social organization, from single-queen (monogyne) to multiple-queen (polygyne) colonies. This selfish genetic element, termed the *Social b* (*Sb*) supergene, contains > 600 genes that collectively promote its fitness by inducing the characteristic polygyne syndrome, in part by causing polygyne workers to accept only queens bearing the *Sb* element (a behaviour termed 'worker *Sb* discrimination'). Here, we employ a newly developed behavioural assay to reveal that polygyne workers, many of which bear the *Sb* element, employ chemical cues on the cuticle of queens to achieve worker *Sb* discrimination, but we found no evidence for such pheromonally mediated worker *Sb* discrimination in monogyne workers, which universally lack the *Sb* element. This polygyne worker *Sb* discrimination was then verified through a 'green beard' effect previously described in this system. We thus have demonstrated that the *Sb* element is required both for production of relevant chemical cues of queens and for expression of the behaviours of workers that collectively result in worker *Sb* discrimination. This information fills a critical gap in the map between genotype and complex phenotype in *S. invicta* by restricting the search for candidate genes and molecules involved in producing this complex social trait to factors associated with the *Sb* element itself.

Introduction

A central focus of modern biology is to explain the genetic basis of evolutionarily significant phenotypic traits. Recent decades have seen great advances using model organisms, comparative genomics, and functional approaches, research that has clarified the genes and developmental/physiological pathways involved in producing important, but often simple, individual phenotypes (Carroll, 2005; Heffer & Pick, 2013). Yet, many phenotypes of interest arise from the action of multiple interacting genes, the effects of which cascade through multiple levels of organization and are dynamic throughout the ontogeny of an organism (Weiss, 2008; Rockman, 2012). These complex phenotypes often are less amenable to the tools of molecular, developmental and functional biology, and consequently are less well characterized than simpler traits, despite their frequent relevance to individual fitness (Weiss, 2008; Rockman, 2012; Travisano & Shaw, 2012).

Eusocial insects — for example, ants, termites and some wasps and bees — display an added level of complexity above the individual organism, providing a further challenge to the mapping of genotype to complex phenotype (Robinson, 1999; Linksvayer, 2015). Central to this challenge is understanding how behavioural interactions of individual workers collectively give rise to distinctive colony-level phenotypes. In ants, worker behaviours regulating higher-level phenotypes such as colony social organization typically are mediated by chemical cues issuing from nestmates or other conspecifics (pheromones) (Hannonen *et al.*, 2002; Richard & Hunt, 2013). Thus, genotype/phenotype mapping of colony-level, superorganismal traits in social insects

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requires investigations ranging from identifying the genetic underpinnings of chemical cues, to cataloging the individual behaviours mediated by these cues, to documenting the collective colony phenotypes resulting from these behaviours (Robinson, 1999; Linksvayer, 2015).

A colony phenotype of major importance in social insects is variation in the number of reproductive females (queens) in the colony (Holldobler & Wilson, 1990; Keller, 1995). In ants, an apparently ancestral 'monogyne' colony social organization, in which a single queen is the reproductive unit of the colony, has given way in many clades to 'polygyne' organization, which features multiple reproductive nestmate queens (Hughes *et al.*, 2008). The fire ant *Solenopsis invicta* displays particularly interesting variation in this regard because polygyne and monogyne social forms exist as interbreeding populations (Tschinkel, 2006; Gotzek & Ross, 2007). Associated with the fundamental difference in colony queen number, the two social forms of *S. invicta* differ also in a number of other important individual and higher-level traits such as mode of colony reproduction, queen longevity and fecundity, and population density, so that a relatively simple difference in number of colony reproductives corresponds with strikingly different life history syndromes within the species (Ross & Keller, 1995; DeHeer *et al.*, 1999; Tschinkel, 2006; Gotzek & Ross, 2007; Lawson *et al.*, 2012).

Remarkably, variation in colony queen number in *S. invicta* is perfectly associated with variation at a single gene termed *Gp-9* (Ross & Keller, 1998; Keller & Ross, 1999; Krieger & Ross, 2002). Two major alleles, *B* and *b*, occur at this gene in invasive populations such as those in the USA, with reproductive queens in polygyne colonies universally bearing the *b* allele (in heterozygous condition; *bb* queens are largely inviable) and queens in monogyne colonies universally lacking it (Ross, 1997; Ross & Keller, 1998; Gotzek & Ross, 2007). Most importantly, the presence of the *b* allele among even a relatively low proportion of colony workers is perfectly associated with expression of polygyne social organization (Ross & Keller, 2002; Gotzek & Ross, 2007). Recently, it was found that the *b* allele occurs within a large chromosomal segment, termed the *Social b* (*Sb*) supergene, that does not undergo recombination with the homologous *SB* region (Wang *et al.*, 2013). The *Gp-9 b* allele thus is inherited with ~ 600 other protein-coding genes as a single Mendelian element, suggesting that many genes associated with it in the supergene are responsible for the wide range of phenotypic differences observed between the social forms (Wang *et al.*, 2013).

The foundation of colony social organization in *S. invicta* is worker regulation of colony queen identity and number (Ross & Keller, 1998; Gotzek & Ross, 2007). The single reproductive queen, as well as all workers, in monogyne colonies lacks the *Sb* supergene, and supernumerary reproductive queens are not tolerated by these workers (Ross & Keller, 1998). However, if even a small proportion of workers bear the *Sb* supergene, a colony assumes the polygyne phenotype, killing queens that lack the supergene and accepting multiple queens that bear it (Ross & Keller, 2002; Gotzek & Ross, 2008). Experiments have shown that queen age, fecundity and social form of origin do not alter exclusive polygyne colony tolerance of queens bearing *Sb* (Ross & Keller, 1998). Therefore, workers in polygyne colonies must employ some robust cue(s), likely chemical (Keller & Ross, 1998), that causes polygyne colonies to collectively accept only queens bearing the supergene—a behaviour we term 'worker *Sb* discrimination'. Understanding expression of social organization in *S. invicta* thus requires understanding what types of queen cues influence worker behaviour towards queens, and how this behaviour collectively produces worker *Sb* discrimination.

Potential queen cues may be found in three classes of cuticular chemicals that differ in terms of their presence or relative quantities between *SB*/*SB* and *SB*/*Sb* queens: piperidine alkaloids, cuticular hydrocarbons, and the GP-9 *B* and *b* allelic protein variants (Eliyahu *et al.*, 2011; D. Gotzek, M. Strand, & M. Beck, unpublished data). Different solvents are expected to extract these components with varying efficacy, forming the basis for experiments to parse the roles of these potential semiochemicals in worker *Sb* discrimination behaviour.

A remarkable behavioural mechanism that may underlie polygyne worker *Sb* discrimination is the 'green beard effect' previously described in this system, in which *Sb*-bearing workers are over-represented among the workers attacking live *SB*/*SB* queens introduced into polygyne colonies (Keller & Ross, 1998). This has been considered a green beard mode of facultative harming (Gardner & West, 2009), because a 'selfish' locus (the *Sb* element in this case) evidently promotes its own fitness both by producing detectable signals and by modifying behaviour towards other individuals bearing those same signals. If individual-level green beard behaviour leads to the group-level phenomenon of polygyne worker *Sb* discrimination, then monogyne colonies, which lack *Sb* and the green beard effect, should not exhibit any preference towards queens based on queen genotype. Thus, in addition to finding queen cues that trigger worker *Sb* discrimination, it is critical to test whether monogyne colonies exhibit worker *Sb* discrimination (e.g. Ross & Keller, 1998) to further elaborate hypotheses that explain how individual behaviours give rise to group-level worker *Sb* discrimination.

Along with the described differences in cuticular chemical profiles of *S. invicta* queens bearing or lacking *Sb*, gene variants and gene expression profiles associated with alternative social organization and/or *Sb*

status have been identified, and the manner by which individual worker behavioural responses to queens of different status collectively regulate social organization is well understood (Krieger & Ross, 2002; Gotzek & Ross, 2008; Wang *et al.*, 2008, 2013; Nipitwattanaphon *et al.*, 2013). What remains to be learned to help complete the map from genotype to complex social phenotype, and ultimately to understand the evolution of social polymorphism in fire ants, is what types of queen cues generate worker *Sb* discrimination and how worker responses may differ between the social forms. Here we employ a newly developed behavioural assay to address these issues. We conclusively demonstrate that worker *Sb* discrimination occurs in polygyne colonies in response to chemical cues associated with queen supergene status, but that such discrimination does not occur in monogyne colonies. We further verify that workers in polygyne colonies respond as expected given a green beard effect mediated via semiochemicals (Keller & Ross, 1998). Together, our results clarify the dual roles of the *Sb* element in determining colony social organization via alteration of queen recognition cues and worker tolerance behaviour, thus elucidating crucial links between genotype and an emergent complex phenotype in this ant model.

Materials and methods

General methods

Solenopsis invicta colonies of each social form were collected in northern Georgia, USA and maintained using standard laboratory procedures (Ross, 1988; Eliyahu *et al.*, 2011). Colony social form was determined by finding single or multiple reproductive queens, then confirmed by GP-9 protein electrophoresis (DeHeer *et al.*, 1999; Gotzek & Ross, 2009). Assay units were established by collecting ~ 5000 adult workers from the foraging areas of single laboratory-colony enclosures and placing them in small plastic rearing trays with a water source, food and a nest. Foraging workers were chosen because such workers presumably would be the first to encounter foreign queens attempting to infiltrate colonies in nature.

All queens used as test material in the assays were collected from laboratory colonies then immediately frozen at −80 °C. Three types of queens were used. Fully reproductive *SB/Sb* polygyne queens are wingless (dealate) queens that occur in multiples within polygyne colonies. Fully reproductive *SB/SB* queens are the single highly fecund dealate queens within monogyne colonies. Virgin incipient-reproductive *SB/SB* monogyne queens were obtained from recently orphaned monogyne colonies (1–5d); orphaning causes virgin winged queens to shed their wings and initiate ovarian development. *Gp-9* genotypes of all polygyne queens and reproductive monogyne queens tested in our assays were confirmed via protein electrophoresis. Homozygous *Sb/Sb* queens were not used because they are effectively nonviable in nature (Gotzek & Ross, 2007). See the Supporting Information for more details on general methods.

Experiments 1A and 1B: Worker *Sb* discrimination assays

Assay units were colony fragments composed of adult workers (foragers) from single polygyne or monogyne colonies, held queenless for 24–72 h before each assay to increase their susceptibility to accept foreign queens. In each worker *Sb* discrimination assay, we simultaneously presented both a polygyne and monogyne queen corpse or queen surrogate and measured relative worker preference for each corpse/surrogate. Queen corpses were employed in Experiment 1A to test whether queen behaviour is involved in eliciting worker *Sb* discrimination and to learn if colonies of both social forms exhibit such discrimination (see Table 1). Queen surrogates soaked in solvent extracts were employed in Experiment 1B to test whether worker *Sb* discrimination behaviour occurs in response to chemical cues from queens and, if so, whether it occurs in both forms and whether the various solvent extracts differ in their ability to elicit such responses (Table 1). Generally, half of the assays of each major type were conducted using polygyne assay units and half were conducted using monogyne units (Table 1).

We employed two alternative pairings of the queen corpses or surrogates presented simultaneously in each assay (polygyne *SB/Sb* paired with monogyne *SB/SB*). Type R/R (reproductive/reproductive) pairings employed corpses or surrogates of fully reproductive *SB/Sb* polygyne and fully reproductive *SB/SB* monogyne queens. Type R/i-R (reproductive/incipient-reproductive) pairings employed corpses or surrogates of fully reproductive *SB/Sb* polygyne queens and virgin incipient-reproductive *SB/SB* monogyne queens (Table 1). These alternative pairings were used to assess effects of differences between polygyne and monogyne reproductive queens that may be unrelated to supergene genotype—specifically, the much higher level of ovarian development and fecundity attained by reproductive monogyne (*SB/SB*) than by reproductive polygyne (*SB/Sb*) queens (Vargo & Fletcher, 1989). Use of both types of pairings means that *SB/Sb* queens were paired with *SB/SB* queens of both lesser and greater reproductive development.

We performed two separate worker *Sb* discrimination experiments: 65 Experiment 1A 'corpse' assays used queen corpses, whereas 324 Experiment 1B 'solvent' assays used methanol, hexane, chloroform, dichloromethane (DCM), hexane and DCM, or all four solvents combined to produce extracts (Table 1). The solvents used in the latter experiment were chosen because their differing solvent properties should yield extracts

Table 1 Overview of behavioral assays conducted to investigate queen chemical signalling of *Sb* supergene status to workers.

Experiment							
Name	Code	Purpose of experiment	Material introduced	Solvent used	Assay unit social form	Type of pairing of introduced material*	Number of biological replicates
Worker *Sb* discrimination assays	1A	Test whether chemical cues from queens or queen behaviors elicit worker *Sb* discrimination	Queen corpses	–	Polygyne	R/R	14
						R/i-R	19
				–	Monogyne	R/R	13
						R/i-R	19
	1B	Confirm that chemical cues from queens elicit worker *Sb* discrimination and test whether different solvents vary in effectiveness of extracting cues	Surrogates† with solvent extracts of queens	Methanol	Polygyne	R/R	14
						R/i-R	14
					Monogyne	R/R	14
						R/i-R	13
				Hexane	Polygyne	R/R	13
						R/i-R	13
					Monogyne	R/R	13
						R/i-R	13
				Chloroform	Polygyne	R/R	13
						R/i-R	14
					Monogyne	R/R	13
						R/i-R	15
				Dichloromethane (DCM)	Polygyne	R/R	13
						R/i-R	13
					Monogyne	R/R	13
						R/i-R	14
				Hexane + DCM	Polygyne	R/R	14
						R/i-R	13
					Monogyne	R/R	14
						R/i-R	12
				Methanol + Hexane + Chloroform + DCM	Polygyne	R/R	14
						R/i-R	14
					Monogyne	R/R	14
						R/i-R	14
Green beard assays	2	Test whether apparent polygyne worker *Sb* discrimination involves green beard effect	Surrogates† with solvent extracts of queens/heterospecific ant corpses	Methanol	Polygyne	Surrogates† with *SB/SB* queen extracts/ heterospecific ant	20
				Hexane	Polygyne	Surrogates† with *SB/SB* queen extracts/ heterospecific ant	20

*Type R/R pairings used fully reproductive *SB/Sb* polygyne paired with fully reproductive *SB/SB* monogyne queen corpses/surrogates while Type R/i-R pairings used fully reproductive *SB/Sb* polygyne paired with virgin incipient-reproductive *SB/SB* monogyne queen corpses/surrogates. Use of both types of pairings means that *SB/Sb* queens were paired with *SB/SB* queens of both lesser and greater reproductive development to control for the higher level of ovarian development and fecundity attained by reproductive monogyne (*SB/SB)* compared to reproductive polygyne (*SB/Sb*) queens.

†Surrogates were small paper slabs containing solvent extracts.

enriched for various cuticular chemicals that differ between *SB/Sb* and *SB/SB* queens (e.g. Eliyahu *et al.*, 2011). Piperidine alkaloids are expected to be highly soluble in polar aprotic solvents such as dichloromethane (DCM), cuticular hydrocarbons in nonpolar solvents such as hexane and chloroform, and the GP-9 proteins in polar protic solvents such as methanol. To test the extracted compounds, we used queen surrogates consisting of small paper slabs marked with fine coloured wire. Frozen queens were soaked in the chemical solvents, after which the queens were removed and the paper slab surrogate was placed in the solvent. After all solvent evaporated, slabs were held at −20 °C until used in an assay.

Assay units were observed over a 6-h period, and behavioural metrics were used to create an 'assay score' that quantifies the degree to which the colony fragment displayed tolerance or intolerance towards the paired introduced corpses/surrogates. Three behavioural metrics contributed to the score. *First in nest* was recorded for the first queen/surrogate, if either, that was moved into the nest within the first 30 min of the assay, with a score of −1 if polygyne and +1 if monogyne. *Final in nest* was recorded for any queen/surrogate that was

found in the nest at the end of the 6 h assay, with a score of −1 if polygyne and +1 if monogyne. *Worker response* was obtained by taking a photograph at the time the first queen/surrogate was moved by workers, then counting the number of dark pixels (represented only by ants) in an equally sized region surrounding the monogyne and polygyne queens/surrogates. *Worker response* values vary from −1 (if 100% of workers were in the polygyne queen image) to +1 (if 100% of workers were in the monogyne queen image), with a value of 0 if approximately equal numbers of workers were in the vicinity of each queen. These metrics were summed to produce a single score for each assay that quantifies the degree to which the colony fragment collectively favoured the *SB/Sb* or *SB/SB* queen corpse/surrogate. Assay scores range continuously from −3 (workers strongly favoured the *SB/Sb* queen corpse/surrogate) to +3 (workers strongly favoured the *SB/SB* queen corpse/surrogate). A score of 0 indicates no overall preference. One-sample Wilcoxon Signed Rank Tests were performed on final assay scores to learn whether polygyne and monogyne units preferred *SB/Sb* or *SB/SB* queens/surrogates. A Benjamini–Hochberg false discovery rate (FDR) control was used to correct for multiple comparisons. See the Supporting Information for more details on worker *Sb* discrimination assay methods.

Experiment 2: Green beard assays

In Experiment 2, green beard assays were conducted using single fully reproductive *SB/SB* monogyne queens extracted with methanol or hexane to produce surrogates introduced into polygyne assay units. Twenty independent assays were performed with each solvent. Previous studies showed that live *SB/SB* queens introduced into large polygyne colonies are immediately attacked and killed, and *Sb*-bearing workers evidently are disproportionately represented among the workers attacking such queens (Keller & Ross, 1998; polygyne colonies contain a mixture of workers with *SB/SB*, *SB/Sb*, and *Sb/Sb* genotypes). Thus, the *Sb* supergene presumably contains alleles that allow *Sb*-bearing workers to recognize queens bearing/lacking the element and that induce these workers to preferentially attack queens lacking *Sb*—a hypothesized green beard mode of facultative harming that favours persistence of the *Sb* element in the polygyne form despite its deleterious effects (Gotzek & Ross, 2007; Gardner & West, 2009; Lawson *et al.*, 2012). For our purposes, an assay based on the phenomenon would confirm queen pheromonal communication of supergene status if two conditions were met: (i) surrogates containing *SB/SB* queen extracts typically are rejected by polygyne colony fragments (demonstrated by the worker *Sb* discrimination assays, see below), and (ii) *Sb*-bearing workers are over-represented among workers aggressively confronting introduced *SB/SB* surrogates.

Green beard assay units were colony fragments composed of adult workers (foragers) from single polygyne colonies held queenless for 24 h (Table 1). For each assay, a reference sample of workers was collected from each colony fragment before the assay started ('R workers'). A single *SB/SB* reproductive queen surrogate and four heterospecific ant workers, *Camponotus* sp., were simultaneously introduced into the assay unit on opposite sides of the tray to start the assay; the alien *Camponotus* were introduced to control for the possibility that an apparent green beard effect could arise not by supergene-based recognition but simply by elevated levels of overall aggression in workers bearing the *Sb* supergene. All 'Q workers' grasping the introduced queen surrogate and 'Ali workers' grasping the introduced alien *Camponotus* ants were collected simultaneously once a sufficient number had accumulated. Supergene (*Gp-9*) genotypes of each collected worker were scored using a multiplex PCR assay. *Solenopsis invicta* workers from large colonies display continuous size variation (Tschinkel, 2006), and supergene genotype has been linked to worker size (Goodisman *et al.*, 1999, 2007; Buechel *et al.*, 2014). Therefore, a subset of workers from the methanol extract assays were classified as large or small on the basis of their individual weights in order to assess whether differences in size between Q and Ali workers may explain any differences between the groups in proportions of *Sb*-bearing workers.

We used a resampling procedure to obtain unbiased estimates of mean supergene (*Gp-9*) genotype proportions. Binomial probabilities were calculated to test whether the numbers of colony fragments with an excess of *Sb*-bearing workers in the Q group compared to the R group depart from the null expectation that half of the units show such excess. Jackknife analyses of supergene proportions were conducted for each worker group in each experiment by systematically omitting each unit's proportion of *Sb*-bearing workers in each of the 20 jackknife samples. The 95%, 99% and 99.9% confidence intervals around each jackknife mean estimate were constructed from the sampling variances (Weir, 1996). Bootstrap analyses were employed to test the null hypothesis that the differences in proportions of *Sb*-bearing workers between the Q or Ali groups and the R group from the same unit differed significantly from zero in each experiment. See the Supporting Information for more details on green beard assay methods and statistical analyses.

Results

Experiment 1A: Worker *Sb* discrimination assays using queen corpses

Our first set of assays using introduced queen corpses demonstrated strong worker *Sb* discrimination with Type R/R pairings (fully reproductive *SB/Sb* polygyne

and *SB/SB* monogyne queens): polygyne units primarily accepted polygyne *SB/Sb* reproductive queen corpses and monogyne units primarily accepted monogyne *SB/SB* reproductive queen corpses (Fig. 1). When Type R/i-R corpse pairings were used (fully reproductive *SB/Sb* polygyne queens and virgin incipient-reproductive *SB/SB* monogyne queens), strong worker *Sb* discrimination again was displayed by polygyne colony fragments, but monogyne fragments exhibited a relatively weaker and nonsignificant (after correction for multiple comparisons) preference for monogyne virgin incipient-reproductive *SB/SB* queen corpses over polygyne reproductive *SB/Sb* queen corpses (Fig. 1). The combined results from this first set of assays indicate that queen corpses invariably elicit worker *Sb* discrimination behaviour in polygyne colonies. On the other hand, the comparative lack of monogyne worker *Sb* discrimination with Type R/i-R pairings makes it impossible to rule out queen reproductive status rather than genotype as the most important determinant of worker preference for queens in this social form (because fully reproductive *SB/Sb* queen corpses were paired with incipient-reproductive *SB/SB* corpses). The worker *Sb* discrimination displayed by polygyne colonies in this experiment shows that behaviour of queens plays no necessary role in this socially important phenomenon and that chemical cues alone appear sufficient to elicit relevant worker behaviour.

Experiment 1B: Worker *Sb* discrimination assays using queen extracts on paper surrogates

We next conducted assays using paper surrogates containing solvent extracts of queens. Polygyne colony fragments displayed significant worker *Sb* discrimination when presented with Type R/R pairings across all four single-solvent and both multiple-solvent experiments (Fig. 2); this discrimination was especially strong with the methanol and hexane extracts. Assay units of this social form also displayed significant worker *Sb* discrimination with Type R/i-R pairings conducted with the methanol, hexane, chloroform and 4-solvent extracts, but not with the DCM or 2-solvent extracts (Fig. 2). These assays demonstrate that polygyne worker *Sb* discrimination is strongly manifested in response to chemicals that can be extracted from queens of dissimilar reproductive state using solvents with diverse solvent properties.

Monogyne fragments presented with Type R/R pairings also displayed significant apparent worker *Sb* discrimination across most solvents (Fig. 2). However, they displayed no such discrimination in any single- or multiple-solvent experiments with Type R/i-R pairings. This result, which parallels the results from the queen corpses, again makes it impossible to conclude that queen *Sb* supergene status, rather than reproductive status, was the main cause of monogyne worker

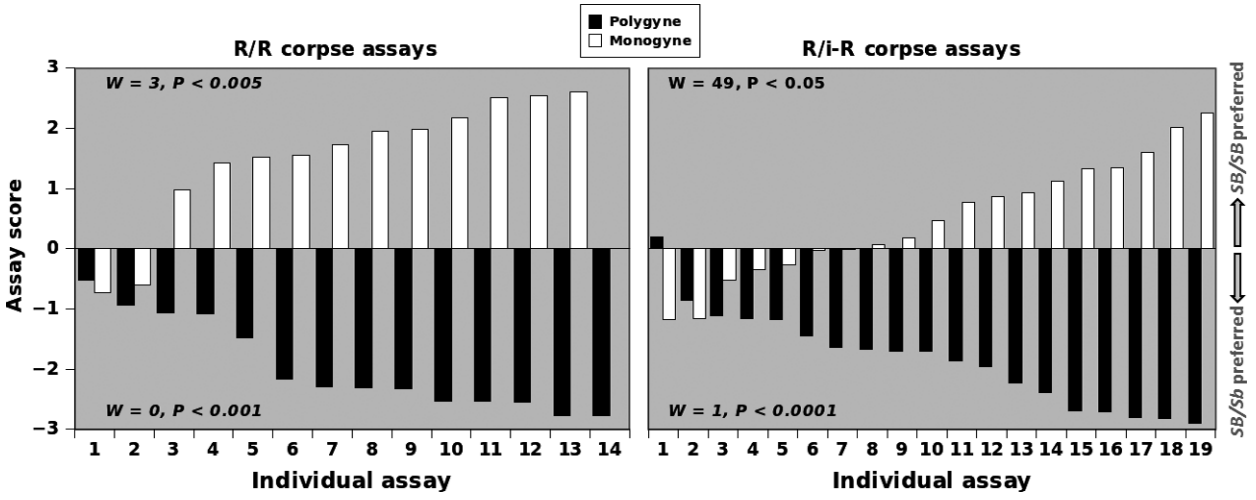

Fig. 1 Results of Experiment 1A, worker *Sb* discrimination assays using queen corpses. Each bar represents a statistically independent assay score of an individual polygyne or monogyne assay unit, with positive scores indicating preference for *SB/SB* (monogyne) queens (maximum possible score = 3) and negative scores indicating preference for *SB/Sb* (polygyne) queens (maximum possible score = −3). Individual polygyne unit scores are arranged in decreasing order (greater preference for polygyne queens) and individual monogyne unit scores are arranged in increasing order (greater preference for monogyne queens). Left-side graph displays results for Type R/R pairings (fully reproductive *SB/Sb* polygyne and *SB/SB* monogyne corpses) and right-side graph displays results for Type R/i-R pairings (fully reproductive *SB/Sb* polygyne queens and virgin incipient-reproductive *SB/SB* monogyne corpses). Wilcoxon W statistics and two-tailed probabilities that score medians do not differ from zero are given in the upper panels for monogyne units and in the lower panels for polygyne units; *P*-values are italicized if significant after Benjamini–Hochberg correction for multiple comparisons with an FDR of 0.05. [Correction added on 20 January 2016 after first online publication: Italicised values in the figure have been corrected. The value, W = 49, P < 0.05, should not have been italicised.]

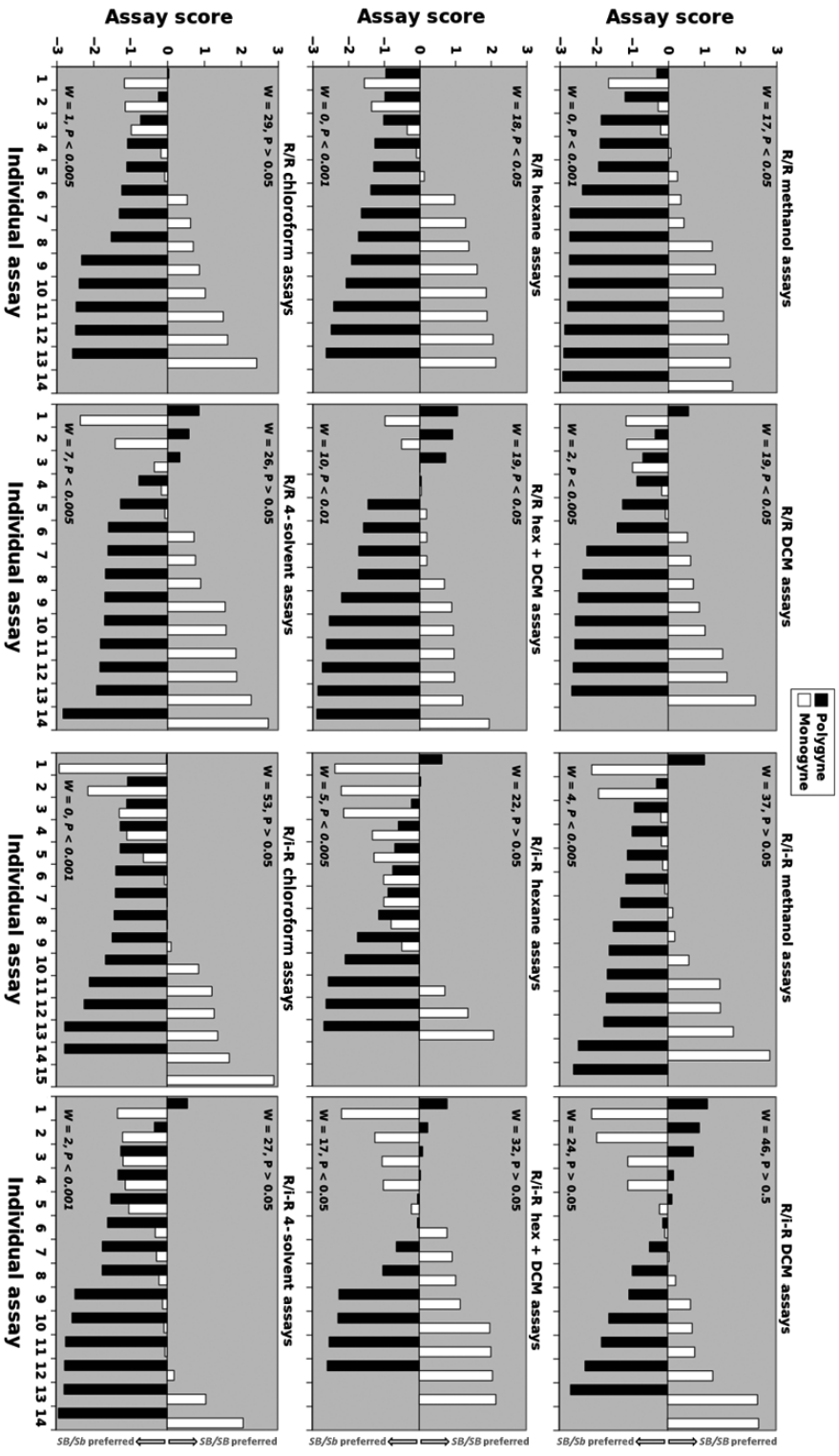

Fig. 2 Results of Experiment 1B, worker *Sb* discrimination assays using queen extracts on paper surrogates. Each bar represents a statistically independent assay score of an individual polygyne or monogyne assay unit for Type R/R (left-side graphs) and Type R/i-R (right-side graphs) pairings. See Fig. 1 caption for additional information. [Correction added on 20 January 2016 after first online publication: Figure 2 has been replaced to reflect the correct italicised values.]

discrimination in the Type R/R solvent experiments in which it was observed.

Experiment 2: Green beard assays

We conducted assays based on the green beard effect associated with the *Sb* supergene to verify that queen pheromonal communication of supergene status revealed in the preceding experiments induces behaviours responsible for polygyne worker *Sb* discrimination and to test whether such discrimination may in fact rely on the green beard effect. We note that because the preceding assays demonstrated that *SB/SB* surrogates receive relatively little worker attention and ultimately are rarely accepted by polygyne colonies, worker behaviour towards such surrogates should be interpreted as aggression in this context. Similarly, the modest numbers of workers clustering on the *SB/SB* surrogates in the green beard assays (mean $n = 5.8$) are assumed to have been behaving aggressively.

Means and confidence intervals for proportions of *Sb*-bearing workers in the Q (attacking queen surrogates), R (reference) and Ali (attacking alien ant species) categories are shown in Fig. 3. The mean proportion of *Sb*-bearing Q workers exceeded the proportion of *Sb*-bearing R workers by 10% and 13% in the two experiments, with the proportion of Q workers with *Sb* exceeding the proportion of nestmate R workers with the element in 13 of the 20 methanol assays (binomial test, $P = 0.132$) and 15 of the 20 hexane assays (binomial test, $P = 0.021$) (combined binomial probability of such an extreme result over both experiments, $P = 0.008$). Both bootstrap and jackknife resampling analyses confirm that Q workers bore *Sb* in significantly higher proportions than did R workers in both the methanol ($P = 0.010$ and $P < 0.001$, respectively) and, especially, the hexane ($P = 0.001$ and $P < 0.001$) experiments (Fig. 3). A greater efficacy of hexane over methanol in extracting the green beard (*SB* supergene) signal is suggested also by the greater number of Q workers seizing the *SB* queen surrogate in the former compared to the latter trials [*t*-test, $t(38) = -5.19$, $P < 0.001$]. The absence of elevated *Sb* proportions in Ali workers compared to R workers using either solvent indicates that a heightened general aggressive tendency of *Sb*-bearing workers cannot explain their over-representation among Q workers relative to R workers.

Interpretation of the results of this experiment potentially is complicated by the fact that supergene genotype is associated with worker size (Goodisman *et al.*, 1999, 2007; Buechel *et al.*, 2014). Our analyses of a subset of workers from the methanol green beard experiment ($n = 502$ workers from 16 colonies) confirm previous reports (Goodisman *et al.*, 2007; Buechel *et al.*, 2014) that *Sb*-bearing workers tend to be larger than other workers (bootstrap resampling test, $P = 0.005$), but this size difference does not generate the observed green beard effect: no difference between the size of Q workers attacking *SB/SB* surrogates and reference R workers was found (two-tailed Fisher Exact Test, $P = 0.492$). On the other hand, Ali workers, which attacked alien *Camponotus* ants, were significantly smaller than R workers (two-tailed Fisher Exact Test, $P = 0.035$), suggesting some linkage between worker size and defensive behaviour against heterospecifics that can also explain the generally depressed proportions of *Sb*-bearing Ali workers in our experiments [bootstrap and jackknife resampling analyses show that

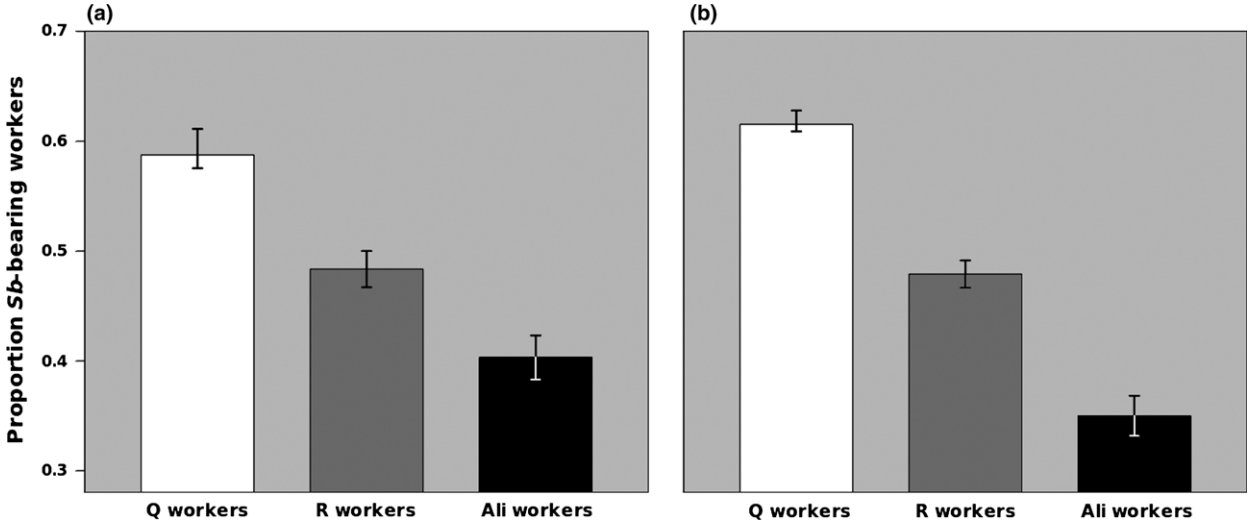

Fig. 3 Results of Experiment 2, green beard assays. Proportions of *Sb*-bearing workers detected in the Q (attacking queen surrogates), R (reference) and Ali (attacking alien *Camponotus* ants) worker categories using methanol (a) and hexane (b) extracts of *SB/SB* queens are shown (with jackknife 95% confidence intervals).

the differences in *Sb* proportions between Ali and R workers are significant in both the methanol ($P = 0.053$ and $P < 0.001$, respectively) and hexane ($P = 0.008$ and $P < 0.001$) experiments; see Fig. 3].

Interpretation of this experiment could be confounded further if recruitment of the generally smaller Ali workers (those attacking the *Camponotus* ants), which disproportionately bear genotype *SB/SB*, led to a depressed proportion of this genotype among the remaining ants, possibly contributing to an over-representation of *Sb*-bearing Q workers (those clustering on the *SB/SB* surrogates). We judge this to be highly unlikely given the large numbers of workers in the assay units (~ 5000) and the comparatively few involved in aggressive behaviours (mean = 11.5 Q workers and 7.7 Ali workers per assay in the two experiments). Indeed, correlation analyses indicate that *SB/SB* proportions are not associated between Ali and Q workers across assays for either the methanol or hexane experiments [Spearman $r_s(18) = 0.139$ and -0.029, $P = 0.559$ and 0.902, respectively]; standardizing the proportions by subtracting R (reference) proportions from both the Ali and Q proportions similarly yields no significant associations [Spearman $r_s(18) = 0.080$ and 0.364, $P = 0.738$ and 0.114, respectively]. Thus, there is no evidence to suggest that supergene genotype proportions observed in Q workers were causally linked to those in Ali workers, that is, that the group responses were not independent.

These results thus robustly confirm a green beard effect manifested, in this case, in response to queen solvent extracts. Demonstration of the effect in this way reinforces our conclusions that queen pheromonal communication of supergene status is responsible for inducing polygyne worker *Sb* discrimination behaviour and that the pheromones mediating such discrimination are soluble in multiple solvents with very different solvent properties. In conjunction with the absence of worker *Sb* discrimination in monogyne colonies, this result also supports the hypothesis that the green beard effect may be the individual behavioural mechanism underlying worker *Sb* discrimination.

Discussion

We employed a newly developed behavioural assay in *S. invicta* to conclusively demonstrate that cuticular chemicals associated with presence or absence of the *Sb* supergene in queens act as cues that elicit worker *Sb* discrimination in the polygyne social form of this ant. Moreover, we confirmed polygyne workers' ability to use such semiochemicals to discriminate between queens of different *Sb* supergene status in a fully independent set of assays based on a green beard effect reported previously in this system. These findings fill a crucial gap in constructing the genotype/phenotype map for a complex, emergent social behaviour responsible for regulating queen identity and number in colonies of this species. For example, gene expression studies have identified candidate genes potentially involved in pathways of cuticular semiochemical synthesis, transport or perception whose expression differs between queens bearing or lacking the supergene (Wang *et al.*, 2008, 2013; Huang & Wang, 2014), whereas cuticular biochemistry studies have revealed differences in the cuticular chemical composition of such queens (Eliyahu *et al.*, 2011). Our results validate these attempts to identify the gene expression patterns, molecular pathways and cuticular chemicals that differ between queens of the two genotypes and encourage the deployment of functional studies that manipulate genes or gene products to definitively identify the specific chemical triggers of worker *Sb* discrimination behaviour. Importantly, such functional studies will rely on efficient assays of the complex social behaviours involved, such as we developed for this study. Progress in completing the map from gene to complex phenotype in *S. invicta* based on functional experiments should yield new insights into the evolution of regulation of colony social organization in this ant and, when combined with appropriate comparative analyses, perhaps in other highly social insects as well (see, e.g. Purcell *et al.*, 2014).

We note that the array of biochemical, physiological and behavioural differences between individuals bearing and lacking the *Sb* supergene are attributed to genes residing in this element and, indeed, the region may be enriched for factors involved in the maintenance of polygyny, for example genes encoding proteins involved in chemical communication and/or transcription factors regulating their expression (Nipitwattanaphon *et al.*, 2013). Studies using up to dozens of presumed neutral nuclear genetic markers have shown minimal genetic differentiation between sympatric social forms in the USA at other genomic regions, as expected given the high levels of gene flow thought to occur between them (Ross *et al.*, 1999; Shoemaker *et al.*, 2006). Nonetheless, the possibility remains that some traits linked to supergene possession are strongly influenced by genes located in 'genomic islands' of differentiation (Cruickshank & Hahn, 2014) positioned elsewhere in the genome that are under form-specific selection and are resistant to introgression (as is the supergene). Comparative high-resolution genome scans of the two forms are required to evaluate this possibility.

Comparisons of the cuticular biochemical profiles of *S. invicta* queens bearing (*SB/Sb*) or lacking (*SB/SB*) the supergene earlier concluded that unsaturated hydrocarbons are strong candidates for the chemicals that signal queen supergene status to workers (Eliyahu *et al.*, 2011), a conclusion reinforced by the finding that many genes involved in hydrocarbon synthesis (desaturase and elongase genes) are up-regulated in *SB/Sb* compared to *SB/SB* queens (Wang *et al.*, 2013; Huang &

Wang, 2014). These nonpolar molecules are highly soluble in the nonpolar solvent hexane, consistent with the overall superior activity of hexane queen extracts in inducing polygyne worker *Sb* discrimination when evaluated across both our discrimination and green beard assays. Remarkably, however, strong polygyne worker *Sb* discrimination also was observed using extracts obtained from methanol (polar protic solvent) and chloroform (nonpolar solvent). These results imply either that the cuticular hydrocarbons acting as semiochemicals may be recovered to a greater or lesser degree in these other solvents or that other, possibly polar, biomolecules may also act as cues eliciting worker *Sb* discrimination. Such multiplicity in chemical social signalling systems is well known in fire ants [e.g. queen pheromones (Tschinkel, 2006), trail pheromones (Tschinkel, 2006) and alarm pheromones (Vander Meer *et al.*, 2010)] as well as other highly social insects [e.g. honey bee queen retinue pheromones (Slessor *et al.*, 2005)]. One possible auxiliary semiochemical involved in worker *Sb* discrimination is the protein GP-9 itself; this polar protein is a member of the odorant binding protein (OBP) family, members of which have been implicated in insect chemical communication (Vogt, 2005; Gotzek & Ross, 2009), and the protein can be recovered in methanol cuticular rinses of *S. invicta* queens (D. Gotzek, M. Strand and M. Beck, unpublished data). The *B* and *b* allelic forms of the GP-9 protein differ at eight of the 134 amino acid residues comprising the mature protein in populations in the USA (Krieger & Ross, 2002).

In contrast to our demonstration of worker *Sb* discrimination in polygyne colonies, we were not able to show that workers in monogyne colonies discriminate among queens on the basis of *Sb* status alone. Specifically, the lack of a preference in monogyne workers, all of which possess genotype *SB/SB*, for queens of the same genotype in our Type R/i-R assays indicates that these workers may prefer replacement queens of higher reproductive condition regardless of their supergene status (Fletcher & Blum, 1981), which could also explain the apparent worker *Sb* discrimination observed in assay units of this form using Type R/R pairings. Fully reproductive and incipient-reproductive *SB/SB* queens do not differ substantially in their cuticular unsaturated hydrocarbon profiles, but these profiles differ markedly from those of reproductive *SB/Sb* queens (Eliyahu *et al.*, 2011), making it possible for polygyne workers to discriminate on the basis of queen supergene status even in the face of strong differences in reproductive development using these chemical cues. The fact that monogyne workers do not do so implies that workers of this form lack the molecular chemosensory or behavioural machinery required to process or act on such cues, which machinery presumably also is encoded in the supergene (Keller & Ross, 1998; Krieger & Ross, 2002; Gotzek & Ross, 2007; Huang & Wang, 2014). Thus, our finding that monogyne colonies do not exhibit worker *Sb* discrimination supports the hypothesis that such discrimination results from the green beard effect operating in individuals that bear *Sb*.

Monogyne workers, rather than relying on queen supergene status, may rely primarily on different sets of cues related directly to queen reproductive state to inform collective decisions about queen acceptance/rejection. Indeed, *SB/SB* queens of dissimilar reproductive development differ in their cuticular composition of a separate class of macromolecules, piperidine venom alkaloids, which has led to the suggestion that these compounds are cues of queen reproductive state (Eliyahu *et al.*, 2011). As may be expected in this scenario, our monogyne assay units performed poorly at worker *Sb* discrimination in Type R/i-R pairings when presented surrogates soaked in extracts obtained using dichloromethane (DCM), a moderately polar aprotic solvent in which piperidine alkaloids are expected to be highly soluble (Eliyahu *et al.*, 2011).

Our observation that monogyne colonies lack worker *Sb* discrimination seemingly contradicts findings of an earlier experiment in which live, reproductive queens bearing the *Sb* supergene invariably were rejected by monogyne colonies that had been queenless for a short period (Ross & Keller, 1998). Assay designs in the two studies differed in many respects, such as assay unit sizes, numbers of queens introduced, lengths of the assays, use of paired or singular introduced material, and perhaps most importantly, the exclusive introduction of living reproductive queens in the previous study. Thus, although we failed to demonstrate *Sb* discrimination in monogyne fragments in which workers were presented paired material for comparative evaluation and discrimination, it may occur under some conditions such as those in the Ross & Keller study (1998), although this is not predicted under the green beard model.

Our study clearly confirms the existence of a green beard effect involving the *Sb* supergene in polygyne *S. invicta*, an effect previously revealed on the basis of a modest number of assays using live queens and incomplete genotyping of attacking workers (Keller & Ross, 1998). Specifically, fire ants display a facultative-harming type of green beard mechanism in which actors bearing the green beard allele (i.e. workers with the *Sb* supergene) use cuticular chemical profiles of prospective or functional queens to determine whether they also bear the allele, destroying them if they do not (Gardner & West, 2009). Green beard effects in general have been suggested to constitute a fundamental class of mechanisms for evolving complex social behaviours such as cooperation (kin selection being another), yet green beard effects have only rarely been observed, especially in animals (Burt & Trivers, 2006; Gardner & West, 2009). Although such effects may in fact be quite common, under most conditions green beard alleles

rapidly drive themselves to fixation and thus become undetectable. In *S. invicta*, recessive deleterious effects of the *Sb* supergene on queens, coupled perhaps with the comparatively high pathogen load and investment in sterile males characteristic of the polygyne form (Ross & Fletcher, 1985, 1986; Valles *et al.*, 2010), constitute negative selection on *Sb* that, in conjunction with gene flow from the monogyne form (Ross & Shoemaker, 1993), maintains *Sb* in an apparent stable polymorphism with the homologous chromosomal region, thus allowing green beard effects to remain visible (Mescher, 2001). Maintenance of the polymorphism also has made it possible to detect other unique, genetically selfish properties of the *Sb* supergene, such as an over-representation of the element among female brood that develop into queens rather than workers (Buechel *et al.*, 2014) and apparent segregation distortion favouring production of *Sb*-bearing eggs in some heterozygous queens (K. Ross and D. Shoemaker, unpublished data). These phenomena hint that many other surprising effects of this genomic element on fire ant social biology remain to be discovered.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Data S1 Supporting information (methods) for study on chemical communication of queen supergene status in *Solenopsis invicta*.

Data deposited at Dryad: doi: 10.5061/dryad.76r80

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