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Male reproductive fitness and queen polyandry are linked to variation in the supergene *Gp-9* in the fire ant *Solenopsis invicta*

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Supergenes are clusters of tightly linked loci maintained in specific allelic combinations to facilitate co-segregation of genes governing adaptive phenotypes. In species where strong selection potentially operates at different levels (e.g. eusocial Hymenoptera), positive selection acting within a population to maintain specific allelic combinations in supergenes may have unexpected consequences for some individuals, including the preservation of disadvantageous traits. The nuclear gene *Gp-9* in the invasive fire ant *Solenopsis invicta* is part of a non-recombining, polymorphic supergene region associated with polymorphism in social organization as well as traits affecting physiology, fecundity and behaviour. We show that both male reproductive success and facultative polyandry in queens have a simple genetic basis and are dependent on male *Gp-9* genotype. *Gp-9*^{b} males are unable to maintain exclusive reproductive control over their mates such that queens mated to *Gp-9*^{b} males remain highly receptive to remating. Queens mated to multiple *Gp-9*^{B} males are rare. This difference appears to be independent of mating plug production in fertile males of each *Gp-9* genotype. However, *Gp-9*^{b} males have significantly lower sperm counts than *Gp-9*^{B} males, which could be a cue to females to seek additional mates. Despite the reduced fitness of *Gp-9*^{b} males, polygyne worker-induced selective mortality of sexuals lacking *b*-like alleles coupled with the overall success of the polygyne social form act to maintain the *Gp-9*^{b} allele within nature. Our findings highlight how strong worker-induced selection acting to maintain the *Gp-9*^{b} allele in the polygyne social form may simultaneously result in reduced reproductive fitness for individual sexual offspring.

Keywords: conflicting selection; facultative polyandry; *Solenopsis invicta*; supergene

1. INTRODUCTION

Many complex adaptive phenotypes are attributed to clusters of tightly linked genetic loci coding for traits that are advantageous in specific allelic combinations [1]. These loci are inherited as a single unit known as a supergene, and tight physical linkage among loci within supergenes minimizes recombination and breaking up of certain allele combinations that potentially lead to maladaptive recombinant phenotypes in offspring [2,3]. Although the existence of supergenes is well documented in a variety of organisms, studies of the evolution and maintenance of supergenes, particularly in systems in which selection operates at different levels, are less common.

One such case involves the supergene region associated with *Gp-9* (*general protein-9*; hereafter referred to as the *Gp-9* supergene region) in the invasive fire ant *Solenopsis invicta* and its close relatives. Evidence supporting the view that *Gp-9* is part of a supergene includes the facts that a plethora of seemingly unrelated traits are governed by this region, that balancing selection maintains the two classes of alleles (*Gp-9*^{b} and *Gp-9*^{B}) within the species and, importantly, that genetic mapping studies reveal the *Gp-9* locus is in complete linkage disequilibrium with a large portion (approx. 5%) of the genome [4] (Yannick Wurm 2012, personal communication). The major effect of the *Gp-9* supergene region appears to be colony-level governance of social form [5]. Two distinct colony social forms exist in *S. invicta* and in its close relatives: monogyne colonies have only a single egg-laying queen, whereas polygyne colonies have multiple such queens. Previous studies demonstrated two classes of alleles, designated as *B*-like and *b*-like alleles, exist within the *Gp-9* region of *S. invicta* and that all polygyne egg-laying queens possess at least one *b*-like allele (*Gp-9*^{Bb}), whereas functional monogyne queens lack such *b*-like alleles and harbour only *B*-like alleles (*Gp-9*^{BB}). Under this system, the presence of *b*-like alleles among colony members appears both necessary and sufficient for polygyny [4,6–9].

Workers in the polygyne social form of *S. invicta* have control over both the sexuals reared in the nest as well as the acceptance of fertilized queens attempting to enter their nests [10–17]. For queen acceptance, worker regulation of queen identity and number is based on *Gp-9* genotypic compatibility: polygyne workers with the *Gp-9*^{b} allele tolerate only sexuals that also have the *Gp-9*^{b} allele (*Gp-9*^{Bb} queens) and execute all queens

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entering nests lacking a *Gp-9^{b}* allele [4]. Thus, polygyne workers will collectively accept multiple queens of the *Gp-9^{Bb}* genotype into their nest, but always reject *Gp-9^{BB}* queens [4,7,18]. For sexuals reared in polygyne nests, most virgin queens (female alates) that lack the *Gp-9^{b}* allele (*Gp-9^{BB}* alates) are executed. *Gp-9^{B}* males reared in polygyne nests presumably are executed as well by polygyne workers, as fertile (haploid) polygyne males are almost exclusively *Gp-9^{b}* [17]. Worker-induced execution of sexuals in polygyne nests, which is induced by a minimum frequency of colony workers carrying the *Gp-9^{b}* allele (approx. 12%) [19], facilitates the maintenance and spread of the *Gp-9^{b}* allele within the fire ant populations. Such worker-induced selection based on *Gp-9* genotype does not occur in monogyne nests, because all individuals are homozygous *Gp-9^{BB}* and monogyne workers reject foreign queens regardless of the *Gp-9* genotype [4].

A myriad of additional advantageous characteristics of the polygyne social form, especially in terms of invasion and spread in new areas, also favour the maintenance of *Gp-9 b*-like alleles. Population densities of polygyne *S. invicta* can be several to many times higher than monogyne populations [20,21], and each colony may have greater long-term survival potential as they can outlive the average lifespan of a single queen (after approx. 7–8 years monogyne colonies fail because the queen runs out of sperm to produce new workers) [22,23]. Additionally, the polygyne social form is considered to be more invasive and more easily spread by human commerce as each colony fragment has a higher probability of containing a viable queen than a monogyne fragment [24]. Indeed, the polygyne social form has spread rapidly throughout the previously monogyne-occupied North American range since the putative introduction of polygyny in the early 1970s [25].

The strong worker-induced positive selection towards individuals harbouring *Gp-9^{b}* alleles and the apparent success of the polygyne social form would suggest that selection favours the spread of the *Gp-9^{b}* allele throughout polygyne *S. invicta*, which in turn potentially could lead to the replacement of the monogyne social form as well. However, the above advantages associated with the *Gp-9^{b}* allele are countered by negative selection on individuals possessing this allele [4]. Foremost is the fact that the *Gp-9^{bb}* genotype is probably lethal or near-lethal, as functional *Gp-9^{bb}* queens are almost entirely absent in nature [6]. Additionally, *Gp-9^{Bb}* queens apparently have lower fitness than *Gp-9^{BB}* queens, the former having smaller body size, lower fecundity and poorer dispersal ability [26–29].

Thus, the *Gp-9* supergene is unique in that selection acting against the *Gp-9^{b}* allele is balanced by selection acting to maintain this allele. Therefore, this gene region provides a unique opportunity to study supergene evolution when 'selfish genes' for colony success interact with linked genes affecting individual fitness. While a trade-off between colony fitness and individual fitness of the *Gp-9^{b}* allele has been demonstrated in females, the effects of *Gp-9* variation on male fitness are poorly understood. The potential conflict between colony and male selection is especially interesting because of the different life history and kin selection dynamics of males [30]. Males in the eusocial Hymenoptera share less DNA with the workers rearing them (in monogyne colonies 25% versus 75% for female sexuals, and less in the polygyne social form), have a very short life expectancy (they typically die during or shortly after a single mating flight) and have little or no involvement in colony maintenance [30–32]. All of these characteristics suggest that selection on male fitness and survival may be relatively weaker than selection on colony fitness. Thus, selection potentially may maintain traits that increase colony fitness despite being associated with detrimental traits to individual male fitness and survival [12,13].

In this study, we investigate the effects of *Gp-9* variation on male reproductive success, polyandry (the inability of males to maintain exclusive reproductive control over their partners), and male sperm counts and mating plug production. Several social and morphological characteristics already are known to be associated with alleles of the *Gp-9* supergene; however, this study is, to our knowledge, the first to investigate the effects of *Gp-9* haplotype on traits involved with sexual selection, mating frequency and male reproductive fitness. In addition to contributing to the growing field of studies examining the evolution and maintenance of supergenes [33], our results provide a unique view into supergene dynamics when selection acts in multivariate and antagonistic ways.

2. MATERIAL AND METHODS

(a) *Field collection of queens and rearing of workers*

Polygyne nests were collected [34] from site D in the study of Fritz *et al.* [17] in north central Florida (FL-P) and Hurley, Mississippi (MS-P). Newly mated monogyne queens were collected near Gainesville, FL, USA (FL-M). Each polygyne queen was reared separately in small nest units provided with 10–12 workers and 0.13 g of brood [34]. Monogyne queens were set up alone. Worker larvae and pupae (20 individuals) were harvested from each polygyne queen unit at a minimum of seven weeks after the start of the experiment to ensure that all sampled offspring were those of the resident queen [35]. Monogyne broods were harvested after the first batch of workers emerged as adults. Samples were stored in 95 per cent ethanol until used for molecular analysis. Sample sizes of mated queens were: FL-P = 83; MS-P = 88; FL-M = 92 (table 1).

(b) *DNA extractions*

Total genomic DNA was extracted using the Puregene DNA Isolation Kit (Gentra Systems) [36]. We performed extractions on each queen, her stored sperm and 16 or more of her offspring (FL-P samples only). We collected sperm samples by removing the spermatheca of each queen under a dissection scope (Zeiss Stemi SV6 stereo microscope) and then removing surrounding maternal tissues, leaving an intact ball of sperm. Results based on sperm and brood samples of FL-P families ($n = 83$ queens) and additional newly mated queens ($n = 20$) revealed sperm alone was 100 per cent accurate in determining whether a queen was singly or doubly mated. Therefore, only queens and spermathecae were sampled from MS-P and FL-M.

(c) Gp-9 *and microsatellite analyses*

Each individual brood, queen and sperm sample was assayed at *Gp-9* using TaqMan allelic discrimination assays [37] to determine the *Gp-9* genotypes of queens and mates. Eleven microsatellite loci (Sol-49, C293PigTail, C21, Sol-55,

Table 1. Summary statistics of populations highlighting *Gp-9* genotype/haplotype of queens and their mates. (MS-P (Mississippi polygyne), FL-P (Florida polygyne) and FL-M (Florida monogyne). For percentages, *Q* refers to queens, *B* to *Gp-9*^{B} males and *b* to *Gp-9*^{b} males. '$Q \times b$' means 'Queens mated to *Gp-9*^{b} males'. '$Q \times b + (B \text{ or } b)$' means 'Queens mated to a *Gp-9*^{b} male also mated to another male of either genotype'.)

	MS-P	FL-P	FL-M
polyandrous queens/total queens	1/88	17/83	3/92
queen genotypes	*Gp-9*^{Bb}	*Gp-9*^{Bb}	*Gp-9*^{BB}
genotypes of males mated to polyandrous queens (*b*,*b* : *b*,*B* : *B*,*B*)	0 : 0 : 1	4 : 12 : 1	1 : 1 : 1
percentage of total matings to *Gp-9*^{b} males	0	31	3.2
percentage of queens mated to *Gp-9*^{b} males with additional mate(s) ($Q \times b + (B \text{ or } b)$)/$Q \times b$	n.a.	57	100
percentage of queens mated to *Gp-9*^{B} males with additional *Gp-9*^{B} mate(s) ($Q \times B + B$)/$Q \times B$	1	2	1

Sol-18, Sol-B8PigTail, i-126, i-114, Sol-6, Sinv-25 and Sol-M3) were assayed and scored as described by Asunce *et al.* [36].

Frequency of matings (number of patrilines among each queen's progeny) was inferred for FL-P colonies using MATESOFT v. 1.0 [38]. Allelic frequencies for each locus were determined using FSTAT, v. 2.9.3.2 [39]. Average paternity skew was also calculated for all polyandrous queens. We also investigated whether *Gp-9*^{bb} offspring from monandrous families with *Gp-9*^{b} fathers exhibited increased mortality, which could alter paternity skew in families with both *Gp-9*^{B} and *Gp-9*^{b} patrilines [17]. However, we found all genotypes, including *Gp-9*^{bb}, present in expected ratios ($p = 0.13$ in pooled families, and even paternity rejected in only one of nine families), thus corrections for paternity skew were not required.

Frequency of polyandrous queens in the FL-M and MS-P populations was inferred from microsatellite analyses of each queen and her stored sperm. The microsatellite analyses were straightforward given the substantial allelic variation and the fact that males are haploid. Indeed, every putative patriline identified was distinguished easily from other patrilines and from the maternal genotype at two or more loci.

(d) *Mating plug fatty acid analysis of* Solenopsis invicta *male reproductive organs*

In an effort to identify phenotypic traits underlying the continued receptiveness to mating experienced after copulation with *Gp-9*^{b} males, we investigated quantities of fatty acids comprising the putative mating plug deposited by males [40] (believed to reduce female receptivity to additional partners) and male sperm load. Fatty acids (linoleic, oleic, palmitic and stearic acids) [40] produced by males and stored in their sperm storage organs (i.e. seminal vesicles and accessory glands) are transferred as a 'sperm plug' upon copulations to inhibit females from subsequent matings. Sperm storage organs from 10 laboratory-reared, mature, haploid, unmated males (five monogyne *Gp-9*^{B} and five polygyne *Gp-9*^{b}) from different healthy colonies were dissected in water (Zeiss Stemi SV6 stereo microscope) for fatty acid analysis. In a second assay, the male reproductive system was separated into three sections: (i) degenerated testes and seminal vesicles, (ii) accessory glands, and (iii) external genitalia. In all cases, the intact reproductive system or sections of each male were placed in a gas chromatography (GC) autosampler vial containing a 250 µl of insert with 50 µl of hexane. Free fatty acids were esterified with BF_3/butanol and then identified and quantified using standard GC (Agilent 6890, Santa Clara, CA, USA) procedures. The total amount of proposed mating plug fatty acids in males of each haplotype was analysed using ANOVA and a Mann–Whitney test in the statistical package R v. 2.13.0 [41]. Fatty acids showed no differentiation between sections (d.f. = 2, $p = 0.6029$), and thus total amounts are reported throughout.

(e) *Sperm quantification*

Sperm storage organs were removed as described above from 10 *Gp-9*^{B} monogyne males and 10 *Gp-9*^{b} polygyne males from healthy colonies free of *S. invicta* viruses (SINV 1, 2 and 3) and *Kneallhazia* infection. Each male reproductive tract (seminal vesicles and accessory glands) was extracted, cleaned in 0.5 M NaCl solution, ground using a pestle in a 1.7 ml tube to ensure the release of all sperm and resuspended in 1 ml of 0.5 M NaCl. Sperm were quantified using a haemocytometer using the average of two chambers per sample. Samples were counted according to the standard protocol: the four corner squares and the middle square were counted, and the total was multiplied by 50 000 to obtain the total quantity of sperm suspended in the 1 ml solution [42]. Sperm were visible using a phase-contrast scope with 40× magnification. A sperm was counted if its head was within the square, regardless of the tail position.

3. RESULTS AND DISCUSSION

Previous studies suggest that queens of the invasive fire ant *S. invicta* predominantly are monogamous throughout their native and introduced ranges ([34,43–45], but see [17]), but our results clearly demonstrate a significant proportion of queens in some areas are polyandrous (up to 20%). Interestingly, our results show that facultative polyandry in *S. invicta* is almost entirely dependent on male *Gp-9* genotype (figure 1; χ^2, d.f. = 2, $p = 0.0029$) and is independent of queen *Gp-9* genotype (χ^2, d.f. = 1, $p = 0.064$): Females mated to *Gp-9*^{b} males routinely seek additional mates (60% of queens mated to *Gp-9*^{b} males have a second mate) and females rarely mate with multiple *Gp-9*^{B} males (1.1% of queens are polyandrous without a *Gp-9*^{b} mate; table 1).

The highest proportion of polyandrous queens and the highest percentage of *Gp-9*^{b} fathers contributing sperm to queens were observed in the Florida polygyne (FL-P) population (table 1). With one exception, every polyandrous queen in FL-P was mated to at least one *Gp-9*^{b} male. By contrast, none of the sampled queens from the Mississippi polygyne (MS-P) population was mated to a *Gp-9*^{b} male and only a single queen was mated to more than a single male (one of 88 queens; table 1). Finally, only three of the 92 queens from the Florida monogyne

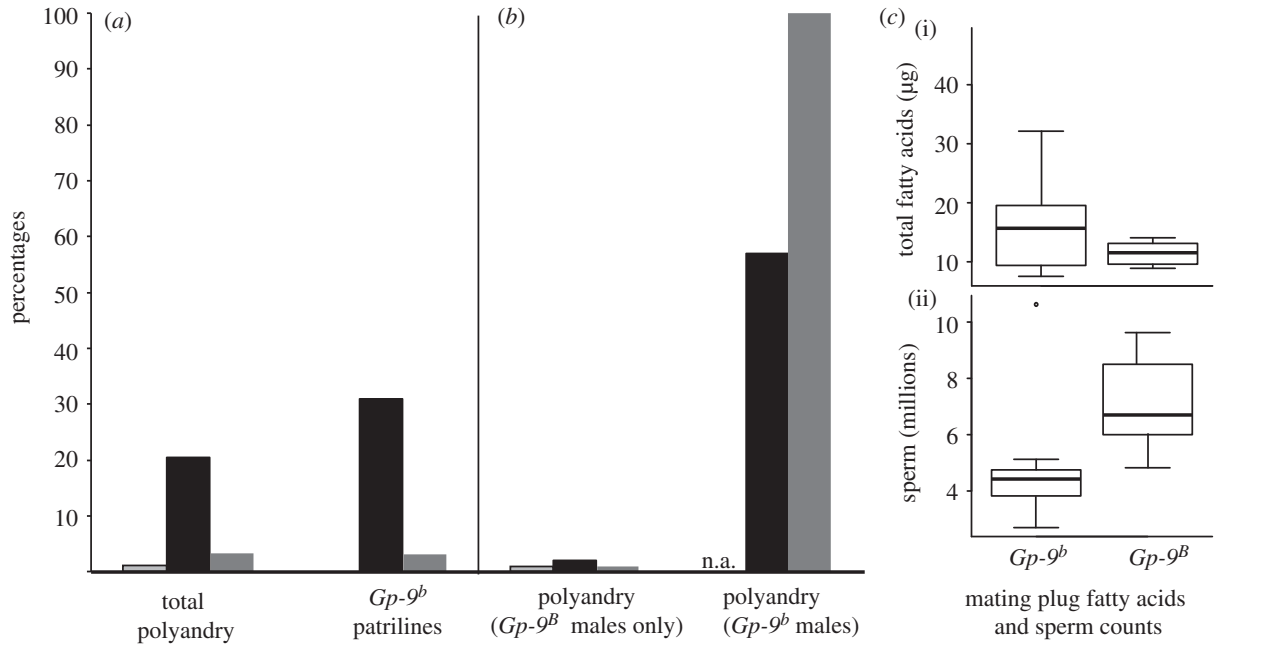

Figure 1. Regardless of social form or location, queens rarely mated with multiple $Gp\text{-}9^B$ males, but were likely to be polyandrous if one partner was $Gp\text{-}9^b$. This difference corresponds to significantly different sperm counts of the two *Gp-9* male haplotypes, but no differences in mating plug production. (*a*) Percentage of polyandrous queens and of $Gp\text{-}9^b$ patrilines in each study population (light grey with dark outline, MS-P (Mississippi polygyne); black bars, FL-P (Florida polygyne); medium grey bars, FL-M (Florida monogyne)). Polyandry and proportion of insemination from $Gp\text{-}9^b$ males covary regardless of social form. (*b*) Association of male $Gp\text{-}9^b$ haplotype and polyandry. Polyandry ($Gp\text{-}9^B$ males only) = (number of queens mated to two $Gp\text{-}9^B$ males)/(number of queens mated to any $Gp\text{-}9^B$ males). Polyandry ($Gp\text{-}9^b$ males) = (number of queens mated to at least one $Gp\text{-}9^b$ male)/(number of those queens with a second mate of any haplotype). (*c*) Total quantities of (i) mating plug fatty acids and (ii) sperm in $Gp\text{-}9^b$ and $Gp\text{-}9^B$ males.

(FL-M) population were multiply mated, two of which were mated to the only $Gp\text{-}9^b$ males sampled in the population (i.e. every mating involving a $Gp\text{-}9^b$ male corresponded to a polyandrous queen). Overall, only three of the 233 queens not mated to a $Gp\text{-}9^b$ male were multiply inseminated (table 1).

We also investigated both putative mating plug composition and sperm counts in males of different *Gp-9* haplotypes as potential traits affecting male reproductive fitness. Fatty acids produced by haploid males of different *Gp-9* haplotypes did not quantitatively differ (figure 1; $Gp\text{-}9^b = 16.85$ µg, s.d. = 9.80 µg, $Gp\text{-}9^B = 11.44$ µg, s.d. = 2.21 µg, $n = 10$, Mann–Whitney test: $p = 0.5941$), implying both $Gp\text{-}9^B$ and $Gp\text{-}9^b$ males produce similar mating plug fatty acids to discourage polyandry. This finding raises some doubt regarding the putative role of these fatty acids as effective mating plugs since polyandry is independent of their relative abundance. Sperm counts, however, did differ significantly between $Gp\text{-}9^B$ and $Gp\text{-}9^b$ males (figure 1; mean sperm count = 7.05 and 4.78 million, respectively; $n = 20$, *t*-test: $p = 0.019$). The link between lower sperm counts in $Gp\text{-}9^b$ males and multiple mating of queens that are mated to a $Gp\text{-}9^b$ male implies that females are able to ascertain sperm load in their mates, and will seek additional copulations if insufficient.

The ratio of expected progeny in mixed broods (i.e. brood from a single female mated to both a $Gp\text{-}9^b$ and $Gp\text{-}9^B$ male) based on sperm count averages was used to test whether paternity skew could be explained by relative sperm contributions alone (χ^2-test of mean sperm counts; 40 : 60 ratio of $Gp\text{-}9^b$ patriline : $Gp\text{-}9^B$ patriline progeny expected versus 28 : 72 observed, $n = 9$ polyandrous queens, 145 worker offspring). We observed a strong paternity skew among offspring of polyandrous queens mated to males of both *Gp-9* haplotypes, wherein $Gp\text{-}9^B$ males always sired the majority (average = 71%, s.d. ± 13%) of larval and pupal offspring (χ^2 of equal paternity: expected is 72.5 from each haplotype; observed is 105 from $Gp\text{-}9^B$ patriline and 40 from $Gp\text{-}9^b$ patriline; $p < 0.001$). While the observed ratios are consistent with fewer sperm being transferred to females from $Gp\text{-}9^b$ males, the paternity skew does exceed slightly the predicted skew based solely on sperm number differences between $Gp\text{-}9^B$ and $Gp\text{-}9^b$ males when the offspring from all queens are pooled (χ^2 of 60 : 40 ratio matching sperm differences; expected 87 from $Gp\text{-}9^B$ fathers and 58 from $Gp\text{-}9^b$ fathers; observed, see above; $p = 0.002$). One interpretation of this latter result is that other factors (e.g. paternal or maternal effects, sperm precedence or lower fitness of offspring from $Gp\text{-}9^b$ males) may also contribute to paternity skew towards $Gp\text{-}9^B$ fathers.

The previously reported worker-induced mortality of $Gp\text{-}9^{BB}$ queens and $Gp\text{-}9^B$ males in polygyne nests [17] acts as a strong selective force to maintain and facilitate the spread of the $Gp\text{-}9^b$ allele in polygyne populations, as well as the spread of this social form throughout the invasive range [27,46,47]. However, the disadvantages associated with harbouring this allele, especially with regard to the fitness of $Gp\text{-}9^b$ males, are obvious. The inability to maintain reproductive control over a mate represents a serious blow to reproductive success on its own, and the additional negative effects found in $Gp\text{-}9^b$ males in terms of lower sperm counts and reduced paternity in mixed broods exemplify the diversity of negative male

traits associated with the *Gp-9*^{b} allele. Together, these traits ensure that polygyne males contribute little genetic material to future generations, such that the vast majority of offspring from queens of both social forms are sired by monogyne *Gp-9*^{B} males.

The maintenance of the *Gp-9* supergene facilitates co-segregation of genes governing social organization in fire ants and concomitantly maintains variation detrimental to individual males. These results suggest that selection for maintaining linkage among genes controlling traits associated with colony social form, survival and maintenance in *S. invicta* apparently is stronger than selection acting on individual males, ultimately resulting in maintenance of variation at the *Gp-9* locus with detrimental individual fitness effects. One might predict selection acting to decouple the negative male fertility traits from the *Gp-9*^{B} supergene locus would be favoured, yet this clearly has not occurred. One simple explanation is that the *Gp-9* supergene is located within a genomic region in which strong selection acts to prevent breaking up of specific adaptive allelic combinations (e.g. inversion or other physical limitations to the chromosomal structural changes, pleiotropic effects or sharing of genetic pathways of some genes within the supergene), and selective pressure for increased male fitness is insufficient to alter this arrangement.

This study represents a first step into investigating the intricacies of supergenes, eusocial insects and conflicting selection pressures, yet more remains to be done to understand this system. Current efforts focusing on detailed analyses of the gene identity and content in this supergene region from numerous fire ant species may provide insight into how traits governing a wide array of phenotypes (e.g. social form, queen size, queen fecundity, queen dispersal ability and male sperm load) all came to be linked in *S. invicta*. Also, by examining the trade-offs between individual fitness and a selfish social gene, one can gain further insights into how cooperative breeding systems such as those found in eusocial Hymenoptera evolve [48] and the effects of these breeding systems on supergene evolution. Clearly, workers, which themselves represent a reproductive dead-end, can directly influence the reproductive individuals that will survive and reproduce [49], and enforce selective pressure on the *Gp-9* supergene region even when at odds with individual male fitness. As more studies emerge documenting pattern of linkage disequilibrium, genetic architecture and selection on supergenes, our understanding of the enigmatic nature of supergene regions and how they form and function will be greatly enhanced.

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