THE PROBLEM OF ESTIMATING SUPERGENE FREQUENCIES IN FUNCTIONAL MALES OF THE INVASIVE ANT *SOLENOPSIS INVICTA*

by

DANIEL HETTESHEIMER

(Under the Direction of Kenneth G. Ross and Brendan G. Hunt)

ABSTRACT

The red imported fire ant, *Solenopsis invicta*, exhibits a unique social structure influenced by genetic variation via a social supergene, a variant of which (*Sb*) is responsible for enhancing its own transmission in polygyne (multiple-queen) fire ant populations. This study expands upon the known selfish effects this supergene has on its transmission within queens by elucidating a significant underrepresentation of *SB* reproductive, haploid males in both pupal and adult stages, contrary to previous models which had relied on the assumption that *SB* and *Sb* males occur in equal proportions in nature. Absence of *SB* male execution by workers in the adult stage suggests the *Sb* supergene exerts selective pressures that distort haplotype frequencies during larval development. Quantification of supergene haplotype frequencies in reproductive males furthers our understanding of evolutionary forces affecting the *Sb* supergene in *S. invicta* and enables calibration of one route of inter-form gene flow.

INDEX WORDS: Ant, Supergene, *Solenopsis invicta*, Green-beard, Haplotype frequency, Gene flow, Population dynamics

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DEDICATION

To Bob, my grandfather, who inspired my love for insects and nature.

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I’m extremely grateful for my faculty mentors, Ken Ross and Brendan Hunt, without whom my academic journey to this point would not have been possible. A special thanks to Joe McHugh who introduced me to and nurtured my love for taxonomy throughout this past year.

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INTRODUCTION

Selfish genetic elements (SGEs) are DNA segments that propagate themselves in a population at the expense of other genes (Ågren and Clark 2018). This is achieved by subverting the normal rules of inheritance, for instance by distorting transmission ratios in their favor during meiosis, helping conspecifics that bear the element or harming those that lack it, and, in highly social insects, by biasing development of totipotent immature females toward fertile queens rather than sterile workers (Chapuisat 2023). These processes can occur even in cases in which they are detrimental to the organisms carrying them (Burt and Trivers 2008). SGEs are highly important in evolutionary terms because they influence individuals carrying them to behave in ways that encourage their transmission at the expense of other genomic elements, giving them the potential to greatly influence evolution within and divergence between populations (Werren, Nur, and Wu 1988; Hurst, Atlan, and Bengtsson 1996). In the red imported fire ant, *Solenopsis invicta*, a selfish genetic element is present in the form of a region of low recombination between structural variants (supergene) on chromosome 16. The supergene has two variants: *SB* and *Sb*, and colony social form varies depending on which alleles (haplotypes) are present in a colony (Wang et al. 2013). Queens carrying one copy of the *Sb* supergene variant (*SB/Sb* genotype) become functional reproductives in polygyne (multiple-queen) colonies, whereas queens lacking a copy of the *Sb* variant (*SB/SB* genotype) establish themselves as the sole female reproductive in monogyne (single-queen) colonies (Shoemaker and Ross 1996; Ross 1997; Krieger and Ross 2002).

In the polygyne form of *S. invicta*, the *Sb* supergene apparently acts selfishly in all life stages in females. At the onset of development, it biases its occurrence in fertile eggs relative to the *SB* variant likely via meiotic drive, although this effect apparently is counteracted by genes linked to *SB* that induce segregation bias in the opposing direction (Ross and Shoemaker 2018). Thus, despite this potential meiotic drive in individual queens, at the population level eggs from polygyne colonies occur in the expected Mendelian ratios of the *SB* and *Sb* supergene variants (50:50, because all reproductive queens of this form are *SB/Sb* heterozygotes). During larval ontogeny, possession of *Sb* increases the likelihood of female brood developing into potentially reproductive queens versus permanently sterile workers, thus enhancing its spread (Buechel, Wurm, and Keller 2014). In the adult stages, the *Sb* allele is present in every reproductive queen as the result of a combination of forces. Most conspicuous, in polygyne colonies, young queens (gynes) lacking *Sb* (*SB/SB* homozygotes) are subject to aggression and eventual execution predominantly by workers bearing *Sb*, discrimination behavior evidently mediated by a semiochemical blend found exclusively on *Sb*-bearing gynes once they reach sexual maturity (Keller and Ross 1998). This blend of cuticular compounds functions as a 'green beard' (Hamilton 1964; Gardner 2019); that is, it is a unique phenotypic feature (akin to a green beard that would allow for easy identification in humans) by which workers recognize and nurture gynes possessing a copy of *Sb*, while recognizing and killing gynes that lack it. In combination with the lethality of the homozygous *Sb/Sb* genotype in queens in the invasive range (Hallar et al. 2007), the result is that all polygyne reproductive queens possess the heterozygous *SB/Sb* genotype.

As in most ants, males of *S. invicta* are greatly understudied despite making up half of the breeding pool of individuals, presumably because they play minimal roles in the social life of the

colony. However, there are signs that the *Sb* social supergene may act selfishly in this sex as well. Fritz et al. (2006) suggested that *SB* males are underrepresented in polygyne colonies within the invasive U.S. range, a finding that would mirror the absence of queens homozygous for *SB*. Fritz et al. genotyped males at the protein-encoding gene *Gp-9*, a marker in complete linkage disequilibrium with the supergene (Ross 1997; Keller and Ross 1999). However, their method of typing males did not allow them to distinguish homozygotes from hemizygotes at *Gp-9*, a significant problem in this study system. Specifically, the unusual circumstance of a strong predominance of sterile diploid males over fertile haploid males characteristic of polygyne invasive *S. invicta* colonies (where 80% or more of the males in polygyne colonies are estimated to be diploid; (Ross and Fletcher 1985; Ross, Vargo, and Keller 1993)) means that the results of Fritz et al. (2006) are difficult to interpret and may not present an accurate depiction of supergene haplotype frequencies in reproductive (haploid) males in nests of the polygyne social form in the U.S.

The reasons for the uncharacteristic pervasiveness of diploid males in invasive polygyne *S. invicta* are many and involve the unique haplo-diploid sex-determination system of fire ants and all other species of the order Hymenoptera, as well as behavioral and ecological factors. In most Hymenoptera, the pairing of alleles at a single sex-determining locus is responsible for the fate of fertilized diploid eggs in a process called complementary sex determination (CSD) (Whiting 1943; 1945; Cook 1993). Under single-locus CSD, the general rule is that individuals with only one variant at the CSD locus are determined to develop into phenotypic males, while individuals bearing two alleles that complement one another (heterozygotes) are determined to undergo development as phenotypic females. The mother (queen) passes on one of her two CSD alleles (she must be heterozygous because she is female) to each of her eggs, which if they

remain unfertilized, give rise to haploid males (CSD hemizygotes), the typical source of male production in Hymenoptera. If the egg is fertilized by a male (sperm) bearing the same variant CSD allele as that contributed by the mother, a circumstance termed a "matched mating" (Adams et al. 1977), there is no complementation in the CSD homozygote, and the individual develops as an atypical diploid male.

The reason such males are atypical is that the CSD locus normally harbors very large numbers of alleles (Adams et al. 1977), so that the chances of a matched mating are low; further reducing the chances is the fact that strong negative frequency-dependent selection against diploid males equalizes CSD allele frequencies, the circumstance in which matched matings are minimized with a given number of alleles (Gloag et al. 2017). The source of such selection is the sterility of diploid males in most taxa with a CSD system and the lack of useful labor contributed to the colony by any males in social species. Being an invasive species, *S. invicta* experienced an extreme bottleneck while colonizing the U.S., losing allelic diversity at many loci, including the sex-determining CSD locus (Ross and Shoemaker 2008). As a result, matched matings are far more frequent in the U.S. than the native range (Ross et al. 1993). Worker fire ants evidently do not discriminate against diploid males to limit their harm to a colony's productivity, in contrast to, for instance, honey bees, workers of which destroy diploid male larvae soon after they eclose from the egg (Woyke 1980). Moreover, male diploidy is absent in monogyne *S. invicta* colonies due to extreme selection associated with the claustral mode of colony founding exhibited by this form, in which a single queen (sometimes more) initiates a colony without foraging (Tschinkel and Howard 1983). The burden of producing males at the expense of workers during the claustral period invariably dooms a young colony with a match-mated foundress to failure. In polygyne colonies, the majority of their multiple reproductive queens have not match-mated, so that

sufficient worker brood is produced to ensure a vigorous and viable colony (Ross and Fletcher 1986).

Our goal in this study was to determine whether haploid *SB* males are underrepresented relative to *Sb* males among the population of polygyne reproductive males in the U.S. as suggested by the study of Fritz et al. (2006). In doing so, we generate accurate supergene haplotype frequency estimates characterizing the male breeding pool in polygyne populations (because diploid males of *S. invicta* are sterile (Krieger et al. 1999) they are not part of that pool). Such robust estimates are central to understanding the complex evolutionary dynamics of the *Sb* supergene in its invasive range, as well as being useful for obtaining estimates of inter-form gene flow, which requires knowledge of haploid male gene frequencies at diagnostic loci (e.g., *Gp-9*). To achieve this goal with confidence requires distinguishing haploid from the far more numerous diploid males in a polygyne colony, necessitating the use of multiple robustly polymorphic genetic markers. By examining both pupae and adults, we hoped to identify any disparity in haplotype frequencies between life stages that might shed light on when *SB* males are lost. We also strove to identify a mechanism that explains the observed male haplotype frequencies and implicates the *Sb* allele in yet another strategy for inequitable self-propagation.

METHODS

Sampling

Whole *S. invicta* nests were excavated from the soil and placed in 19-liter buckets at various sites in Athens-Clarke Co., Georgia, U.S. during the spring of 2021. In total, 40 polygyne colonies were collected from the field and returned to our laboratory. Colonies were maintained in a controlled environment rearing room at a temperature of 29-32° C, relative humidity of 40-

60%, and a constant photoperiod of 14 hours of light followed by 10 hours of darkness (Ross, 1988). Ants were held in large (54 x 42 cm) plastic trays, the walls of which were coated with a polytetrafluoroethylene preparation, 60 wt. % dispersion in H_2O (MilliporeSigma, Burlington, MA) anti-traction compound to prevent escape. The trays held 14 cm diameter petri dishes containing moistened plaster bottoms that served as nests. Ants were fed several different food mixtures: a high-sugar mixture, a high protein mixture, and freeze-dried insects homogenized with water in a blender (Zeng 2022).

Male ants were collected by aspiration from each colony within three days of establishment in the laboratory in order to obtain a representative sample of the natural population of males in polygyne colonies in northern Georgia. Once collected, males were frozen (-80°C) in bulk in labeled, screw-cap microcentrifuge tubes corresponding to each colony pending measurement and genetic analysis.

Distinguishing haploid and diploid males

Our main objective was to obtain unbiased estimates of the social supergene haplotype frequencies for *S. invicta* haploid males from polygyne colonies in the invasive U.S. range, a challenging task because the great majority of polygyne males in this area (80-95%) are diploid (Ross and Fletcher 1985; Ross et al. 1993, 1996). Thus, our first goal was to parse out as many diploid males in the bulk collection as early in the pipeline as possible in order to reduce the potential time and expense of genotyping every male collected. According to Ross and Fletcher (1985), diploid and haploid males comprise two distinct but partly overlapping size distributions when comparing area of the mesoscutum (a hard cuticular plate on the dorsum of the mesosoma [thorax]). Their investigation also suggested that diploid males typically weigh more than their haploid counterparts, although again, weights of the two types overlap to some extent. Further

complicating matters, haploid males with the *SB* haplotype are significantly larger and heavier than those possessing the *Sb* haplotype (Goodisman et al. 1999).

Frozen males chosen for sub-sampling were thawed, blotted dry, then weighed individually to ±0.01 mg. Initially, males were selected haphazardly to create a preliminary weight histogram. This histogram depicted a bimodal distribution, expected to represent haploid (lighter) and diploid (heavier) males. This was confirmed by genotyping males at *Gp-9*, a marker on the *Sb* social supergene of *S. invicta* (Gotzek and Ross 2007; Wang et al. 2013) as well as at four microsatellite loci, as described below. Microsatellite genotypes of each weighed male showed that the frequency of haploid males approaches zero at 8.0 mg, a value in agreement with Ross and Fletcher (1985), who found that 100% of haploid males they collected fell below a weight of 8.4 mg. As a result, we sampled every male below 8.0 mg. During our initial sampling to create the weight histogram, several haploid males measured heavier than 8.0 mg, encouraging us to sample subsets of 20 males for every 0.25 mg increment between 8.0 mg and 10.0 mg to ensure that we did not truncate the upper tail of the *SB* weight distribution. Of the 160 additional males genotyped above the 8.0 mg threshold, no haploids were found. The importance of sampling the entire range of different-sized adult haploids is to avoid unintentionally excluding the largest haploid (*SB*) males and thus biasing our haplotype frequency estimate for polygyne males in the wild (Goodisman et al. 1999).

Pupal males were collected opportunistically along with the adults. Only white-bodied, pink-eyed pupae were collected in order to eliminate pupal age (and water content) as a factor affecting their weight. Because there were relatively few males of this life stage present in the 40 colonies, all that were collected were weighed and genotyped.

DNA extraction and Gp-9 genotyping

Gasters (post-petiole abdominal segments) were removed from adult males after weighing and DNA was extracted from the remainder of the body using the QIAGEN Puregene DNA extraction kit. DNA was extracted from the whole bodies of pupal males.

Extracted DNA was amplified using primers Gp-9_24bS, Gp-9_25bAS, Gp-9_26BS, and Gp-9_16BAS (Valles and Porter 2003). A multiplex PCR was run in 15 uL reactions containing 7.50 uL of Taq DNA polymerase (TaKaRa Premier Ex Taq HS, Cat# RR030), 0.15 uL each of Gp-9_24bS and Gp-9_25bAS primers at 50 uM, 0.30 uL each of Gp-9_26BS and Gp-9_16BAS primers at 50 uM, 4.50 uL of water, and 2 uL of DNA template (diluted 1:4 in water) (modified from Valles and Porter 2003). The reactions took place in a BIO-RAD T100 thermal cycler using the following program: 94°C, 2 min; 34x (94°C, 15 s; 55°C, 15 s; 68°C, 30 s); 68°C, 5 min; 10°C until terminated. The *Gp-9* genotype (haplotype) of each male was scored after running the PCR product out on a 1.5% agarose gel stained with ethidium bromide. The PCR product of the *B* allele of *Gp-9* (*SB* supergene haplotype) is 517 bp, while the product of the *b* allele (*Sb* supergene haplotype) is 423 bp (Valles and Porter 2003).

Microsatellite genotyping

Microsatellite genotyping was conducted using adult and pupal male template DNA in order to distinguish diploid homozygous from haploid hemizygous males, both of which yield single bands (PCR products) on gels in the *Gp-9* PCR assay. Male DNA served as templates for genotyping at four amply polymorphic microsatellite loci: *Sol42, Sol49, C536,* and *cassidy* (Ascunce, Bouwma, and Shoemaker 2009). PCRs were performed using primers Sol-42_for_VIC, Sol-42_rev, Sol-49_for_Fam, Sol-49_rev, Sdag-C536-F_PET, Sdag-C536-R,

cassidy_F_VIC, and cassidy_Rp. Amplification was conducted in a BIO-RAD Peltier Thermal Cycler-100 under the following program: 94°C, 90 s; 60°C, 45 s, -5°C per cycle; 72°C for 1 min; repeat cycle 9 times. 94°C, 30 s, 55°C, 45 s; 72°C, 1 min; repeat 24 times. 72°C, 1 min; 10°C until terminated. After the PCR was completed, the products were sequenced by the commercial service GENEWIZ (Azenta Life Sciences, Burlington, MA). Chromatograms were scored with the aid of Thermo Fisher's online cloud fragment analysis tool. Given the number and variability of the microsatellite markers (Suppl. Table S1), we estimate that among the males with single PCR products (bands) in the *Gp-9* assay, the probability of classifying a diploid homozygote at this gene falsely as a haploid hemizygote using the four microsatellites is 0.5%.

Correcting for social structure

There are a number of factors linked to social systems that have the potential to influence the results of population surveys. In our case, we wished to consider several factors that vary between colonies, such as colony-level effects on weight (due to any of a number of potential factors that vary between nests), genetic non-independence of samples (nestmate relatedness > 0), and uneven sampling of colonies, all of which can potentially skew the weight distributions or haplotype frequencies that we generated.

To account for genetic non-independence, we first calculated the genetic relatedness between all pairs of nestmate pupal and adult haploid males using the program PolyRelatedness V1.11b (Huang et al. 2015) and weighting individuals equally. The same microsatellite genotypic data used to determine ploidy of males were used to estimate haploid male nestmate relatedness, with the reference (base) population allele frequencies obtained from a sample of 172 wingless (reproductive) polygyne queens and 166 of their male mates from 22 nests collected in the same area where the study males were collected (see Wang 2014 and Weir et al.

2006 for discussions of the importance of a reference set). To visualize the potential importance of uneven sampling, we generated a bar graph depicting the numbers of samples of haploid males of each haplotype that we obtained from each of the 40 study colonies (Suppl. Fig. 1).

To account for the effects of genetic non-independence and uneven sampling on our haplotype frequency estimates, we took a resampling approach that weighted colonies equally (rather than individual males) while making full use of our data. We selected one male per colony at random to calculate population haplotype frequencies, then repeated this process (with replacement) 499 times. The 500 resampled values were averaged to estimate population haplotype frequencies, with the 2.5th and 97.5th percentile values taken to bound the 95% confidence intervals (CIs).

Male execution assay

We performed an assay to monitor the fate of males of both supergene haplotypes in the presence of polygyne workers. We targeted small to medium-sized males in order to perform the assay with as many haploids and as few diploids as possible. Adult males were sampled from fall-collected newly-established polygyne colonies in the laboratory, weighed, then individually isolated inside 0.6mL PCR tubes. A small hole in the bottom of each tube allowed workers to move freely into and out of the tube. Holes were made large enough to allow even major workers to pass in and out of the tube but not so large that males could pass through them.

Males placed inside the tubes were immediately returned to their colonies of origin by placing the tubes in the large foraging area of the tray outside of the nest. Worker/male interactions were monitored once every hour for the next 12 hours to determine how the males were treated by nestmate workers. If workers were observed biting or stinging a male, we removed the tube and scored the trial outcome as an execution. We also removed tubes with

intact or partially dismembered dead males that had been executed during the time between observation points. Attacked/executed males as well as males that survived until the end of the 12-hour observation window were frozen for subsequent DNA extraction and genotyping at *Gp-9* and the four microsatellites. We ran an additional execution assay using monogyne males in monogyne assay colonies to learn if our laboratory rearing conditions invariably provoke execution of males and whether there are general differences between the social forms in their tolerance of adult males.

RESULTS

Effect of supergene haplotype on male weight

We genotyped a total of 790 adult males and 188 pupal males at *Gp-9*; of these, 478 adults and 113 pupae with single stained bands (PCR products) in the assay gels, the ploidy of which was uncertain, were genotyped at the four microsatellite loci. The resulting 530 confirmed adult and pupal haploid males of each haplotype are depicted according to their weight distributions in Fig. 1, as are the remaining sampled (diploid) males.

Adult haploid males possessing the *Sb* supergene weigh significantly less than *SB* males; on average, *Sb* males weighed 6.12 mg while *SB* male weighed 7.07 mg ($p < 0.0001$, t-test). This result is expected, as Goodisman (1999) and Lawson et al. (2012) reported larger size and weight, respectively, of *SB* males compared to *Sb* males in polygyne colonies. It should be mentioned that because we avoided sampling the largest males in this study, we cannot accurately estimate the average weight for diploid males. However, among the males in our truncated sample, the average diploid male weighed more than the average haploid male (8.15 mg vs 6.28 mg, [$p < 0.0001$, t-test]) (Fig. 1).

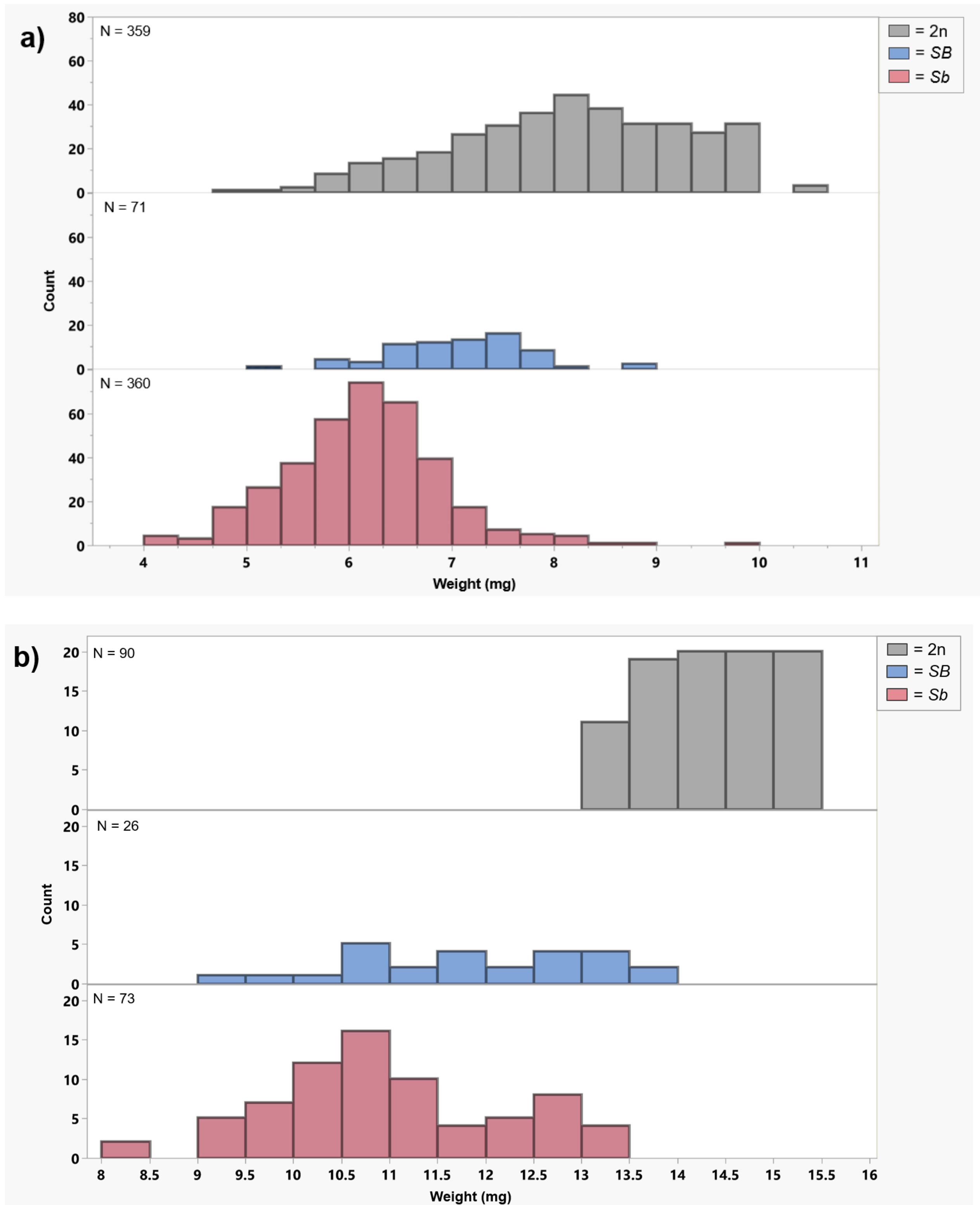

Figure 1. Male Weight. Male weight and supergene genotype for adults (a) and pupae (b) of the polygyne form of invasive *S. invicta*. The adult diploid (2n) histogram represents only a fraction

of such individuals originally collected due to our restricted sub-sampling of the highest-weight individuals.

Supergene haplotype frequencies in polygyne haploid males

Among the confirmed haploid males sampled from polygyne colonies in our study, those carrying the *SB* variant at the supergene are greatly underrepresented while those carrying *Sb* are greatly overrepresented (Table 1). In the adult stage, the observed uncorrected haplotype frequencies are less than 17% *SB* and greater than 83% *Sb*, far from the 50:50 ratio expected ($p < 0.0001$, binomial test) given that every polygyne reproductive queen in the invasive range is an *SB/Sb* heterozygote and males are impaternate (they represent the products of unfertilized haploid eggs). A similar but slightly less extreme imbalance in the supergene haplotype frequencies is seen in the pupal stage (Table 1). The sampled population of haploid pupae consists of fewer than 27% *SB* males and over 73% *Sb* males, frequencies that again differ significantly from 50% ($p = 0.0001$, binomial test). The differences in adult and pupal haplotype frequencies, although modest, are statistically significant (0.030, Fisher's exact test).

The haplotype frequencies corrected for non-independence of the genetic data and uneven sample sizes per colony (see Suppl. Fig. 1) by resampling the data are slightly less extreme than the uncorrected frequencies, but again are highly significantly different from 50%, judging from the 95% CIs (Table 1).

Table 1. Estimated male haplotype frequencies at the supergene in polygyne *S. invicta.* Adult and pupal haploid male supergene haplotype frequencies in the polygyne social form of invasive *S. invicta*. Both uncorrected and resampled frequencies are listed (with 95% CIs in parentheses). CIs for uncorrected data were estimated by bootstrapping the mean with 5000 replicates, while those for resampled data were obtained as described in the main text.

	Uncorrected data		Resampled data	
Life stage	*SB*	*Sb*	*SB*	*Sb*
Adult	**0.167** (0.136, 0.200)	**0.833** (0.798, 0.866)	**0.218** (0.214, 0.224)	**0.782** (0.776, 0.787)
Pupae	**0.263** (0.180, 0.350)	**0.737** (0.646, 0.818)	**0.314** (0.310, 0.319)	**0.686** (0.681, 0.690)

Testing for colony-level effects on adult male weight, we found that colony of origin has a similar magnitude of influence as the supergene haplotype of a male, with both factors highly statistically significant (both $p < 0.0001$, two-way ANOVA, $F = 6.1$ and 71.3; $df = 24$ and 1 respectively). Notably, of the total of eight haploid males heavier than 8.0 mg we discovered, four were from the same colony, an observation consistent with the presence of strong colony-level effects on male weight.

We estimated the average genetic relatedness of haploid male nestmates to be 0.198 (95% CIs: 0.192, 0.205) for adults and 0.169 (0.109, 0.220) for pupae, with the pupal estimate significantly lower than the adult estimate (tested by bootstrapping difference using single-colony values; 5000 iterations). Considering that brothers have a pedigree-based relatedness of 0.5 with a male-haploid genetic system, our estimates illustrate that at least some haploid male nestmates are quite close kin. Indeed, using equation (2) from Kümmerli and Keller (2007) and

assuming relatedness of zero for nestmate queens (Hale Walker et al. 2024), we estimate that effectively 2.5-3.0 queens per colony can explain the genetic diversity of nestmate haploid males included in this study, emphasizing again the genetic non-independence of nestmate males.

It is apparent that different polygyne colonies produce vastly different numbers of haploid males (Suppl. Fig. 1). In fifteen of the forty colonies obtained for this study, no haploid males were present, despite a mean of 10.8 haploid males obtained per colony. The two largest colonies accounted for 41.5% of the total haploid males sampled in the study. This highly uneven sampling likely explains most of the differences between the uncorrected and resampled allele frequency estimates.

Male execution assay

Results from our male execution assay showed that polygyne workers executed more than half of haploid males presented to them, while monogyne workers executed virtually none ($p < 0.0001$, Fisher's exact test) (Fig. 2). Among the polygyne haploid males, polygyne workers did not execute *SB* and *Sb* males at significantly different rates ($p = 0.255$, Fisher's exact test; *SB*: n = 13, *Sb*: n = 15). Thus, our male execution data do not reveal strong discrimination by polygyne workers against adult males lacking the *Sb* variant at the supergene. Unexpectedly, we found that polygyne workers executed a significantly higher proportion of haploid males (0.538 [95% CIs 0.357, 0.714]) than diploid males (0.198 [0.123, 0.274]) ($p = 0.0007$, Fisher's exact test). In addition, polygyne workers executed diploid males with the *Sb/Sb* genotype (0.469 [0.200, 0.733]) at the social supergene at a significantly higher rate than those possessing the *SB/Sb* genotype (0.144 [0.078, 0.222]) ($p = 0.008$, Fisher's exact test) (*SB/SB* males were too rare for statistical comparison with other diploid males). The absence of discrimination between adult haploid males based on their supergene haplotype, together with the quite similar haplotype

frequencies in pupal and adult haploid males, suggest that the haplotype frequency imbalance observed in both stages is likely to be imparted at earlier (e.g., larval) developmental stages. The lack of aggression against males noted in monogyne colonies indicates that male execution in the polygyne colonies is not an artifact of some feature of the artificial rearing environment.

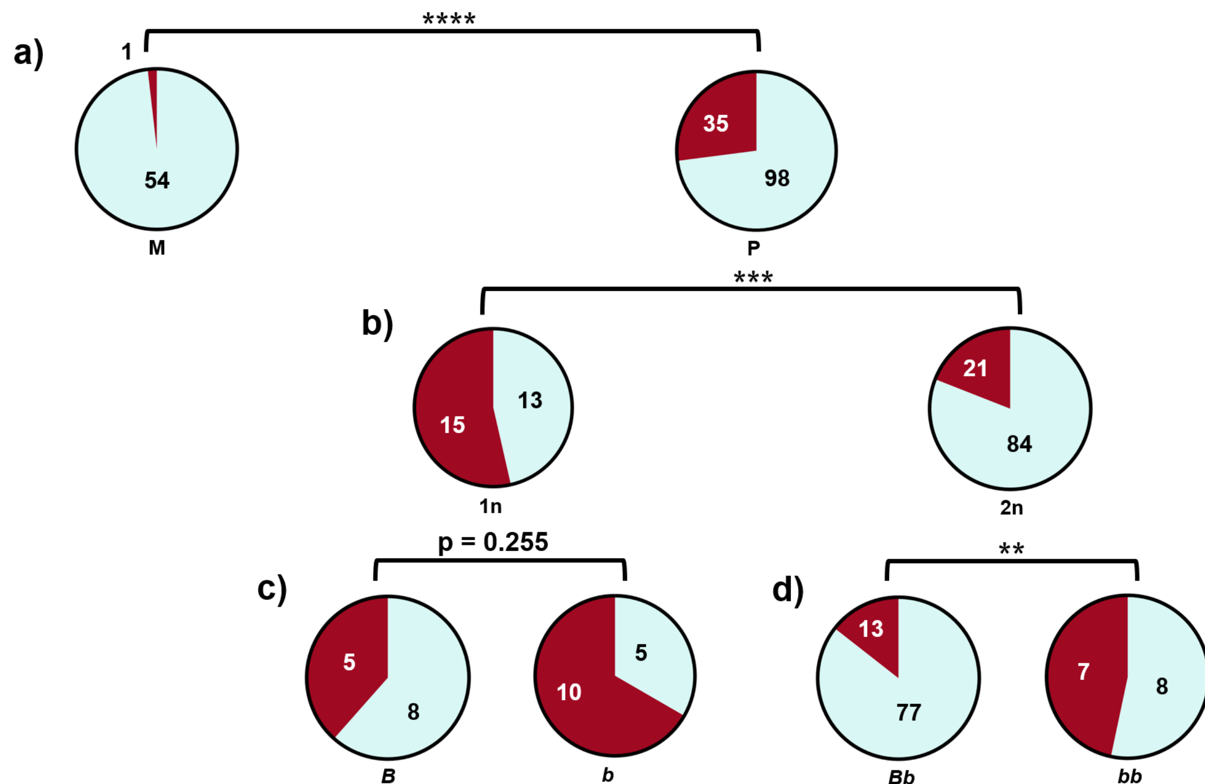

Figure 2. Male execution by workers. In each pie chart, the red sector represents the number of the total that were executed and the aqua sector represents the number not executed. a) Male execution rates in monogyne (M) versus polygyne (P) colonies of invasive *S. invicta*. b) Male execution rates for haploid versus diploid males within polygyne colonies. c) Male execution rates for the two supergene haplotypes within polygyne colonies. d) Male execution rates for two supergene genotypes within polygyne colonies. Fisher's exact tests: ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$.

DISCUSSION

Earlier studies of the genotype and allele frequencies in the different sexes and life stages of polygyne *S. invicta* have been instrumental in inferring how the selfish *Sb* supergene in this ant perpetuates itself and spreads within and between the social forms via the fundamental forces of selection, gene flow, and transmission bias (e.g., Goodisman et al. 2000; Ross 1997; Ross and Shoemaker 1997; DeHeer, Goodisman, and Ross 1999; DeHeer 2002; Ross and Shoemaker 2018). Such knowledge can in turn be useful in hypothesizing macroevolutionary pathways for the appearance and success of such selfish genetic elements using comparative methods with related taxa (e.g., Gotzek and Ross 2007, Chapuisat 2023). Woefully missing from this body of knowledge in fire ants is a basic population genetic parameter, frequencies of the selfish supergene variant in fertile males of the polygyne social form.

With much information detailing discrimination against *SB/SB* gynes by *SB/Sb* (polygyne) workers (Keller and Ross 1998; Ross and Keller 1998), Fritz et al. (2006) examined whether an analogous phenomenon might occur in reproductive males. The suggestive findings from that study motivated us to conduct a more rigorous study in which we accounted for the high frequency of male diploidy in the U.S. to accurately estimate the supergene haplotype frequencies among polygyne males in two life stages.

Determining the frequencies of this genetic element in polygyne males is important for helping complete the full picture of the dynamics of *Sb* transmission and spread, especially for calibrating estimates of the strength of one notable force in this system, gene flow between the social forms mediated by polygyne queens mating with monogyne males (e.g., Ross 1992, Shoemaker and Ross 1996, Gotzek and Ross 2007). An early diagrammatic model of the population dynamics by Ross (1997) generated to explain the observed genotype and allele

frequencies in the different female castes and life stages, as well as a formal population genetics model by Goodisman et al. (2000) intended for the same purpose, both assumed that the supergene haplotypes *SB* and *Sb* occurred at equal frequencies in polygyne haploid males in the breeding pool; this assumption was reasonable given that males are the haploid offspring of queens that invariably are heterozygotes in invasive polygyne populations. The authors of the formal model, in an attempt to explain its failure to require any role for the one route of inter-form gene flow strongly implicated by empirical studies, monogyne males mating with polygyne queens, conjectured that viability selection on polygyne males may distort the haplotype frequencies from 50:50, thus misdirecting the model output.

This particular route of inter-form gene flow had been predicted to be of central importance in the population dynamics based on several empirical observations: (1) monogyne colonies produce many more sexuals than polygyne ones (Vargo & Fletcher 1989) and the overwhelming majority of males produced by polygyne colonies are sterile diploids incapable of fertilizing a queen's eggs (Ross & Fletcher 1985, Krieger et al. 1999), (2) polygyne queens that mate with a *Sb* (polygyne) male almost invariably remate with a *SB* (typically monogyne) male, while polygyne queens that mate with *SB* males rarely remate (Lawson et al. 2012; Hale-Walker 2024), (3) proximity of abundant monogyne colonies predicts both the mating success of polygyne queens (a variable but substantial proportion remain permanently unmated) as well as the likelihood that they mate with a male of the monogyne form rather than their own form. Quantifying the magnitude of such inter-form gene flow relies on accurate estimates of haplotype frequencies in fertile males of both forms at informative markers such as the *Sb* supergene. Our robust empirical estimates of *Sb* supergene frequencies in males of the polygyne

form suggest that previous studies have underestimated the magnitude of gene flow between sympatric populations of the two forms (Suppl. Fig. 2).

The haplotype frequencies we estimated suggest that the *Sb* allele acts as a selfish genetic element during and/or before the adult and pupal stages of haploid males in polygyne *S. invicta* colonies. The underrepresentation of *SB* males in both life stages accords with the conclusions of Fritz et al. (2006), who did not distinguish haploid from diploid (sterile) males. The majority of the underrepresentation of *SB* males must be established during the larval stages since the haplotype frequencies are equal in the eggs (Ross & Shoemaker 2018) but significantly diverged from the expected 50:50 ratio by the early pupal stage.

The discrepancy between adult and pupal haplotype frequencies we found may be a sign of continuing selection that takes place sometime between the pupal and adult stages. This may involve a "green beard" signal associated with the *Sb* genotype (likely a cuticular compound (Zeng et al. 2022)) becoming more conspicuous to workers as males mature as adults, a phenomenon observed among queens (Keller and Ross 1993). However, this does not explain how the majority of discrimination against *SB* males is mediated in larvae, for instance by execution or neglect, although presumably another semiochemical linked to the *Sb* supergene is involved. Again, we note that the frequency of each haplotype in haploid eggs from polygyne queens is 50% (Ross & Shoemaker 2018), implicating selection at the larval stages as the main source of the observed haplotype skew in the adult males. Since our haploid frequencies suggest that the majority of the prejudice against *SB* males occurs before the pupal stage, the next step would be to explore the larval stages for a disparity between larval and pupal haplotype frequencies. Unfortunately, male larvae are indistinguishable from worker and queen larvae by

appearance alone and intense sampling and genotyping would need to be done in order to filter down to even a modest sample of haploid male larvae.

We were unable to determine the proximate cause of the haplotype skew in our execution assay designed to do so. Within haploid polygyne males, there was no statistically significant difference in execution rates between the two haplotypes. We expected to observe some bias against *SB* males by polygyne workers analogous to the well-documented phenomenon of such workers executing *SB/SB* adult gynes (Keller and Ross 1998). However, it is perhaps not surprising that we were unable to detect a difference, considering that most of the haplotype skew originates prior to the adult stage.

Most unexpected, however, was the disparity between execution rates of haploid versus diploid males in polygyne colonies, as workers killed diploids significantly less frequently than haploids. We surmise that this may be a result of physiological feminization of diploid males throughout development, a common phenomenon in diverse taxa with haplo-diploid sex determination (Giorgini, Monti, and Caprio 2009; Hitchcock, Gardner, and Ross 2022; Duchateau and Mariën 1995; Smith and Wallace 1971). This might include formation of an incomplete reproductive tract, development of genitalia bearing some female-like characteristics (Kerr and Nielsen 1967), and gene expression patterns similar to those of queens early in diploid male development that translates into semiochemical similarity (Hung et al 1974, Nipitwattanaphon et al 2014). The difference in execution rates between *SB/Sb* and *Sb/Sb* males is difficult to explain, even speculatively, due to *Sb/Sb* being a lethal genotype in queens that does not appear frequently in polygyne colonies, so there is no basis for how workers would be expected to treat *Sb/Sb* reproductives relative to other genotypes. The lack of male execution in our monogyne control colonies suggests that the execution observed in polygyne nests is

unlikely to be an artifact of the artificial environment in which the colonies were kept but likely represents some real biological phenomenon.

In *S. invicta*, the difference in weight observed between *SB* and *Sb* males, as well as between gynes with different supergene genotypes, appears to reflect more limited dispersal capabilities and reduced fecundity for reproductive individuals carrying the polygyne-affiliated (*Sb*) supergene variants (DeHeer, Goodisman, and Ross 1999; Keller and Ross 1999; DeHeer 2002). Such associations with limited dispersal and fecundity are also apparent for the independently evolved supergene system underlying polygyny in *Formica selysi* (De Gasperin et al. 2024).

With our findings, we have a more complete knowledge of the polygyne *S. invicta* breeding pool in the invasive U.S. range. We now know that the *Sb* supergene allele exerts additional evolutionary pressure on *S. invicta* by skewing the haplotype frequency of reproductive males in favor of males carrying a copy of *Sb* despite their inherent fitness disadvantages. Associating deleterious phenotypic traits with the advantageous transmissive properties of the *Sb* supergene variant is important for demonstrating the effectiveness of the supergene in controlling social structure and driving evolution in *S. invicta*, as well as for understanding why a supergene may spread despite harboring deleterious mutations that would otherwise hinder spread of an allele.

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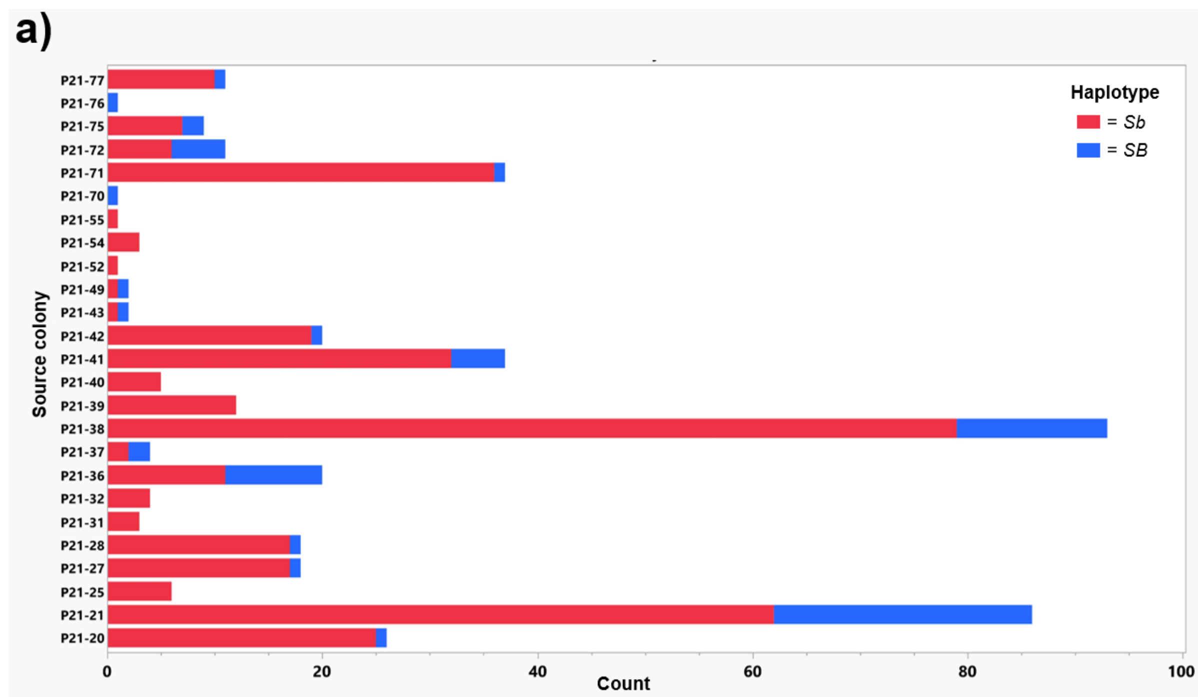
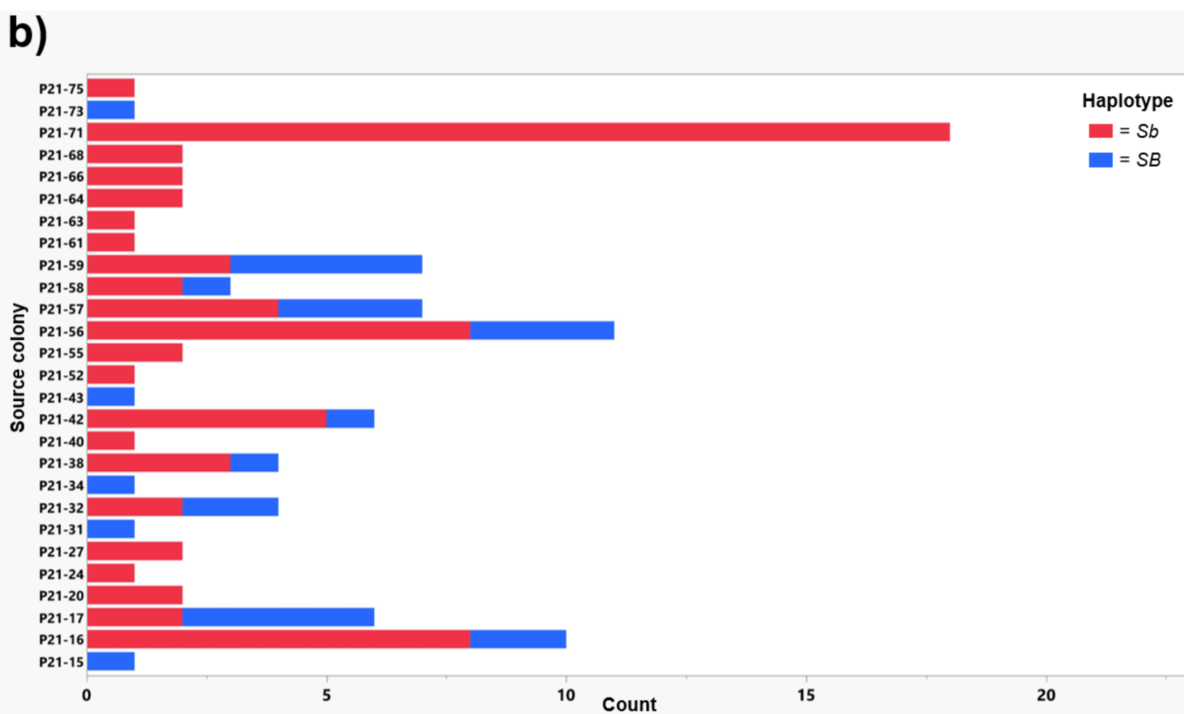
APPENDIX

Supplementary Materials





Suppl. Fig. 1. Haploid male distribution across colonies. Haploid a) adult and b) pupal males sampled from each colony.

Suppl. Table 1. Characteristics of microsatellite loci. Shown in the table are various microsatellite loci used to differentiate hemizygote haploids males from homozygote diploid males at *Gp-9*.

Locus	Count of alleles observed	Expected heterozygosity (H_{exp})[1]	Estimated prob. of HWE (H_{obs}=H_{exp})[2]	Estimated prob. of null allele[3]	Estimated genotyping failure rate[3]	Chromosome
Bertha	**7**	**0.643**	**0.682**	**0**	**0**	**3**
C27	**3**	**0.328**	**0.192**	**0.018**	**0.011**	**6**
***C294*[4]**	**6**	**0.696**	**0.000**	**—**	**—**	**16**
C536	**9**	**0.809**	**0.456**	**0.001**	**0**	**6**
Cassidy	**5**	**0.654**	**0.865**	**0**	**0.012**	**7**
i_109	**6**	**0.729**	**0.963**	**0**	**0**	**14**
i_114	**7**	**0.716**	**0.077**	**0**	**0**	**5**
i_120	**7**	**0.707**	**0.511**	**0**	**0.017**	**10**
***i_126*[4]**	**8**	**0.749**	**<0.001**	**—**	**—**	**16**
i_129	**5**	**0.323**	**0.413**	**0**	**0.075**	**4**
Sol_42f	**16**	**0.770**	**0.388**	**0.001**	**0.012**	**15**
Sol_49	**8**	**0.753**	**0.907**	**0**	**0.012**	**8**
Sunrise	**5**	**0.498**	**0.558**	**0**	**0.006**	**14**

$y_M = -([q_m/q_{m(P)}]-1)$

y_M is proportion of polygyne queen matings with monogyne males
q_M is composite frequency of *Sb* in mates of polygyne queens
$q_{m(P)}$ is frequency of *Sb* in haploid males of polygyne form

y_M	q_m	$q_{m(P)}$	*Study*
0.96	0.02	0.50	Fritz et al. (2006), *Site A*, 50:50
0.98	0.02	0.83	Fritz et al. (2006), *Site A*, 17:83
0.42	0.29	0.50	Fritz et al. (2006), *Site D*, 50:50
0.65	0.29	0.83	Fritz et al. (2006), *Site D*, 17:83
0.80	0.10	0.50	Ross (1997), 50:50
0.88	0.10	0.83	Ross (1997), 17:83
0.76	0.12	0.50	Ross & Shoemaker (2018), 50:50
0.86	0.12	0.83	Ross & Shoemaker (2018), 17:83

Suppl. Fig. 2. Effect of fertile polygyne male supergene haplotype frequencies on estimates of inter-form gene flow. Shown are comparisons of estimates of the proportion of matings of polygyne queens attributable to monogyne males (y_M) from three studies that illustrate the effect on this parameter of varying polygyne male haplotype frequencies ($q_{m(P)}$). The top row in each comparison shows the results when the haplotype frequencies are assumed to be equal, while the bottom row shows the results when haplotype frequencies are those calculated for adult polygyne males in this study.