MATERNITY APPORTIONMENT IN MULTIPLE-QUEEN COLONIES OF THE FIRE ANT *SOLENOPSIS INVICTA*

by

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(Under the Direction of Kenneth G. Ross)

ABSTRACT

Organisms have utilized group living as a strategy for enhancing their survival and reproduction for millennia. A notable species displaying the most advanced form of group living, termed eusociality, is the fire ant *Solenopsis invicta.* This species is socially polymorphic, having two social forms genetically determined by a large supergene. Our goal is to explore the evolutionary implications of *S. invicta* colonies having multiple egg-laying queens (polygyny). We show that: 1) reproductive skew, or unequal sharing of maternity among nestmate queens, invariably occurs when there is more than one reproductive queen contributing to offspring production; 2) skew is significantly more pronounced for daughters of the sexual caste than daughters of the sterile worker caste; and 3) overrepresentation of the *Sb* supergene occurs in the sexual daughter caste, but not the worker caste. This study illuminates important elements of queen reproductive hierarchies and colony genetic structure in polygyne *S. invicta*.

INDEX WORDS: Reproductive skew, *Solenopsis invicta*, Polygyny, Eusociality, Supergene, Caste biasing

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INTRODUCTION

Group living occurs widely in the animal kingdom, with species as diverse as lions, naked mole rats, emperor penguins, honeybees, and snapping shrimps displaying social habits (Rubenstein & Abbot, 2017). Factors influencing the origin and maintenance of social behavior have intrigued biologists since Darwin, with the major benefits of social life thought to be increased protection against predation, increased efficiency in obtaining scarce or unique resources, and the general benefits of division of labor in accomplishing tasks crucial for survival and reproduction (Hamilton, 1971; Alexander 1974; Rubenstein & Abbot, 2017). Along with the benefits attributed to social behavior come costs, most notably, the increased risks of disease transmission and the emergence of reproductive conflict and competition within groups (Cremer et al., 2007; Bourke & Ratnieks, 1999; Nonacs, 2011; Ratnieks et al., 2006). Importantly, such conflict is expected in any group not composed of genetically identical individuals because of the divergence in reproductive interests between non-clonal colony-mates, with the strength of selection for cheating intensifying with increasing genetic dissimilarity.

Social behavior has evolved repeatedly in insects (Wilson, 1971; Hughes et al., 2008; Ross & Matthews, 1989; Costa et al., 2006), and its most extreme form, eusociality, is found in termites, ants, some bees, and some wasps. The defining feature of eusociality, division of reproductive labor, entails the more-or-less permanent sterility of the majority of group members, and in many eusocial Hymenoptera it is accompanied by extreme specialization and morphological differentiation between the reproductive and sterile individuals (queens and workers, respectively, in these functionally all-female societies) (Hölldobler & Wilson, 1991).

Such dramatic modifications for social life represent a crucial landmark in the evolution of biological complexity on earth (Maynard-Smith & Szathmáry, 1995), one result of which has been the unrivaled evolutionary success and ecological dominance of eusocial insects (Wilson, 1990).

Fire ants are advanced eusocial insects notable for their populous, long-lived colonies and remarkable degree of caste dimorphism (differentiation between queens and workers), which features a complete absence of the ovaries, and consequent permanent sterility, in individuals of the worker caste (Tschinkel, 2006). The red imported fire ant, *Solenopsis invicta*, is an invasive species in the U.S. and other Pacific Rim countries that has emerged as a model for studies of social evolution for several reasons. The ants are abundant in the invasive range, easy to collect, and are amenable to experimental manipulation, and an extensive background literature on their social biology has been produced over the past few decades owing to their significant pest status (Tschinkel, 2006; Ross et al., 2023). Most important for its role as a model organism for research on social evolution, *S. invicta* and related fire ant species exhibit two distinct forms of colony social organization with well characterized genetic underpinnings. The presumed ancestral type, termed the monogyne form, features colonies containing only a single fertile egg-laying (reproductive) queen, while the alternate, polygyne, form features colonies with several to many dozens of such queens (Gotzek & Ross, 2007; Yan et al., 2020; Kay et al., 2022). The differences between the two forms, which include numerous life-history, reproductive, and behavioral traits in addition to the difference in colony queen number, are associated with a well-established genomic distinction at one of their 16 haploid chromosomes.

Genomic studies of invasive *S. invicta* populations over the past decade have revealed that colony social form invariably is associated with the presence or absence of a supergene on the so-called 'social chromosome' (chromosome 16) (Figure 1). The derived *Social b* (*Sb*) supergene variant of Chr16 is found in most members (in all reproductive queens) of polygyne fire ant colonies but is missing among the inhabitants of monogyne colonies, in which the alternate, ancestral-type haplotype (*SB*) is fixed (Ross, 1997; Ross & Keller, 1998; Wang et al., 2013). The variant *Sb* haplotype consists of three inversions that together span ~11.4 Mb on the distal arm of chromosome 16 and combined contain almost 500 genes. Because the *Sb* variant contains these major chromosome structural rearrangements, it rarely recombines with *SB*, meaning that the two social forms are reproductively isolated and on separate evolutionary trajectories at this unique portion of their genome (Figure 1). Importantly, presence of the *Sb* haplotype is associated with many individual traits of workers and sexuals, some of which appear to decrease individual fitness (Tschinkel, 2006; Ross et al., 2023). This same genetic basis for the alternate social forms of *S. invicta* is known to occur in a half-dozen closely related South American fire ant species (Yan et al., 2020; Kay et al., 2022; Stolle et al., 2022).

Because fire ant queens are effectively singly mated, colonies of the monogyne form are simple families composed of closely related (r=0.75) full sisters, while colonies of the polygyne form have a more complex genetic structure, consisting of numerous matrilines, each usually composed of full sisters that are unrelated to members of other matrilines (Figure 2) (the nestmate queens heading each matriline usually are unrelated in the invasive range [Ross et al., 1996; Ross & Shoemaker, 2018])[1]. The strong asymmetries in relatedness within and between matrilines signal colony genetic structure conducive to the emergence of reproductive conflict. Indeed, the expected balance of conflict and cooperation embodied in the stable existence of these multiple-queen societies has been viewed as recapitulating the origin of eusociality with

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[1] Males play no meaningful role in the social life of a fire ant colony and we do not consider them here.

respect to the need to overcome the costs of reproductive competition to realize the benefits of cooperation among individuals of varying genetic relatedness (Crozier, 1994; Ross & Keller, 1995; Keller, 1993; Ross et al., 2023).

An important common element in the origin of rudimentary social behavior as well as the emergence of polygyny secondarily in eusocial animals is the role played by uneven sharing (apportionment) of reproduction among potential or actual reproductive individuals in a society. A large body of theory and empirical work united under the umbrella term 'Reproductive Skew Theory,' has emerged over the past several decades with the goal to explain patterns of skew by analyzing fitness payoffs for individuals comprising the groups. Although the enterprise is widely interpreted as having failed to produce a unified theory that is broadly predictive of variation in apportionment of parentage, it provides a useful conceptual and operational framework for conducting descriptive empirical studies that can yield otherwise useful data on life histories, breeding systems, and social selection (Azevedo-Silva et al., 2020; Ross et al., 2020; Dahan et al. 2021).

The existence of vast differences in potential breeding success (reproductive skew) among nestmate queens in polygyne *S. invicta* was already noted soon after the discovery of polygyny in invasive populations of this species. Most obviously, fully one-third of wingless 'reproductive' queens of the polygyne form are unmated and thus incapable of producing daughters in this male-haploid species (Fletcher et al., 1980; Ross et al., 1996). Although such queens potentially could produce most or all of a colony's haploid males from their unfertilized eggs, a laboratory study indicated that most of the eggs produced by these queens are inviable (non-embryonated or trophic eggs) (Vargo & Ross, 1989). Their contribution to male production in natural polygyne colonies, if any, has not been determined, but will be the subject of a subsequent report based on data from the experiment described here. A second group of potential reproductively compromised queens are those that mated with a male sharing one of their alleles at the CSD (complementary sex determination) locus, a mating that results in half of the queen's fertilized eggs being predetermined to develop as sterile diploid males rather than workers or queens. An earlier laboratory study of semi-natural polygyne colonies (similar in design to the experiment described here) indicated that such diploid-male-producing queens are not necessarily at a disadvantage, relative to other mated queens, in terms of production of fertile sexual offspring (Ross, 1988). This same study suggested that strong differences in lifetime reproductive success, manifested as persistent reproductive skew in the maternity of daughter queens, might occur even among mated queens not producing diploid males, a conclusion consistent with results of a follow-up field study of the genetic structure of polygyne colonies (Ross, 1993).

In this project, we adopted a conceptual framework rooted in reproductive skew theory to design a comprehensive experiment assessing the nature, causes, and consequences of skew in maternity apportionment in polygyne *S. invicta* colonies. Among the several advantages of this study system are the ability to compare skew patterns between the worker (sterile) and queen (reproductive) offspring derived from replicate sets of nestmate mother queens, and the ability to track these patterns over time. As expected, if reproductive competition is important, we document substantial skew in our study colonies, especially with respect to the production of sexual relative to worker daughters, with the observed skew sufficiently stable that it likely translates into lifetime fitness differences that distinguish reproductive 'winners' and 'losers.' We attempt to link such fitness differences to measurable traits of queens such as weight and fecundity, in order to advance understanding of the proximate causes of skew and the nature of selection acting on these factors (Nonacs & Hager, 2011). Additionally, we integrate data on the proposed effects of the *Sb* supergene on caste representation within matrilines (Buechel et al., 2014) to better understand how this unique layer of added complexity is superimposed on existing patterns of maternity skew. Together, our results depict a complex system of social, genetic, and behavioral factors that interact to shape colony social and reproductive structure in multiple-queen colonies of *S. invicta*.

METHODS

Ant Collection and Rearing

Ten polygyne *S. invicta* colonies were collected in their soil nests in 5-gallon talc-dusted buckets in the field in Athens-Clarke Co., Georgia in February of 2018. Each nest was spaced at least 20m from other collected nests. Upon return to the laboratory, the ants were separated from the soil by slowly dripping water into each bucket to force the ants to float on the water's surface, enabling transfer of the relatively intact colonies into plastic enclosures (26×40×8 cm trays) containing 14-cm-diameter round plastic nests with dark covers and moistened plaster bottoms (Jouvenaz, 1977; Ross, 1988). The enclosures with their live ants were maintained in a controlled-environment insectary in which the photoperiod (14hrs L : 10hrs D), temperature (32°C), and humidity (85% RH) were held constant. Colonies were provisioned with water and food (a high-protein diet [tuna/dog food/peanut butter mix], a high-carbohydrate diet [assorted vegetables/granulated sugar mix], and freeze-dried crickets and mealworms) (Ross, 1988; Trible & Ross, 2015). Each experimental colony was confirmed to be of the polygyne social form by finding multiple wingless (reproductive) queens; 58 of the 65 such queens chosen for the experiment were recovered at its end or shortly after their death during the experiment, and each of these was found to possess the polygyne-specific *Sb* supergene haplotype, as revealed by the presence of the diagnostic $C294^{092}$ allele upon microsatellite genotyping (Ross & Shoemaker, 2018).

Experimental Procedures

Colony Set-up. Once the colonies were habituated to the insectary (2-3 days), all sexual forms (males and gynes[2] in the 4th instar larval, pupal, or adult stages) were separated and removed from each colony by sieving the ants with a #14 grade geological sieve (1.4mm openings). To further prepare the ten colonies for use in the experiment, the number of reproductive queens was then reduced to five in half of the colonies and to eight in the remaining half. The 65 reproductive queens retained were uniquely marked using a single or two spots of Markal® Ball Paint (Wojcik et al., 2000) on the dorsum of the alitrunk to serve as a unique identifier by which each could be distinguished through the course of the study.

Four major sampling periods spanned the length of the study, with each period culminating in a sample point at which worker and gyne pupae were collected for genotyping (Figure 3). At the start of the first sample period in April 2018, all ten colonies were sieved a second time to remove all sexual adults and brood that had appeared since the initial sieving. This was done to ensure that all of the subsequently appearing gyne pupae and young adults were daughters of the focal (paint-marked) queens rather than offspring carried over from previous resident queens. The time of this second sieving is considered as Day 1 of the experiment. The period between each pupal sampling point was approximately 45 days. This duration was chosen to balance the need for relatively even sample coverage over the expected 6-9 month study with a desire for sufficient temporal resolution to detect significant short-term changes in queen fecundity or productivity, as were found in a similar earlier study (Ross, 1988).

Sample and Data Collection. At each sampling point marking the end of a sample period, white, pink-eyed pupae of both workers and gynes were collected. These even-age cohorts were known

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[2] We use the term gyne to refer to a morphological queen that has not yet shed her wings, mated, or begun laying eggs (any life stage).

to be derived from eggs laid approximately 20 days (workers) or 27 days (gynes) previously based on reported brood developmental periods at temperatures similar to those in our insectary (Markin et al., 1975). Therefore, we weighed all queens, measured their oviposition rates, and sampled their eggs 20 and 27 days prior to each collection of the worker and queen pupal samples, ensuring that we tracked each queens' condition at the time eggs yielding her genotyped worker or gyne daughters were laid. Oviposition tests were performed by isolating queens in small specimen cups with moistened plaster bottoms for 4 hours (Fletcher et al., 1980), at which point the eggs were counted then frozen at -80°C.

For each of sample periods 2-4, we instituted an additional weigh-in of the queens at a point about halfway between the previous pupal sampling point and the subsequent weigh-in/oviposition test linked to the next pupal sample (Figure 3). The additional weigh-in was done to improve temporal resolution of the dynamics of queen oviposition rates without stressing queens unduly with extra oviposition tests (weight and fecundity are highly correlated in *S. invicta* reproductive queens—Ross, 1988) (Figure 4).

Either 95 (5-queen colonies) or 190 (8-queen colonies) pink-eyed pupae of each caste were collected at each of the four principal sampling points; in some instances, sufficient numbers of gyne pupae were not available, so dark pupae or newly emerged alates were collected to make up the difference. The resulting range of age differences of up to about 10 days is unimportant for most analyses; for others in which it is important, the analyses were done both with and without these older individuals included. Upon completion of pupal sampling, all remaining sexuals of all stages were removed from colonies to prevent carry-over to the next sample point.

Following conclusion of the fourth sampling period in October 2018, additional samples were collected at varying intervals from most colonies on an opportunistic basis. Up to three of these supplemental samples were collected (in two of the colonies) before most focal queens in a colony had died and the colony was weakened to the point of failure. The two longest-surveyed colonies were sampled seven times each over a period of 15 months. In this report we focus on the first four (principal) samples.

Colonies were inspected daily to discern the general health of the colony based on the amount and type of brood, as well as to locate every queen to ascertain her well-being. Queens that died between observations or that clearly were being attacked by workers (which inevitably leads to their death; Keller & Ross, 1998) were immediately collected and frozen at -80°C pending subsequent genotyping. Seven queens (10.8% of total) died without their bodies being recovered, so genetic data are not available for them or the sire(s) of their female offspring.

Microsatellite PCR and Genotyping. All collected samples designated to be genotyped were transferred individually into single PCR-microplate wells for genomic DNA extraction performed using a modified high-throughput procedure (Turelli & Hoffmann, 1995). This stock genomic DNA solution was diluted 10:1 then used as template in multiplex PCR reactions to obtain multilocus genotypes at 13 variable microsatellite loci (Ascunce et al., 2011). One primer of each locus primer pair was labeled at the 5' end with one of four fluorescent dyes (FAM, PET, NED, VIC; Applied Biosystems). Primer pairs were combined in multiplex reactions by taking into account PCR thermal cycling profiles, dye labels, and expected size of the PCR products. The complete set of 13 loci was amplified in three separate 12µL PCR reactions, each containing Hot-Start Taq 2X Mastermix (Denville Scientific), 0.06-0.4µM of each member of 2-5 pairs of primers, 2µL of DNA, and water. The thermal cycling profile was as follows: one cycle at 94°C (60s); followed by 35 cycles at 94°C (30s), primer-specific annealing temperature (45s), and 72°C (60s); followed by a single final extension at 72°C (40min). Resulting PCR amplicons were diluted (1:100 to 1:200) and pooled into a single plate for sequencer injection. GeneScan 600 LIZ size standard (0.1µL) was added to all pool-plex dilutions, which subsequently were run on an ABI-3730XL 96-capillary sequencer (Applied Biosystems). One well in each 96-well microplate contained water in place of DNA template to serve as a negative control.

Genotypes of most of the focal queens as well as their mates were obtained by extracting DNA from the queen heads and from the contents of their spermathecae (Lawson et al., 2012). Because polygyne *S. invicta* generally mate with a single haploid male (91% of the queens in Ross & Shoemaker [2018] and in this study), it is relatively straightforward to predict their female offsprings' multilocus genotypes using the genetic data from one or both parents.

The 13 microsatellite loci exhibit considerable variation, with each possessing 3-11 alleles and expected heterozygosities in the range 0.29-0.82 (Table 1). Moreover, 11 of these loci occur on ten of the 15 non-supergene chromosomes, ensuring their largely independent segregation (confirmed in Ross and Shoemaker, 2018) and, thus, enhancing their information content for matriline assignments. The remaining two loci are linked to the *Sb* supergene, *C294* completely (it occurs within one of the supergene inversions) and *i-126* less so (it occurs near the centromere on the non-supergene arm of Chr16) (Figure 1) (Ross & Shoemaker, 2018). Thus, these two markers could be used as well to study the interaction of *Sb*-induced caste bias with patterns of caste bias stemming from reproductive conflict and skew in maternity apportionment.

Microsatellite genotypes were scored from sequence chromatograms with the aid of the software GENEMARKER (SoftGenetics). All chromatograms were inspected to confirm the accuracy of the software genotype calling, with special attention paid to suspect calls. We note that artifactual errors in genotype scoring caused by allelic drop-outs (non-amplification of one allele in heterozygotes) or by DNA contamination of samples must be very rare in our study.

Careful examination and analyses of the patterns of multilocus genotypes in progenies typically fathered by a single haploid male, as we performed, would readily reveal evidence for such artifacts. This, combined with the substantial allelic variation at the microsatellite loci (Table 1), limited number of potential parents, and, in most cases, availability of the parental genotypes, gives us confidence in our ability to assign virtually all genotyped offspring to the correct matriline (and patriline).

Data Analyses

Matriline Assignments. The matriline composition in each colony initially was hypothesized based on patterns of pairwise genetic relatedness (*r*) obtained for all nestmate daughters sampled, combined with information on the potential parental genotypes for the colony. Maximum likelihood estimates of relatedness were obtained using the program MLRelatedness (Kalinowski et al., 2006), with a specified separate reference population consisting of a set of 174 polygyne reproductive queens and 169 of their haploid male mates from northeastern Georgia (from Ross and Shoemaker 2018 and this study). The pairwise estimates of *r* were sorted and displayed graphically in relatedness "heatmaps" in order to recognize and reconstruct preliminary family groupings (Figure 5). A series of "if-then" statements in Microsoft Excel based on diagnostic multilocus genotypes were then used to determine the likely parental genotypes for each family, which were matched to the empirically determined genotypes for the potential parents in that colony when these were available. Additional checks that such assignments were correct included manually verifying mendelian segregation ratios in hypothesized matrilines at loci for which the putative mother queen was heterozygous (patrilines in the few instances of polyandrous queens) and affirming invariant homozygosity for an allele when it was the sole allele recorded at that locus in the putative parents of each daughter. The few unresolved matriline assignments remaining after these procedures generally involved aberrant genotypes at one or very few loci; in such cases, the original chromatograms were inspected to verify or reject suspect genotype calls by the Genemarker software. Information on each mother queens' longevity and egg production rates through the experiment were helpful in assigning or excluding these problem specimens. Because most specimens left unassigned by this point in the process were missing data at multiple loci, all specimens with five or more locus dropouts were excluded from further analysis. In the end, only 1.8% of specimens with sufficient genotypic data could not be assigned confidently to a matriline (Table 2) and were omitted from further study.

Measurement of Maternity Skew. A variety of different measures of variation in parentage among reproductive individuals in social groups have been proposed (Nonacs, 2000; Ross et al., 2020). We chose two complementary metrics. The S_3 skew statistic (Crozier & Pamilo, 1996) is a simple static variance estimator with values that range from zero (reproduction shared equally among nestmates) to 1.0 (one individual completely monopolizes reproduction). The M statistic of Ross et al. (2020) is a multinomial likelihood estimator that is unbiased with respect to differences in group or sample sizes, and has an added advantage that the skew metric can be measured over extended time periods even with dynamic group composition. This is ideal for our purposes, because we wished to assess the likely existence of long-term reproductive "winners" and "losers" for colonies in which queens died sporadically throughout the course of the experiment. Mortality information from the daily colony inspections, combined with the relatively fine-grained resolution of our queen weigh-ins and oviposition tests, allowed us to parse out the residency periods (relative exposure times) for each queen, during which they could produce eggs, and thus to obtain reasonable skew estimates relevant to individual lifetime reproductive fitness. Values of *M* range from negative (more even sharing of reproduction than predicted from a random multinomial null model), to zero (reproduction is shared as equally as expected under a null model), to positive values (uneven apportionment of parentage). Finally, the genetic impact of the observed maternity skew was assessed by comparing values of the metric Q_E, the effective level of polygyny (or effective queen number) (Crozier & Pamilo, 1996). Q_E is a hypothetical quantity corresponding to the number of queens that would produce the mean observed colony genetic diversity assuming queens shared equally in reproduction (no maternity skew).

Variation in maternity apportionment in our experiment can be of several types; nestmate queens may differ from one another in their shares of reproductive success (gyne production), that is, classic skew; individual queens may differ in their allocation of daughters to each caste (within-matriline caste skew), and both of these types of skew may be dynamic, such that a queen's production of daughters of either caste can vary across sampling points (temporal skew). To help unravel the importance of the temporal dimension relative to the single-timepoint concepts of skew, we assessed values of the *M* statistic for nestmate queens at single sample points and compared these to *M* values obtained for each queen individually across sampling points. Only queens present in the same sets of at least four individuals across at least three consecutive sampling points were used for this analysis. A hierarchical randomization test in which the groups were reallocated within each colony (1000 iterations) was used to assess the significance of differences between means for the two sources of variation (between queens and between samples) separately for each offspring caste (Lock et al., 2021).

Predictors of Queen Reproductive Success. We examined the relationship between oviposition rate and proportional reproductive shares estimated from the relevant samples of worker and gyne offspring using simple least-squares linear regression of the individual queens' maternity apportionment on fecundity. Proportion data usually are not suitable for linear regression, but we justify its use in this case on the following bases: 1) our model does not predict impossible values, with predicted y-values falling between 0.109 and 0.920 across the range of observable x values for the worker plot; and 2) when the extreme values of fecundity (<0.10 or >0.90; 31% of the data points) were omitted, the slope and y-intercept remained virtually unchanged from what was obtained for the full worker data set, indicating that the regression line does not flatten and deviate much from linearity at extreme values of x (fecundity), thus retaining its predictive ability across the range of x values.

We next assessed the ability of several different individual queen characteristics monitored throughout the experiment to predict the condition of queens that displayed unusually high gyne maternity apportionment at particular sampling points, termed 'gyne producers.' These variables included queen weight, oviposition rate, time of death relative to the focal sample, relative production of workers in the preceding sample, and various derivatives or composites of these simple variables (see Table 3). Queens selected for the analyses met the following criteria: 1) they produced more than twice the number of gyne pupae in the focal sample than the preceding sample, 2) they produced more gyne than worker pupae at the focal sample, and 3) they did not substantially increase their worker production from the preceding to the focal sampling point. For comparison, we selected other queens that we term 'worker producers.' These queens: 1) produced one third or more of the colony's total worker output at the focal sampling point, and 2) produced fewer than half as many gynes as workers among their offspring at the focal sampling point. A preliminary survey of the 15 variables for their predictive potential employed a series of simple Mann-Whitney U-tests conducted on a preliminary, incomplete data set; the four variables yielding significant or near-significant results in this first round were then formally analyzed using our full data set with both Mann-Whitney and bootstrap tests (5000 iterations).

Assessment of *Sb* Supergene Association with Offspring Caste. An earlier laboratory study of *S. invicta* by Buechel et al. (2014) reported a significant bias in the segregation ratios of supergene haplotypes for gynes within colony fragments headed by single queens. Specifically, the *Sb* haplotype was consistently over-represented in gynes from eight full-sister progenies (significantly so in five), whereas it segregated in the expected 1:1 ratio with the homozygous S*B*/*SB* genotype in workers from the same progenies (the eggs provided to each unit were derived from single polygyne mothers, all of which are S*B*/*Sb* heterozygotes [Gotzek & Ross, 2007]). Although compelling, the findings of that study can be strengthened by replication featuring a more natural experimental design and larger sample sizes; our experiment offers the ability to test this important result by estimating segregation ratios in single matrilines in a more natural social context. Our experimental colonies preserved normal polygyne colony social, genetic, and age structure and were reared in seminatural conditions in the world's premier fire ant breeding facility. Because all reproductive polygyne queens in the U.S. are heterozygotes for *Sb*, all of those that mated with a single *SB* male (88% of queens in this study) are expected to produce female offspring of both castes in a 1:1 ratio of *SB/Sb* heterozygotes to *SB/SB* homozygotes. The atypical queen that mates with a *b* male generally mates subsequently with a second male bearing the *B* allele (Lawson et al., 2012). In the case of such polyandrous queens, one must look at the segregation ratio in the *SB*-sired patriline rather than the entire matriline.

Our test of the Buechel et al. results was feasible because the microsatellite locus *C294*, located on the distal arm of Chr16 within the supergene region, has an allele ($C294^{92}$) only and invariably found in the variant *Sb* haplotype (i.e., they are in complete linkage disequilibrium [LD]) (Ross & Shoemaker, 2018). A second locus, *i_126*, also resides on Chr16, but is located outside of the area containing the inversions, on the opposite arm near the centromere (Figure 1). It, too, is in LD with the *Sb* haplotype, although the association is weaker than for *C294*, with the allele most commonly encountered on *Sb* (i_126^{230}) occurring in only 67% of *Sb* haplotypes (Ross & Shoemaker, 2018). A low rate of recombination in the proximal centromeric region containing *i-126* (but greater than at the inversion regions) is the likely explanation for this pattern.

We counted the number of gyne and worker offspring that bore the $C294^{92}$ allele (*Sb* haplotype) in each relevant progeny of sufficient size; relevant progenies were sired by *SB* males and comprised the great majority (90%) of full-sib families in this study. We also counted the pupae of each caste that bore allele i_126^{230}; strong predictions unique to this marker can be made, if the *Sb* haplotype is indeed involved in biased caste representation, owing to the fact that the marker is not as tightly linked to *Sb* as is *C294*. Statistical analyses thus were conducted to test the following hypotheses:

1) Genotypes with $C294^{92}$ and i_126^{230} should occur in Mendelian (1:1) ratios in full-sister progenies (matrilines, patrilines) of the worker caste derived from heterozygous mothers,
2) *C294* and *i_126* genotype proportions should deviate significantly from Mendelian ratios in favor of alleles $C294^{92}$ and i_126^{230} in full-sister progenies of the gyne caste,
3) *C294* and *i_126* genotype proportions in gynes of a progeny should deviate from the proportions in the worker caste of these same progenies, with i_126^{230} occurring at a population frequency intermediate to 0.5 and the frequency of the $C294^{92}$ allele in this caste.

All statistical tests used matriline (patriline) or colony-level genotype proportions as input data, with a matriline included in the tests at this level only if >10 pupae of each caste had been collected. The fact that most progenies analyzed consisted of both castes provides added power to our analyses by virtue of the matched nature of the data.

RESULTS

Scope of the Data

Information about the primary data generated for this study is shown in Table 2. Female offspring in ten colonies were sampled at four points, with the final sample being taken at day 185 after the start of the experiment. A low but steady rate of queen mortality occurred over this 6-month timespan, with 47 of the original 65 marked reproductive queens (72.3%) alive at the time of the final sample. Eight of the original queens were unmated; these queens and the issue of parentage of males in the study colonies are not considered further in this report. All queens were subjected to oviposition tests and were weighed at each of the four sampling points, with an additional three weigh-ins conducted at midpoints between the regular four. Over 5000 pupal worker offspring and over 3000 gyne offspring were sampled and successfully assigned to matrilines on the basis of their multi-locus genotypes at 13 microsatellite loci.

Patterns in maternity apportionment

Distributions of maternity skew estimates over the entire experiment using two different metrics are shown separately for each offspring caste in Figure 6. The distributions clearly are not centered at zero (no skew) using either estimator, nor are many values for individual mother queens close to zero; instead, the distributions are offset positively, with many values considerably greater than zero, especially with respect to gyne offspring. Considering that positive values for both indices scale according the degree of skew, it is clear that maternity skew is prominent in these colonies and evidently is greater for the maternity of sexual than worker daughters, consistent with the conclusions of earlier laboratory and field studies (Ross, 1988, 1993).

Dissection of the various components of overall skew is important for understanding its proximate causes and genetic significance. Gynes are the currency of fitness in fire ants (workers are obligately sterile), so it is imperative to consider skew separately for the two female castes, to disentangle factors that may influence skew in both castes from those acting uniquely on one caste or the other, as well as to appreciate how differential skew can shape patterns of colony genetic structure (nestmate relatedness). Estimated values for the *S3* skew metric at each sampling point reveal a consistent trend of greater maternity skew for the gyne than the worker offspring produced by a colony at a given point in time (Figure 7 a-c), mirroring the general results above, and the many cases of such differential skew occur in all colonies and at all sampling points. Estimates of the *M* skew statistic calculated for single sampling points yield a similar result (Figure 7d), while the estimates of *M* that cover the course of the experiment, accounting for individual queen residency times over that period, indicate that every colony's long-term skew in its gyne production exceeds that in its worker production (Figure 7e).

The impact on colony genetic structure of such differential maternity skew according to offspring caste can best be understood by estimating the effective queen number, $Q_\mathbf{E}$, a hypothetical quantity corresponding to the number of queens that would generate the observed genetic diversity (relatedness) in a colony were there is no skew (Crozier & Pamilo, 1996; Pamilo & Crozier, 1996). Estimates of $Q_\mathbf{E}$ typically are substantially lower than the actual queen number (Figure 8a), again implicating maternity skew in most samples, with the estimates derived from worker pupae generally (79%) greater than those derived from gyne pupae, as expected (binomial test for equal representation; p<0.001). On average, $Q_\mathbf{E}$ derived from worker pupae is 68% of the actual queen number, while $Q_{\mathbf{E}}$ derived from gyne pupae is 51%. From a population genetic diversity perspective, the effect is as if only half of mated reproductive queens contributed to the subsequent Mendelian generation (daughter gynes). In terms of colony genetic structure, nestmate worker relatedness increases from the expected r=0.25 with no skew to an estimated r=0.34 in the low-queen-number (3-queen) colonies, but gyne relatedness rises to r=0.43 in these same colonies, due solely to differential maternity skew (Figure 8b) (see Ross [2001] for methods of calculation). In higher-queen-number (7-queen) colonies, the rise due to skew is from the expected r=0.11 to r=0.16 for workers but to r=0.28 for gynes. Clearly, maternity skew is an important factor determining patterns of intra-colony relatedness in polygyne fire ants (Ross, 1988, 1993).

While measurement of skew and effective queen numbers indicate widespread, well developed inequities in maternity apportionment in these multiple-queen societies, they do not reveal how this skew arises. Is the large overall skew in gyne production due mainly to strong but transient dominance of maternity by one or a few queens, or are patterns of maternity usually relatively stable, signaling durable differences in the shares of gyne production among nestmate queens that can be equated with lifetime reproductive fitness? Assessment of skew through time in individual colonies is essential in order to answer this question. As a first approach, we compared skew estimates for nestmate queens in single samples with skew across sampling points for individual queens. The results in Figure 9 show that the temporal component of variation is generally small compared to the inequities seen between nestmate queens at any given point in time, consistent with the common occurrence of relatively stable, long-term differences in the reproductive competence of individual queens. The disparity is much stronger for gyne than worker offspring, consistent with the notion of long-term dominance or subordinance patterns being more common with respect to gyne than worker production. Inspection of individual queen reproductive patterns in all colonies (Figure 10) reveals that persistently strong production by individual queens does indeed occur with some frequency (e.g., Colony P18-1, Queen C; P18-4, E; P18-9, E), but low or even no gyne production (e.g., P18-3, H; P18-9, B; P18-12, H) is of equal or even more importance; both patterns contribute to the relatively lower across-sample than between-queen skew estimates. Importantly, occasional short-term flushes of gyne production by individual queens also can be discerned (e.g., P18-3, F; P18-4, C; P18-12, E), a fact we exploit below to evaluate possible correlates of short-term reproductive success. Such brief, intense bouts of sexual production were also reported in the earlier study of Ross (1988), where they often were associated with prior weight loss and subsequent death of the mother queen. We identified six queens that exhibited all of the features of this 'weight loss - death' syndrome, including a surge in production of gynes, in our experiment (Figure 10).

A general problem in quantifying the variable patterns described above is that the skew statistics, as commonly used, consider neither the identity of reproductive individuals nor temporal dynamics in these individuals' productivity. Because different configurations of reproductive hierarchies can yield similar skew-values, such limited analyses cannot directly confront the issue of whether relatively high queen reproductive success is due to long-term gyne-maternity specialization of individual queens, to brief, perhaps terminal, attempts to dominate gyne production, or both. Long-term monitoring of identifiable reproductives in a colony, such as in our experiment, solves the problem but at the cost of complete naturalness of the experiment (e.g., queen number must be limited). We quantified relative gyne production (reproductive success) specifically in colonies whose pools of (mated) queens survived for the entire term of the experiment (P18-3, -4, -7, -9, and -12). Funnel graphs depicting the results for these five colonies (Figure 11) reveal extraordinary skew in the long-term reproductive success of queens, with the single top ‘winner’ queens in each group of 4-7 responsible for 38-54% (mean=46%) of the colony’s total gyne production. The two most successful queens in a colony were responsible for 67-78% of this production (mean=75%). The poorest-faring queens typically produced fewer than 10% of all daughter gynes, with some producing none at all. Some of these same ‘loser’ queens produced prodigious numbers of worker pupae (e.g., Colony P18-3, Queen H; P18-4, D; P18-7, C; P18-9, C; P18-12, D), contributing to the disparity in skew-index values between worker and gyne offspring.

Comparison of Figures 10 and 11 reveals that ‘winner’ queens, those with the highest overall gyne production, only occasionally had this production concentrated at one or a couple adjacent sampling periods (e.g., P18-4, C; P18-9, A; P18-3, G). That is, high-fitness queens generally were responsible for production of a large share of gynes over a substantial portion of the experiment. Importantly, many of the ‘loser’ queens, which by definition produced very few gynes, also consistently failed to produce many worker offspring over the course of the experiment. The combination of many queens with simultaneously high worker and gyne production and queens that are poor producers of either can explain the seemingly contradictory finding that production of the two castes by individual queens relative to the overall population is significantly correlated at each of the four sampling points (Spearman r=0.32 to 0.42; n=44-56; all p<0.01) despite the strong differences in degrees of skew between the castes. In total, our results suggest that the question of whether ‘winner’ queens display overall differences in lifelong fitness-related traits linked to gyne specialization or, instead, represent individuals surveyed across snapshots of concentrated gyne production, is perhaps naïve; both “strategies” appear to exist. Nonetheless, the presence of temporal dynamics in gyne production evident for some individual queens provides us the ability to investigate possible biological correlates of such short-term reproductive success.

Predictors of Short-term Queen Reproductive Success

We conducted a series of analyses to identify measured factors predictive of short-term reproductive success of queens. A simple first step was to ask whether production of workers, gynes, or both is a mere function of oviposition rate, a possibility given that individual queen's gyne and worker production covary significantly at each sampling point (see above). We examined the relationship between oviposition rate, which varies widely among nestmates at any time (Figure 4), and proportional reproductive shares estimated from the relevant samples of worker and gyne offspring. Simple linear regression fits a line with a slope near 1.0 and *y*-intercept near 0.0 to the worker production data very well—over 65% of the variance in the data is explained by this simple model (Figure 12a). The implication of this striking relationship is that the number of eggs produced by a queen strongly predicts her relative contribution to the colony worker force in the near term, suggesting a seemingly minor influence of the nursing workers on the matrilineal composition of the worker brood. In contrast, a simple linear model fits poorly the relationship between oviposition rate and gyne production (Figure 12b), suggesting that any of a myriad of factors other than queen fecundity significantly influence matriline representation among these sexuals.

Further investigation was guided by the earlier work of Ross (1988), who reported bouts of gyne overproduction by 'winner' queens preceded by queen weight (fecundity) loss and followed shortly thereafter by their deaths. Our investigations made use of several characters monitored throughout our experiment but not measured in the previous study. Variables included queen weight, oviposition rate, time of death relative to the focal sample, and various derivatives/ combinations of these simple metrics (see Table 3), along with the queen's relative share of worker pupae in the preceding sample. The latter can be equated with the portion of colony workers of an age appropriate to act a brood nurses (Tschinkel, 2006) to the pupae in the focal sample, the importance of which is that workers in the majority may be able to act nepotistically to favor sexualization of their full sisters during larval feeding (sexual larvae of *S. invicta* are fed more, or different, food than worker larvae during development [Tschinkel, 2006]). Queens identified as 'gyne producers,' which individually produced a disproportionate number of sexuals at a particular time compared to their production otherwise, were identified based on criteria other than those contributing to the variables in Table 3. For comparative purposes, we identified 'worker producer' queens that were responsible for much of the worker production at a given sampling point (see Methods). Individual queens did not appear more than once in either group.

Among the more than a dozen potential candidate variables, only four showed any promise as predictor factors during preliminary surveys and, among these, only one showed a statistically significant relationship with concentrated, elevated gyne production. This variable was the change in queen fecundity, relative to that of her nestmates (Figure 13), from the preceding sampling point (variable $\Delta rf_{0\text{-}1}$ in Table 3). Gyne producers exhibited significantly lower values than worker producers (bootstrap test, 5000 iterations, one-tailed *p*=0.011; Mann-Whitney U=154.5; *p*=0.037), pointing to below average fecundity gains over the prior 6-week period compared to their nestmates, or even (for 11 of the 20 queens) fecundity declines. Notable for their lack of association with gyne production are queen weight and fecundity at the time of the sample, consistent with the regression results presented above showing a weak relationship between fecundity and subsequent gyne production. Mortality just before or after the focal sample point also was not associated with high gyne production, consistent with the relative rarity of queens displaying the weight loss-death syndrome described by Ross (1988).

Importantly, when the six queens identified as displaying this syndrome in our experiment are considered separately from the remaining 14 gyne producers, three additional predictors become significantly different between the two classes of queens. All of these relate to changes in fecundity or weight in the weeks leading up to the focal sample, with the joint implication that queens exhibiting the syndrome experience relative declines in fecundity but not proportional weight changes just prior to their high gyne production. The total of six such queens out of the 57 possible (mated) queens in our study (10.5%) corresponds well to the frequency in the Ross (1988) study, in which eight of 70 mated queens (11.4%) fit the description.

Association of Sb *Supergene with Offspring Caste*

A by-product of this study of maternity apportionment is segregation data fortuitously obtained for two *Sb* supergene-linked microsatellites in single-family progenies. The production of such data is relevant because, among the various selfish features of this green-beard supergene (Ross et al., 2023), it has been implicated in biasing development of *Sb*-bearing female larvae toward the queen rather than the worker caste (Buechel et al., 2014). With our study design, we have available large numbers of colonies, mother queens, and progenies comprising both castes that afford us considerable power to test possible biased segregation ratios for the *Sb* supergene using the completely linked *C294* locus. As well, data for a second microsatellite locus, *i_126*, which displays weaker linkage disequilibrium with *Sb* than does *C294*, allows us to test other strong predictions of the *Sb*/caste bias hypothesis.

The distributions of daughters of each caste bearing allele $C294^{92}$ (*Sb*) in individual matrilines (or patrilines), colonies, and the entire population are presented in Figure 14a-c. A comparison of the population distributions for alleles $C294^{92}$ and i_126^{230} is shown in Figure 14d. With respect to the hypotheses posed in the previous section:

1) In workers, proportions of genotypes with either $C294^{92}$ or i_126^{230} occur in Mendelian (1:1) ratios in 91-100% of full-sister progenies derived from heterozygous mothers (binomial tests, *C294*: *n*=41 of 45 progenies; *i_126*: *n*=15 of 15 progenies) (Figure 14a). As a point of comparison, for the 11 non-*Sb*-linked loci in this study, daughter progenies (castes combined) derived from heterozygous queens segregated in Mendelian ratios in 90.4% of cases (*n*=384 of 425 progenies).
2) In gynes, proportions of genotypes with $C294^{92}$ or i_126^{230} occur in Mendelian ratios in only 19% (*C294*) and 50% (*i_126*) of full-sister progenies (*C294*: *n*=29 of 36 progenies; *i_126*: *n*= 5 of 10 progenies). The deviations are invariably in favor of these *Sb*-linked alleles.
3) Genotype proportions for both loci in gynes differed from the proportions in the worker caste at the population level, highly significantly so for *C294* and marginally so for *i_126* (1-tailed bootstrap tests for paired values, *n*=37 and 13, $p<0.001$ and $p=0.049$, respectively). Over 70% of gynes at the population level possessed allele $C294^{92}$ (*Sb*) compared to about the predicted Mendelian 50% found for workers; also as predicted, i_126^{230} occurs in gynes at a frequency almost exactly intermediate to these two values

(61%) and, again, in workers it occurs at near 50% (Figure 14d; note the much smaller sample size for *i_126* than *C294*)[3].

The population frequency of gyne pupae bearing *Sb* (78%) from the earlier study of Buechel et al. (2014) does not differ significantly from our estimate of 68-72% obtained using *C294* (one-way fixed effects, 2-tailed randomization ANOVA test, 1000 iterations, p=0.36) . These authors also found the frequency of workers with the *Sb* haplotype to be close to the expected 50%. The combined results from the two studies show unequivocally that the *Sb* element in invasive polygyne *S. invicta* consistently is overrepresented among gyne but not worker progeny with respect to the normal laws of inheritance.

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[3] Sample sizes are considerably smaller for this locus than for *C294* because all polygyne reproductive queens are $C294^{92}$ heterozygotes that typically mate with males lacking this allele and thus produce offspring segregating the appropriate genotypes for this analysis (a heterozygote with the focal allele and a homozygote without it).

DISCUSSION

This work comprises an extensive investigation of reproductive skew, that is, how maternity shares are apportioned, among nestmate queens in the multiple-queen (polygyne) form of the fire ant *Solenopsis invicta*. Sexual (gyne) and sterile (worker) daughters produced in ten laboratory colonies were assigned to individual matrilines headed by 5-8 resident queens at each of four sampling points distributed evenly across the 6-month study period, so that maternity apportionment (skew) among queens could be quantified periodically. Ancillary information on queen condition and survivorship was collected in coordination with the progeny sampling. Reproductive skew is considered to be a prominent factor molding the selective forces at play during social evolution, primarily through its effect on within-colony genetic (kin) structure (Reeve & Ratnieks, 1993; Crozier & Pamilo, 1996 book; West et al., 2007; Hodge, 2009; Ross et al., 2020; Nonacs & Hagar, 2011). Social Hymenoptera are especially prone to asymmetries of relatedness between subgroups within a colony because of their male-haploid genetic system, setting the stage for intra-colonial conflict and competition between genetically dissimilar groups with divergent evolutionary interests; this conflict plays out in many different sectors, including offspring sex-investment ratios, timing of production of new sexuals, the role of workers in producing sons, and the partitioning of reproduction among queens or their male mates (Reeve & Keller, 2001; Crozier & Pamilo, 1996; Bourke, 2015; Southon et al., 2019). Reproductive skew theory consequently has emerged as a body of theoretical and empirical work to address the role of reproductive skew and the strength of its influence on social evolution (Ratnieks et al. 2006; Nonacs & Hagar, 2011).

The evolution of polygyny from monogyny in ants is often considered to recapitulate the origin of eusociality with respect to the evolutionary forces involved (Crozier, 1994; Ross & Keller, 1995; Keller, 1993; Ross et al., 2023), meaning that studies such as ours may be useful in identifying such forces. Colony social and genetic structure in fire ants is in many ways ideal for assessing the magnitude and nature of maternity skew. Fire ant workers are obligately sterile, so investment in them can yield only indirect fitness returns to queens. Nestmate queens generally are singly mated and unrelated in our study population, which maximizes the relatedness asymmetry between workers in the same (r=0.75) and different (r=0) matrilines, and provides powerful incentives for workers to practice nepotism and queens to behave selfishly (cheat). Yet the colonies appear outwardly to function as efficient, well-lubricated superorganisms.

Reconciling this apparent contradiction requires detailed examination of the reproductive apparatus of the superorganism as an arena for both competition and cooperation among different kin-groups. While reproductive skew theory arguably has effectively explained relationships between unequal sharing of parentage and such factors as genetic relatedness of group members, ecological conditions, and opportunities to manipulate competitors, such theory has been less successful in predicting how the opposing tendencies of competition and cooperation are actually resolved in terms of allocation of reproduction (Nonacs & Hager, 2011). Thus, renewed emphasis has been placed on characterizing well developed model systems on a case-by-case basis in order to bolster the empirical backdrop on which the theory rests (e.g., Hodge, 2009). In so doing, it is important to disentangle the observed components of skew, including the temporal dimension. Our study is well suited to the task, employing a long experimental period, frequent large samples, relevant ancillary data, and assessment of skew separately in the two offspring castes. We note that the need to identify predictors of skew based on measurable characteristics of the individuals involved has been emphasized as an important step forward in any system (Nonacs & Hager, 2011).

We found, in agreement with earlier laboratory and field studies, that reproductive skew is extensive in polygyne fire ant societies. Prominent skew among nestmate queens occurred in their production of daughters of each caste at single points in time and, to a lesser extent, variation (skew) occurred in the reproductive output of single queens across the six-month study period. Most importantly, we confirmed the finding of an earlier, prototypical laboratory experiment (Ross, 1988), and supported by a field study (Ross, 1993), that the extent of skew was considerably more pronounced for gyne (future queen) daughters than for worker daughters, a phenomenon reported as well in other social Hymenoptera (Queller et al., 1993; Pamilo & Seppä, 1994; Kümmerli & Keller, 2007; see also Rüppell et al., 2002). This pattern is important for several reasons. First, because workers of *S. invicta* are sterile, production of gyne offspring is the only route to direct fitness returns through daughters in polygyne colonies. Thus, when coupled with the fact that nestmate queens generally are unrelated (Ross et al., 1996; Ross & Shoemaker, 2018) so that indirect fitness returns to them are small, it may be expected that competition for maternity of gynes is intense in these colonies. Second, differential skew by caste results in different colony genetic structures for workers and queens, with effectively fewer queens represented as mothers for the pool of sexual daughters (e.g., Figure 8). Such a difference in this central arena for genetic conflict may trigger the action of unique selective forces acting on the breeding system, resulting in a feedback loop of spiraling, increasingly selfish, genetic interactions among queens; one potential outcome of the resulting intensifying reproductive cheating could be the spawning of social parasitism and inquiline species (Dahan & Rabeling et al., 2022). Finally, the differences in levels of maternity skew between progeny castes raise the important question of what mechanisms might be responsible.

In addressing the issue of mechanisms of differential caste skew, it is helpful to characterize the individual queens that contribute to such skew. We expected, based on the earlier results of Ross (1988), that queens dominating gyne production in their colonies would predominantly do so via concentrated pulses of gyne production that coincided with reductions in their maternity of workers. In fact, while we did identify over a dozen such queens, including six that apparently conformed to the weight loss-death syndrome described by Ross (1988), similar numbers of queens with high reproductive success over the term of the experiment had substantial numbers of their gyne offspring reared over most of the sampling periods, often accompanied by many worker daughters as well. Thus, there may be at least two alternative strategies for high lifetime reproductive success in these ants, one involving one or more pulses of high gyne production coupled with reduced worker maternity and the other featuring sustained high levels over long periods, not necessarily with a concomitant reduction in worker production. We speculate that the few examples we found of queens exhibiting the weight loss-death syndrome may have been older queens embarking on their final of a series of gyne-intense bouts of reproduction (see also Jaimes-Nino et al., 2022), but the variable (and often long) periods of survival of many such queens after producing a flush of gynes hints that this is not a simple age-related phenomenon. All these scenarios would seem to require that queens exert some influence over offspring caste, that is, that maternal effects play a role.

The possibility of maternal effects bears on the nature of the complex mechanisms of caste determination in *S. invicta*, which involve variation in the eggs produced by queens (e.g., trophic vs. embryonated eggs, haploid vs. diploid embryonated eggs, diploid embryonated eggs homozygous vs. heterozygous at the CSD locus) combined with worker intervention during brood rearing by feeding and grooming or cannibalism (see Passera et al 2001). The temporal dynamics to skew in gyne production that we found for some queens, featuring discrete periods of higher and lower success, allowed us to infer if any of the measures of queen condition we obtained were associated with flushes of gyne production. We found that, among many variables examined, drops in the relative fecundity of queens in the few weeks preceding laying of the eggs for the focal sample were strongly associated with flushes of gyne production. This decline in the pace of oogenesis compared to nestmate queens in the period running up to overproduction of gynes is consistent with the suggestion that queens can affect the caste fate of fertilized eggs by provisioning them with some RNA, protein, and/or hormone supplement(s) (Petersen-Braun 1977; Ross 1988), the process of which presumably requires a slowdown in egg production. The finding of another variable that predicts gyne production in the restricted group of queens displaying the weight loss-death syndrome hints that the relative slowdown or absolute drop in fecundity in gyne producers is not related to changes in weight (nutrient reserves) in the same way as in the worker-producing group; the higher values for the gyne producers suggest that weight changes are much less responsive to fecundity changes in this group, pointing to possible fundamental differences in the energy dynamics involved in egg production in the two groups.

This evidence for a blastogenic component to caste determination does not obviate a role for workers in influencing the caste fate of fertilized eggs. Indeed, an interaction between queens and workers mediated by queen-produced pheromones acting in aggregate to alter worker behavior toward potential sexual brood has been demonstrated in *S. invicta* and other ants (Vargo & Fletcher, 1986; H. Zeng unpublished manuscript), and such a system could be manipulated in a particular queen's favor. However, the seemingly most likely mode of worker intervention is nepotism, whereby workers preferentially feed larvae of their own matriline and/or cannibalize larvae of other matrilines to bias colony gyne production in favor of their sisters (especially likely in view of the colony kin structure of invasive polygyne *S. invicta*). A straightforward prediction of this scenario is that a pattern of relative over-representation of sexual progeny of a given queen should be preceded by relative over-representation of worker progeny of the same matriline in the earlier sample. No evidence for such a relationship was found in the study of Ross (1988), nor did we find it here; specifically, no difference exists between the two queen groups with respect to their relative success in worker production in the period preceding the focal sample, regardless of the exact composition of the gyne-producer group (Mann-Whitney U tests, all $p>0.27$; bootstrap tests, 5000 iterations, all $p>0.31$).

An unusual feature of maternity skew in *S. invicta* is the superposition of caste biasing associated with a selfish genetic element (the *Sb* supergene) reported by Buechel et al. (2014) and confirmed here. While our estimate of the magnitude of the bias using the marker *C294* is somewhat less than the original estimate (68-72% vs. 78% of gyne progeny bearing the *Sb* haplotype; Figure 14c), both studies show near perfect Mendelian ratios (1:1) of *Sb*-bearing and non-*Sb*-bearing worker pupae. In addition, our study reveals a similar but weaker bias at a second marker located on the social (*Sb*) chromosome but outside of the inversions comprising the supergene (*i_126*), yet near 1:1 ratios predominate at this locus in workers, and at the 11 other loci located on different chromosomes in both castes. Moreover, the frequency of the *Sb*-linked *230* allele of *i_126* in gynes is exactly intermediate between the frequency of the completely linked $C294^{92}$ allele in gynes and the expected 50%, which occurs in workers using any of the markers (Buechel et al. [2014] used a third marker locus for the supergene, *Gp-9*, which is in the same inversion as *C294* and, unsurprisingly, is in complete LD with the *Sb* haplotype). These results leave little doubt as to the reality of the phenomenon and raise two important points.

The first is that the caste bias is yet another phenomenon demonstrating the selfish genetic tendencies of the *Sb* element, which previously has been implicated in greenbeard-like worker discrimination among queens (Keller & Ross, 1998; Trible & Ross, 2016; Zeng et al., 2022) transmission ratio distortion in diploid embryos (Ross & Shoemaker, 2018), reduced fertility of *Sb* compared to *SB* males (Lawson et al., 2012), and, possibly, polygyne worker discrimination against *SB* males in favor of *Sb* males (Fritz et al., 2006). Second is the issue of how this bias is achieved. Both our study and that of Buechel et al. (2014) surveyed the pupal stage; this leaves open the possibility of either intrinsic differences in the developmental tendencies of the eggs laid by queens or differential behavior of the workers in sexualizing female larvae containing the *Sb* element (or both) as proximate causes. Additional experiments on earlier larval stages will be required to distinguish these possibilities, difficult as they will be given the abundance of diploid male brood in polygyne colonies and lack of markers to distinguish sex prior to the pupal stage and female caste prior to the fourth larval instar. This problem of identifying the proximate cause(s) of biased cast representation of the *Sb* element parallel the problems discussed above with respect to the causes of maternity skew, so that progress on one front can be expected to facilitate progress on the other.

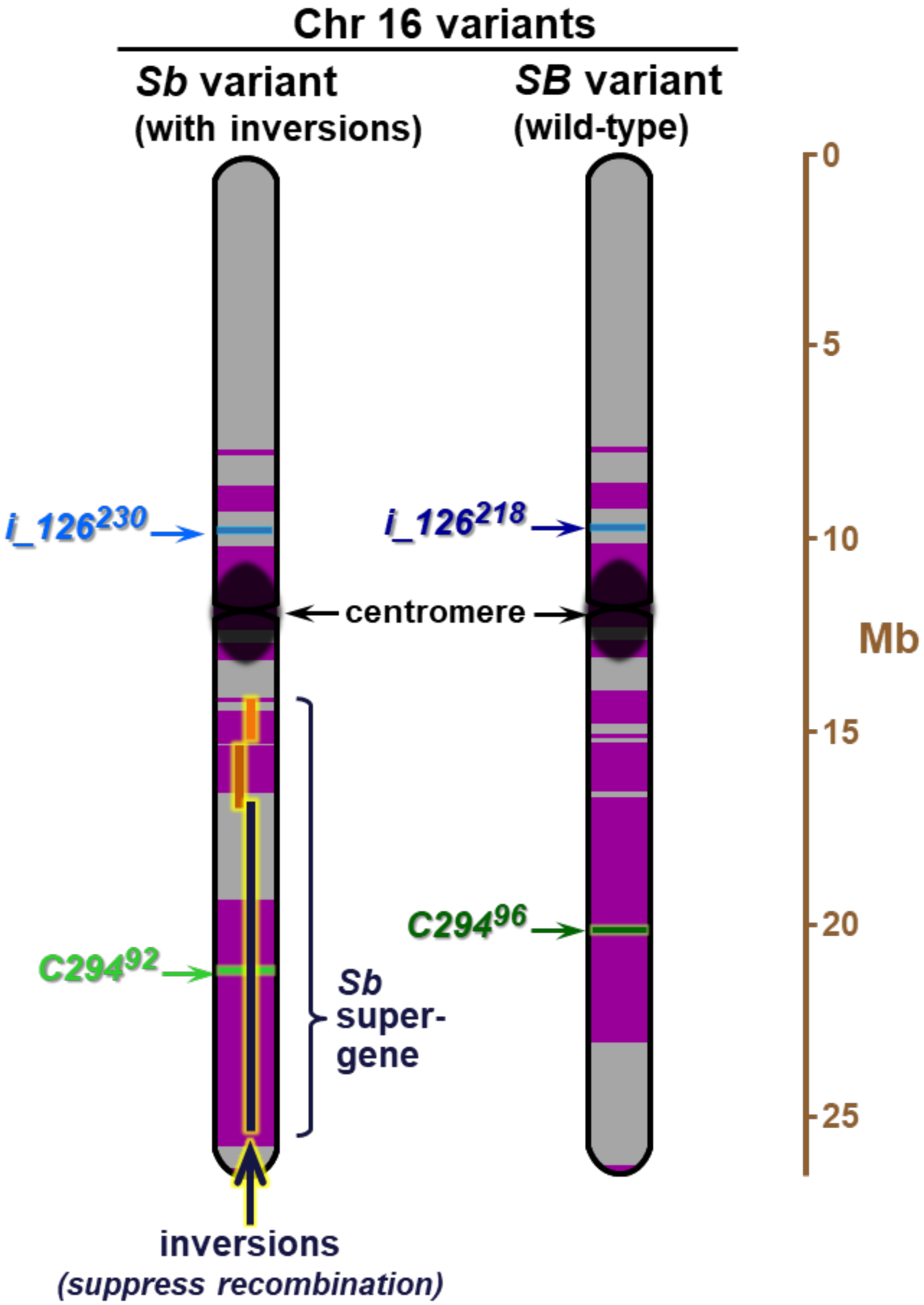


Figure 1. Chromosome 16 of *S. invicta.* The *Sb* chromosomal variant is depicted on the left and the *SB* variant on the right. The loci *C294* and *i_126* are positioned based on the latest complete genome assembly.

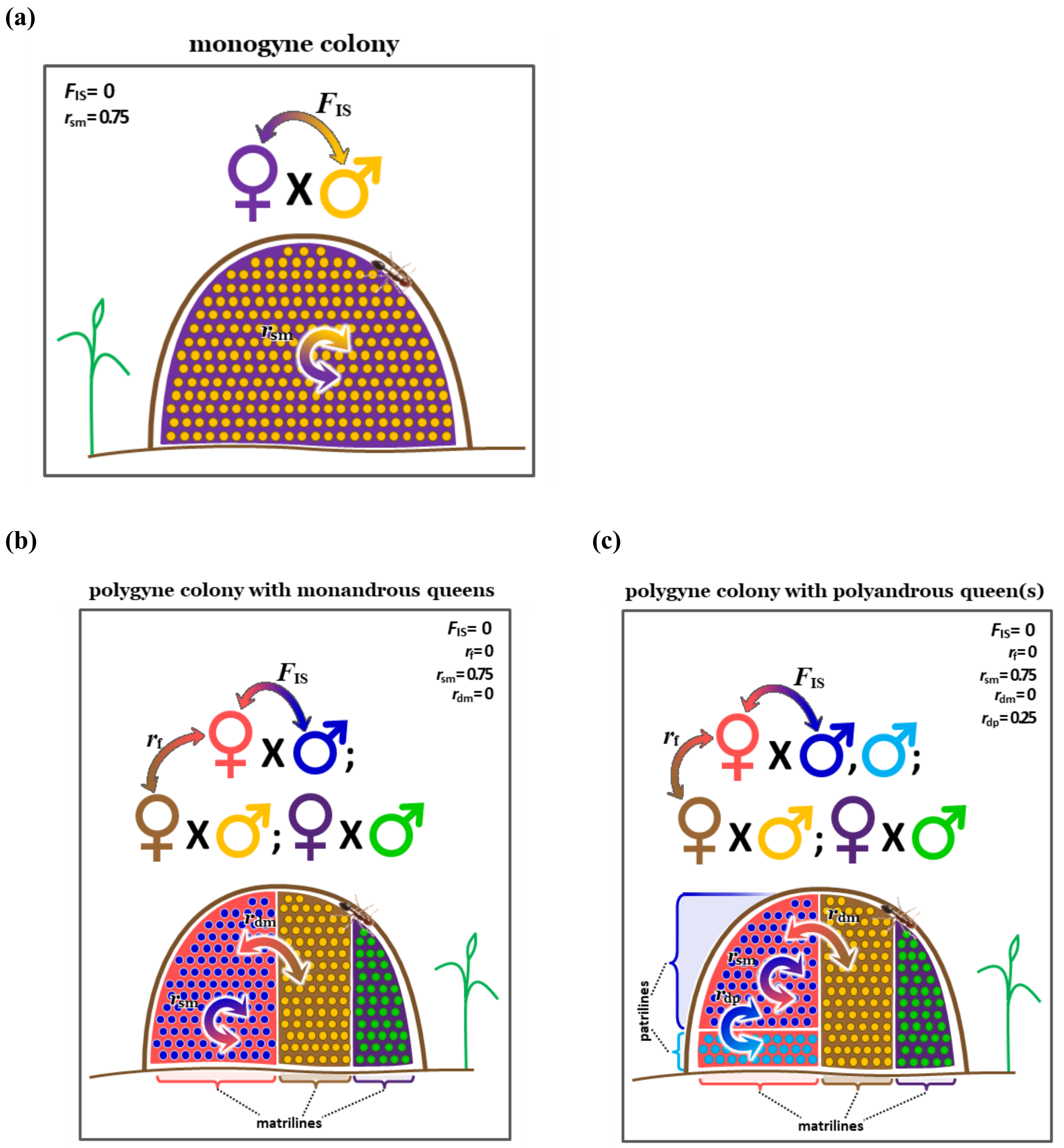


Figure 2. Genetic (kin) structure of monogyne and polygyne fire ant colonies. (a) Structure of a monogyne fire ant colony comprising one matriline (purple) and one patriline (yellow), as is typical for the singly-mated queens of this form. **(b)** Structure of a polygyne colony with three matrilines (red, brown, purple) that are coincident with the single patrilines of the once-mated queens (dark blue, yellow, green). **(c)** Structure of a polygyne colony with three matrilines (red, brown, purple), two patrilines (dark blue, light blue) nested within the matriline of a queen that mated twice and two patrilines (yellow, green) coincident with the matrilines of two singly mated queens. Arrows depict relatedness between families or subfamilies within the colonies and between different classes of breeding individuals.

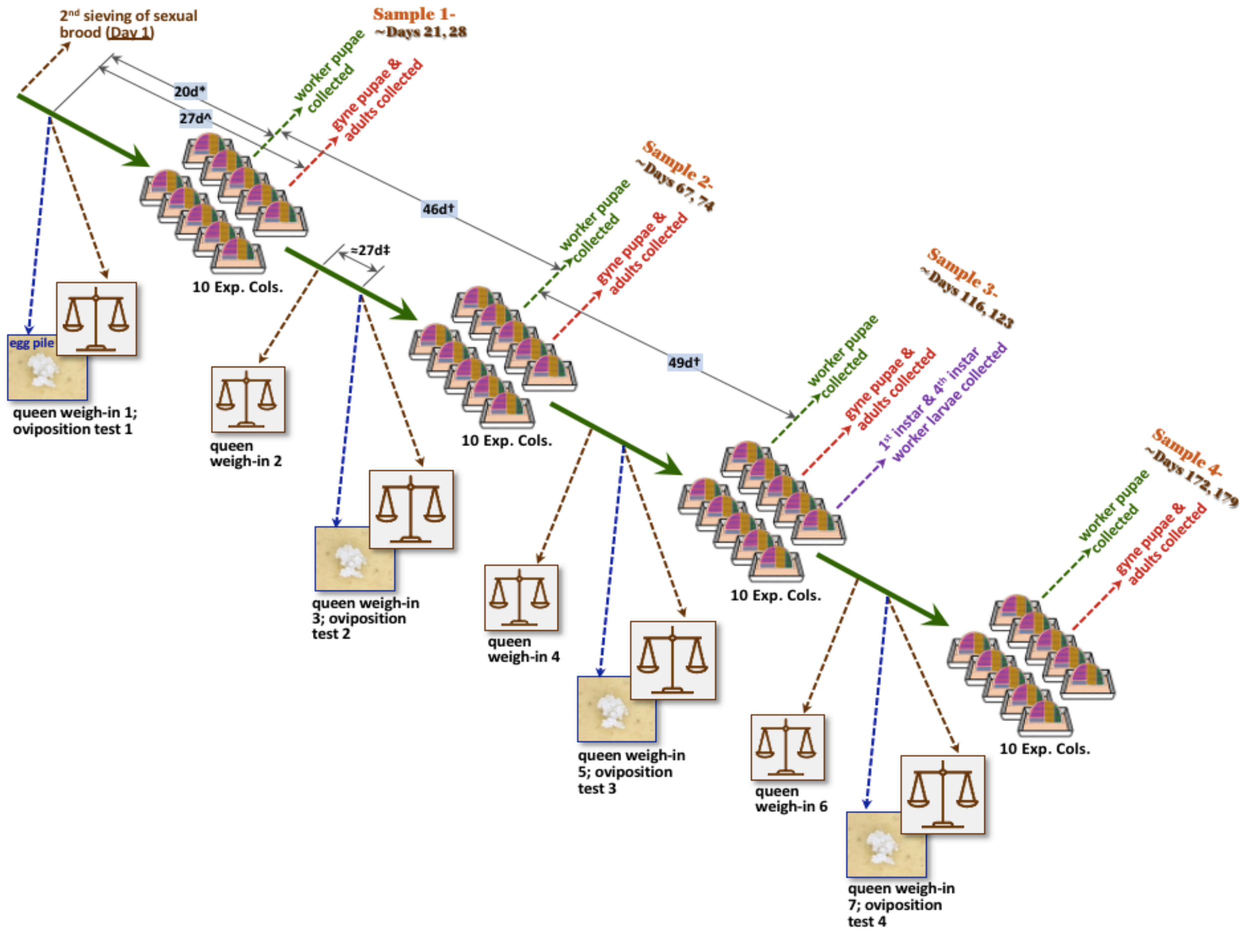


Figure 3. Timeline of the study. Depiction of key events across the sampling timeline for the four principal sampling events, including progeny sampling points, times of queen weigh-ins, and times of oviposition tests are shown.

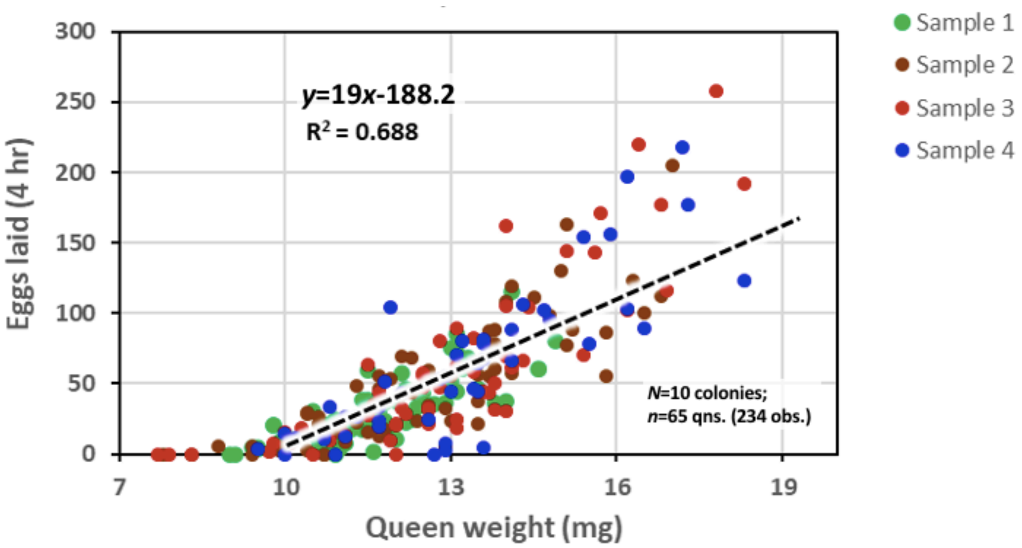


Figure 4. Relationship of queen weight and oviposition rate. Scatter plot depicting the relationship between queen weight and oviposition rate as assessed over a 4 hour time period. The four sampling points are represented by differently colored dots.

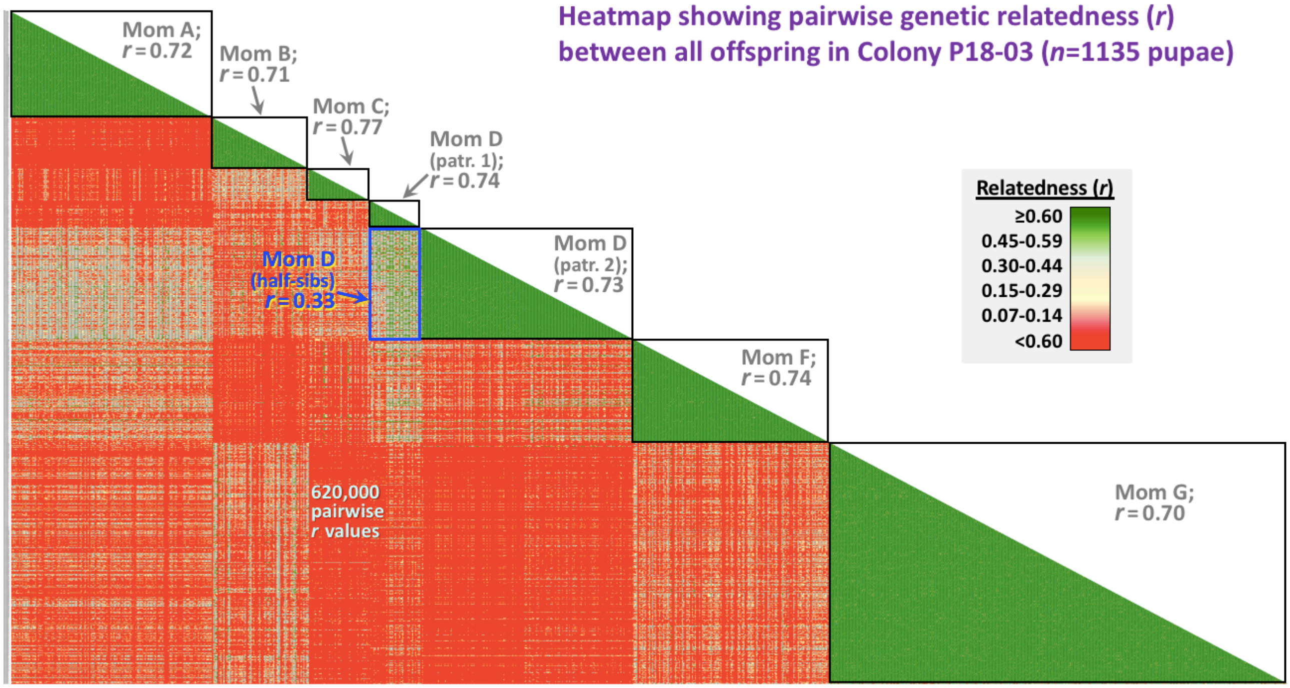


Figure 5. Relatedness heatmap for exemplar study colony P18-03. Pairwise genetic relatedness values (r) between 1135 worker and gyne pupae, totaling 620,000 dyads are shown. Black rectangles demarcate full-sib families or subfamilies (expected r=0.75), while the blue rectangle bounds half-sibs that are members of different patrilines nested within the single matriline headed by twice-mated Queen D (expected r=0.25). Long-term maternity skew is apparent from the greatly varying sizes of the green triangles corresponding to matrilines.

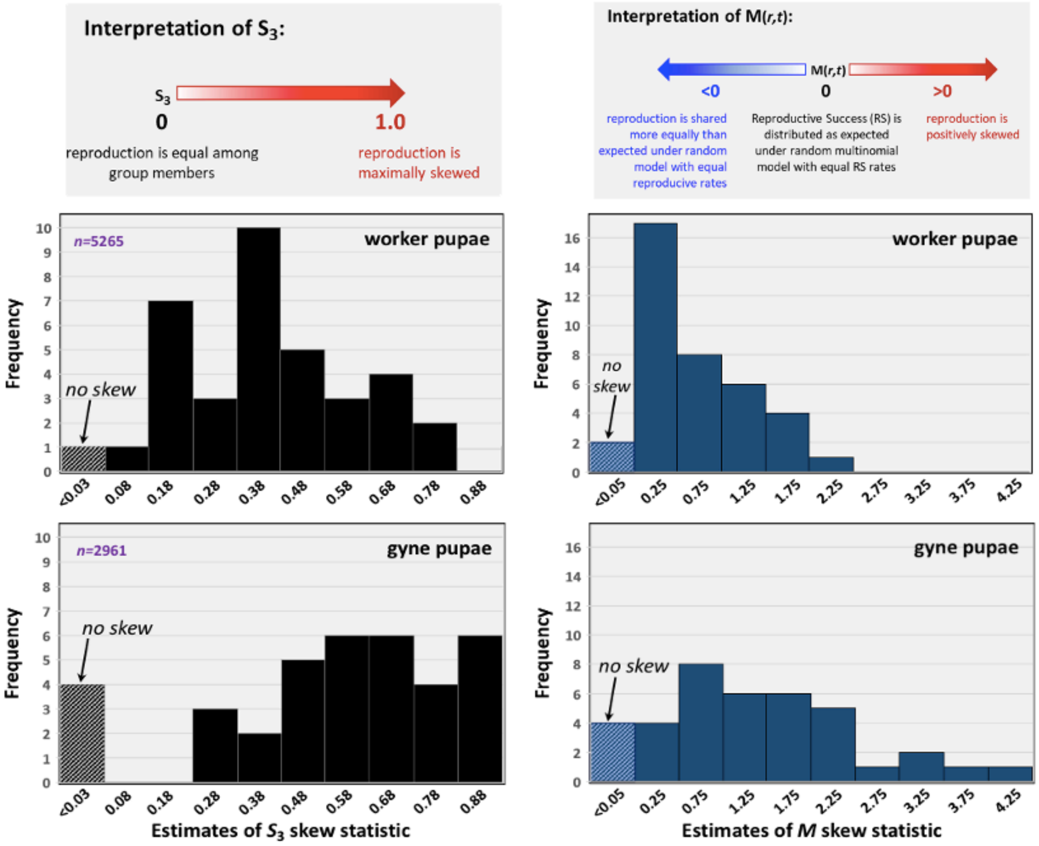


Figure 6. Overall estimates of maternity skew using *S3* and *M* statistics. Values for the two metrics are shown for each female caste in ten polygyne study colonies (all 4 sample points included). Very few of the estimates are close to zero, indicating widespread skew in reproductive output among nestmate queens at single sampling points.

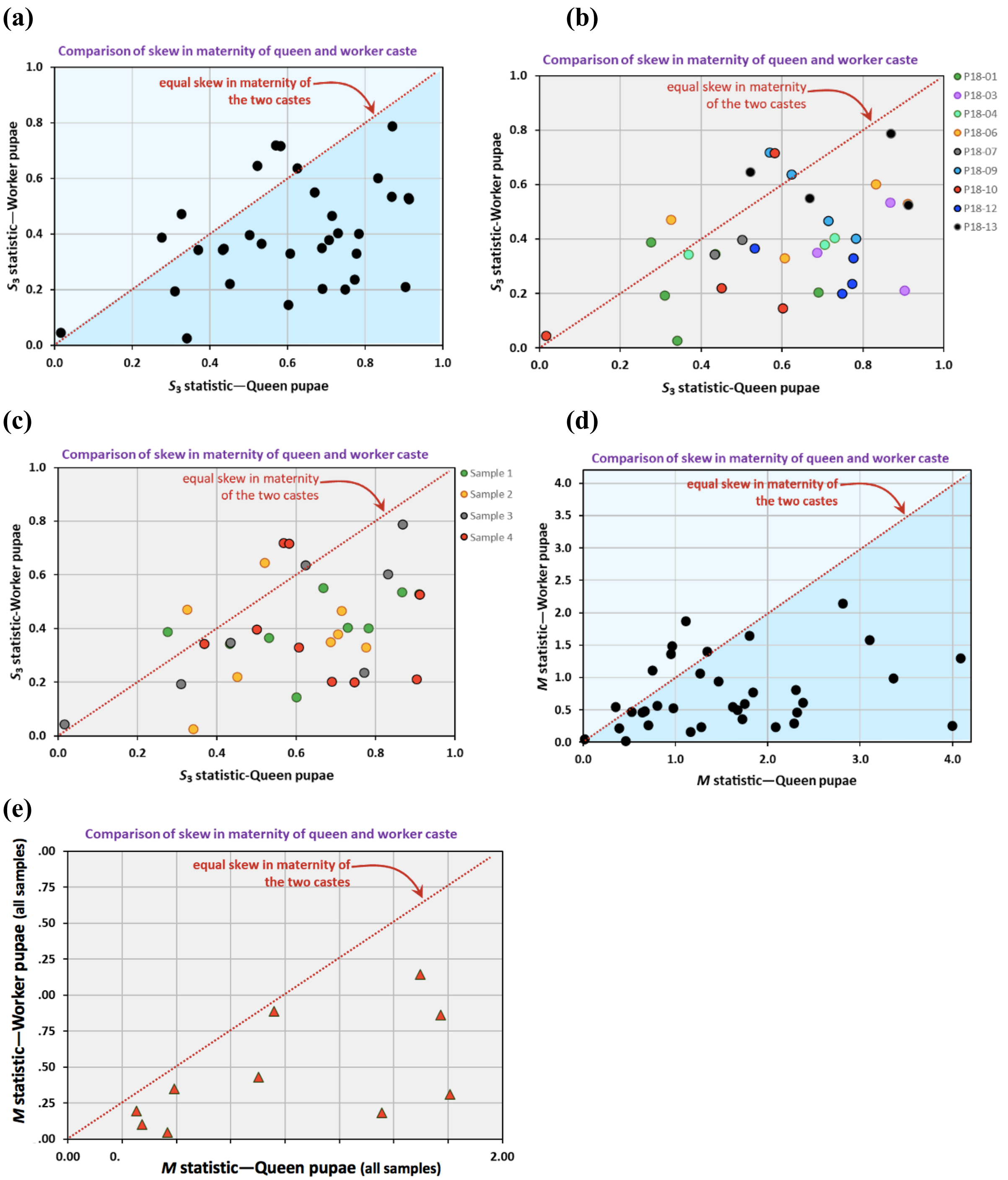
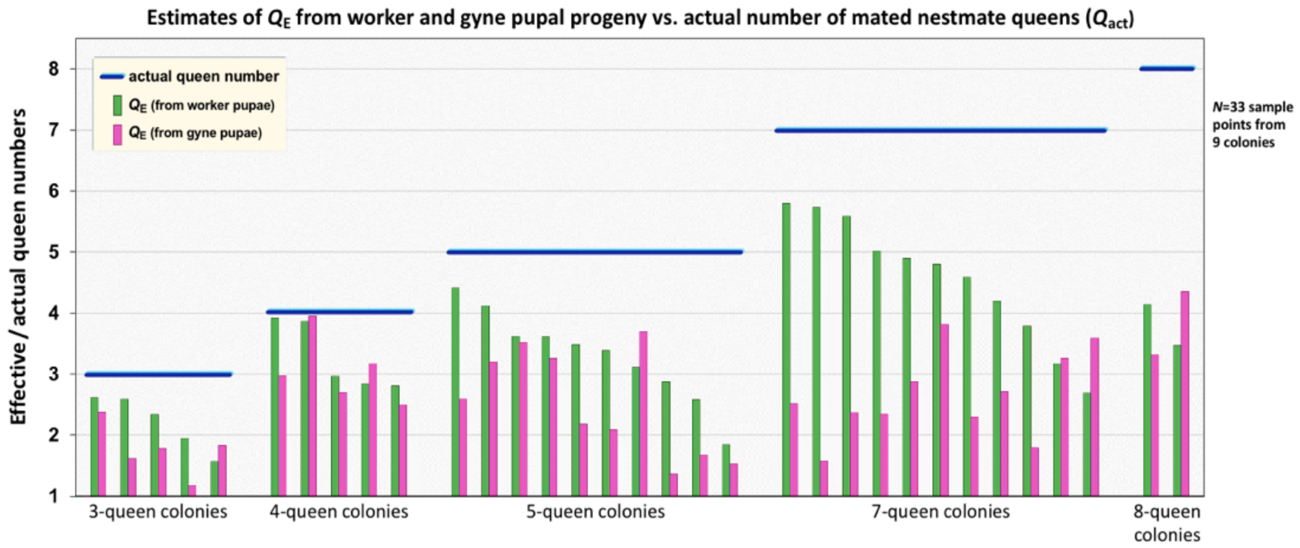


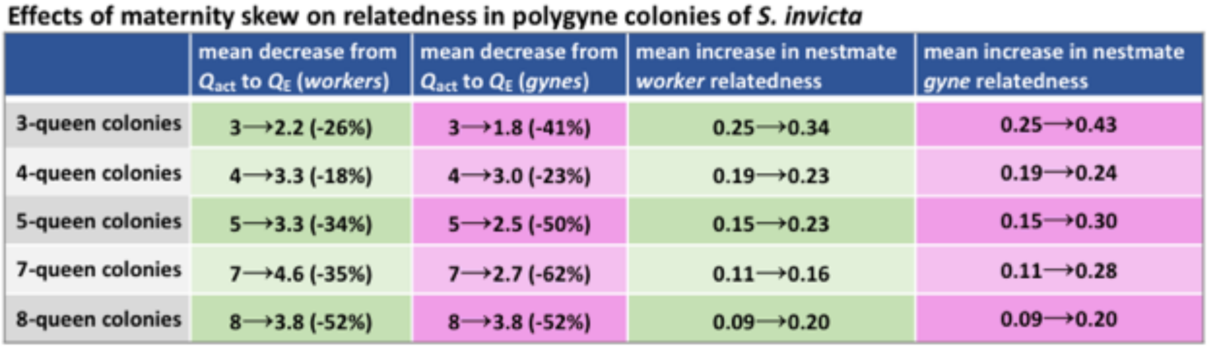
Figure 7. Sampling point estimates of *S3* and *M* statistics. (a) Comparison of skew in maternity of gynes and workers, showing colony **(b)** and sample **(c)** of origin for the *S3* skew statistic. **(d)** Comparison of skew in maternity of gynes and workers at single sampling points, and **(e)** over the entire experiment, for the *M* skew statistic.

(a)



Estimates of Q_E from worker and gyne pupal progeny vs. actual number of mated nestmate queens (Q_{act})

(b)



Effects of maternity skew on relatedness in polygyne colonies of *S. invicta*

	mean decrease from Q_{act} to Q_E (*workers*)	mean decrease from Q_{act} to Q_E (*gynes*)	mean increase in nestmate *worker* relatedness	mean increase in nestmate *gyne* relatedness
3-queen colonies	3⟶2.2 (-26%)	3⟶1.8 (-41%)	0.25⟶0.34	0.25⟶0.43
4-queen colonies	4⟶3.3 (-18%)	4⟶3.0 (-23%)	0.19⟶0.23	0.19⟶0.24
5-queen colonies	5⟶3.3 (-34%)	5⟶2.5 (-50%)	0.15⟶0.23	0.15⟶0.30
7-queen colonies	7⟶4.6 (-35%)	7⟶2.7 (-62%)	0.11⟶0.16	0.11⟶0.28
8-queen colonies	8⟶3.8 (-52%)	8⟶3.8 (-52%)	0.09⟶0.20	0.09⟶0.20

Figure 8. Estimates of effective queen numbers (Q_E). (a) Effective and actual queen numbers for worker pupae and gyne pupae are shown for 3-, 4-, 5-, 7-, and 8-queen colonies. **(b)** Effects of maternity skew on relatedness in workers and gynes, including average decreases from Q_{act} to Q_E, and average increases in nestmate worker and gyne relatedness.

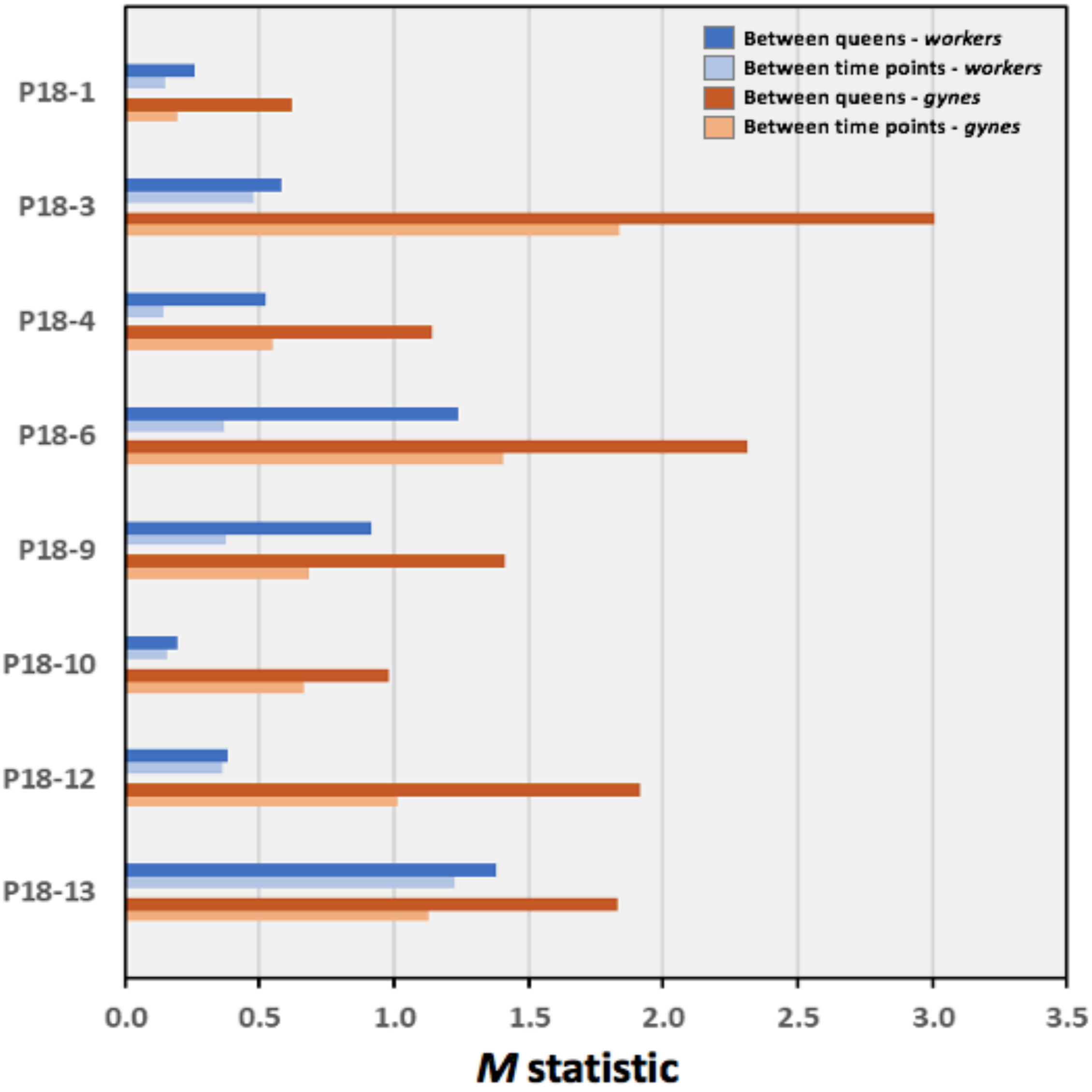


Figure 9. Comparison of between-queen and between-sample maternity skew calculated using the *M* statistic. Bars for the same caste are paired, with light bars showing values calculated across sampling points for single queens and dark bars showing values calculated between queens (matrilines) at single points.

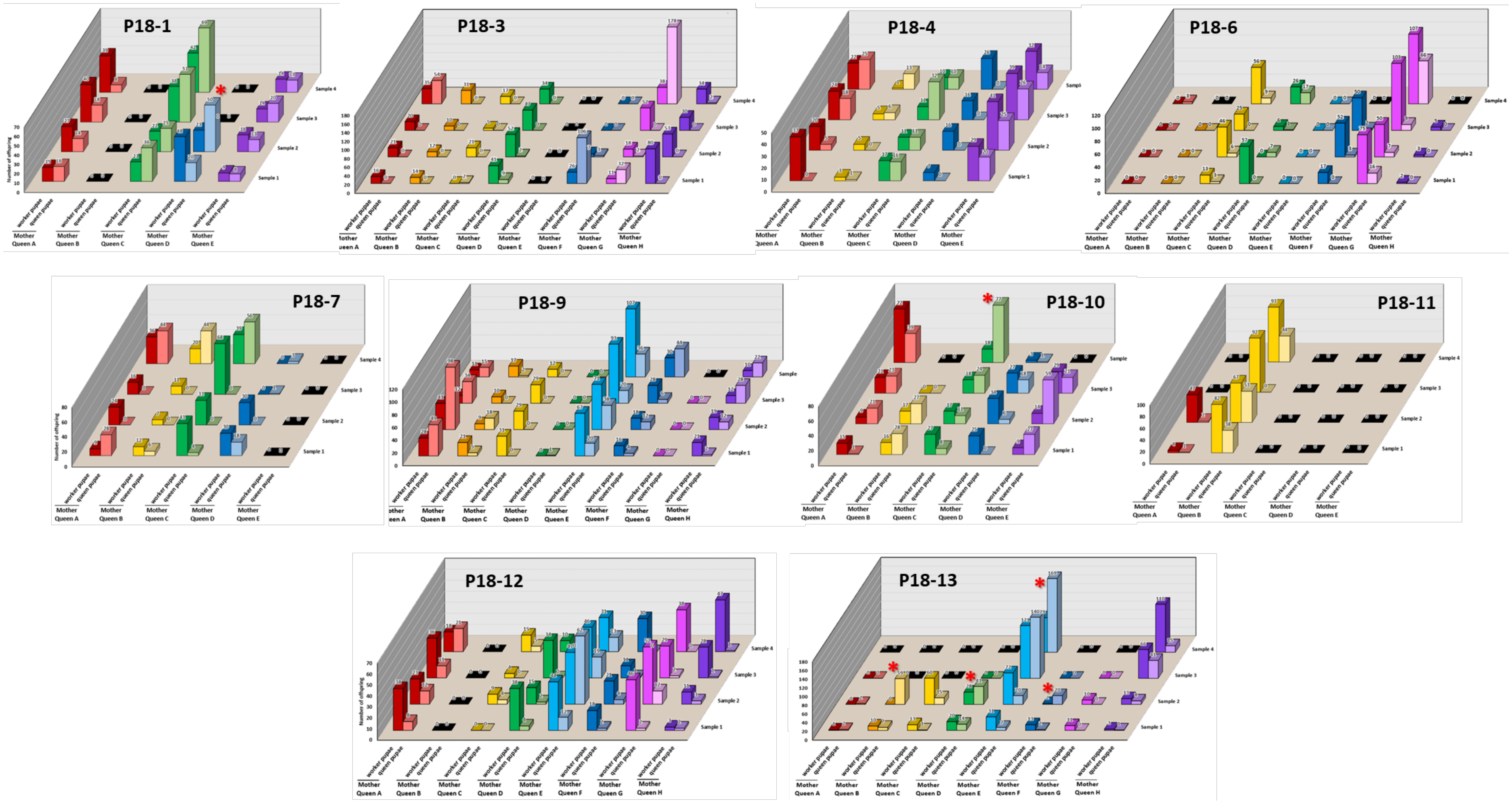


Figure 10. Maternity apportionment colony graphs. The column graphs depict maternity apportionment for worker and gyne offspring of 5-8 queens in ten experimental polygyne colonies of *S. invicta*. Black rectangles represent unmated queens or mated queens that died during the experiment. Six queens displaying the weight loss-death syndrome associated with a bout of gyne production are indicated with asterisks.

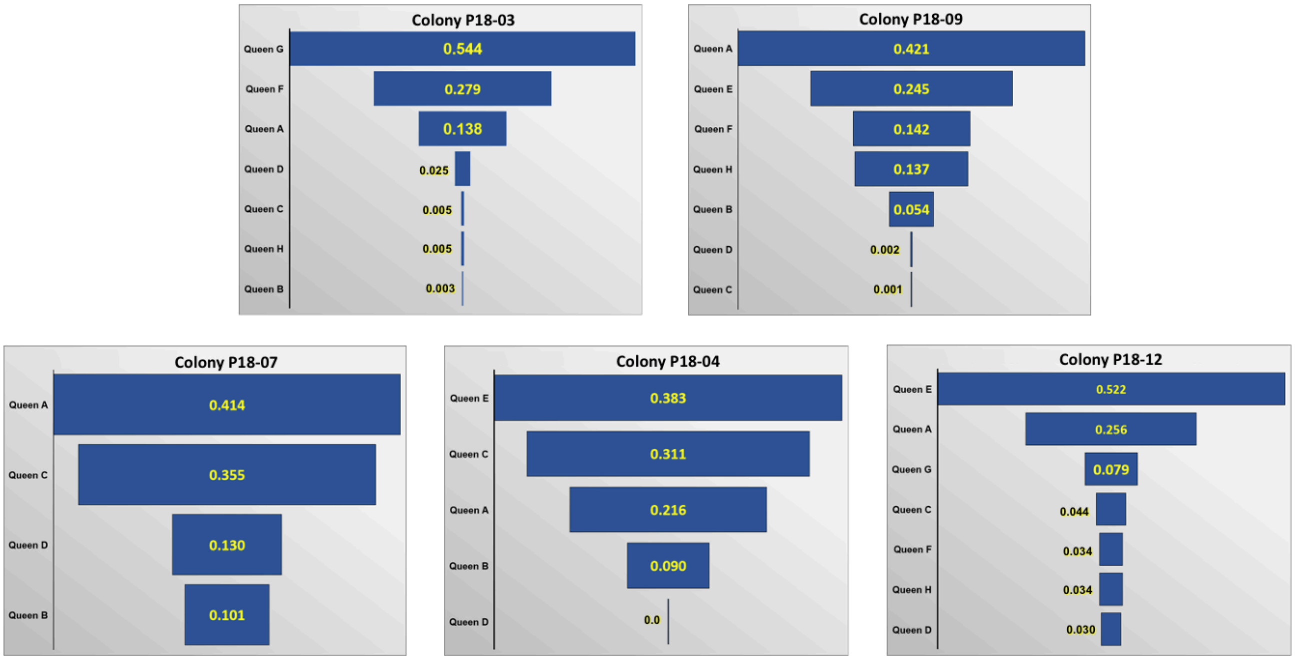
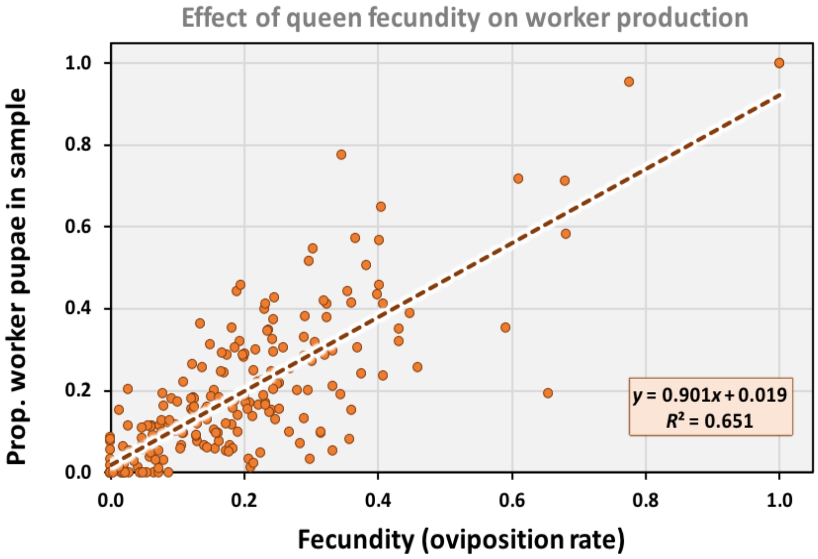
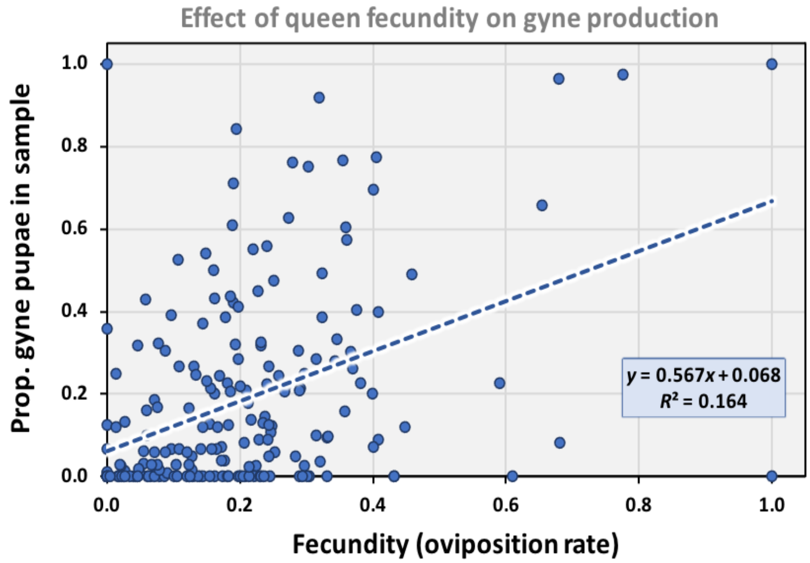


Figure 11. Long-term queen reproductive success. Funnel graphs depict the proportions of a colony's total gyne production over the experimental period attributable to each nestmate queen. Only the five colonies in which all mated queens survived this entire period are included.

(a)



	slope	y-interc.	R²
Mean	**0.901**	**0.019**	**0.651**
95% CI	(0.896-0.905)	(0.018-0.020)	(0.650-0.660)



	slope	y-interc.	R²
Mean	**0.567**	**0.068**	**0.164**
95% CI	(0.557-0.577)	(0.066-0.069)	(0.158-0.167)

(b)

Figure 12. Relationship of fecundity and offspring production. (a) Linear regression of fecundity (measured via oviposition rate) vs. the proportion of worker pupae produced in a sample. There is a strong positive linear relationship between fecundity and worker production. **(b)** Linear regression of fecundity vs. the proportion of gyne pupae produced in a sample. The poor fit of the regression line to the two variables indicates that there is not a strong linear relationship between fecundity and gyne production. Tables show 95% confidence intervals for the regression parameters obtained by bootstrapping.

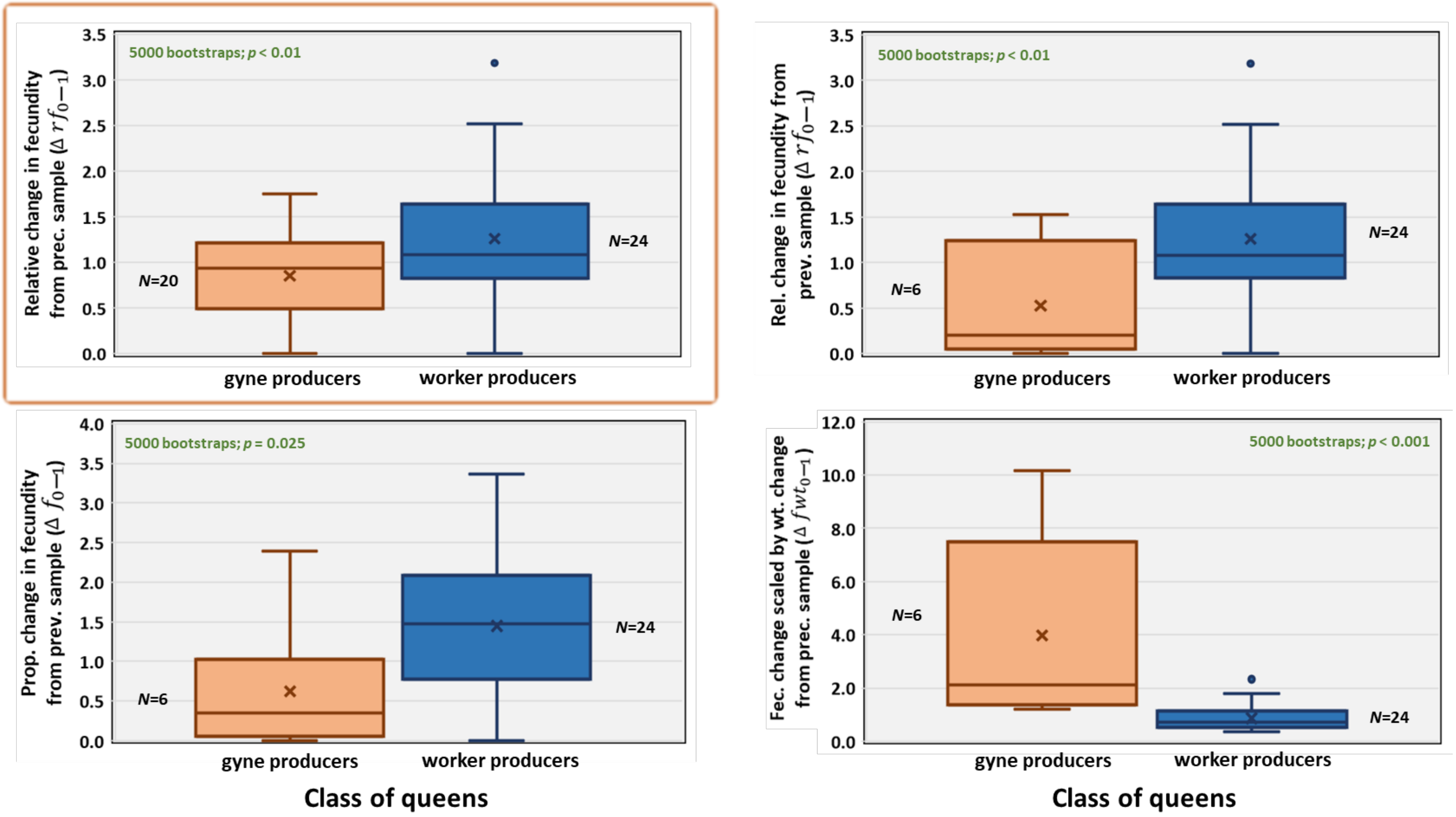
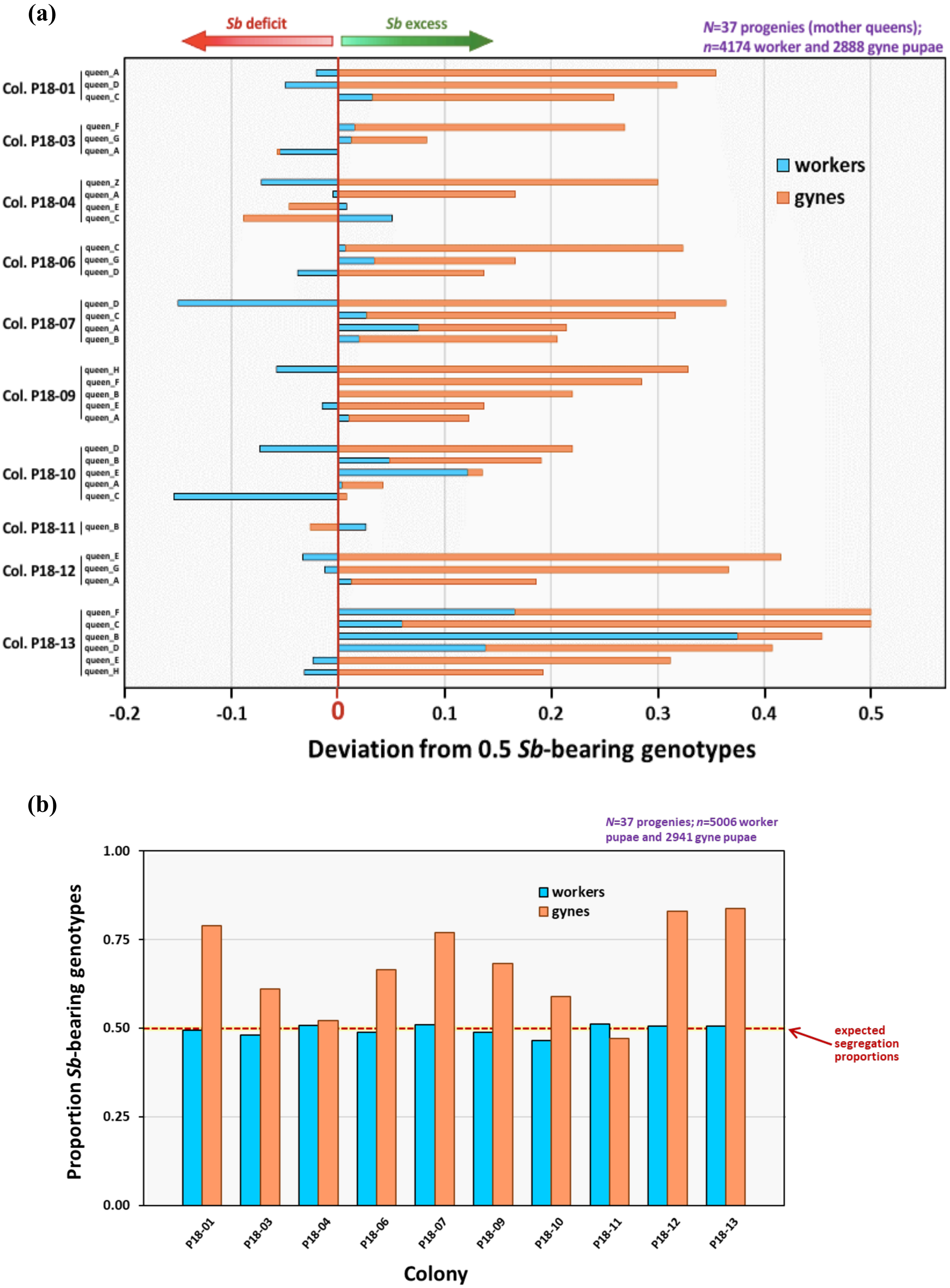
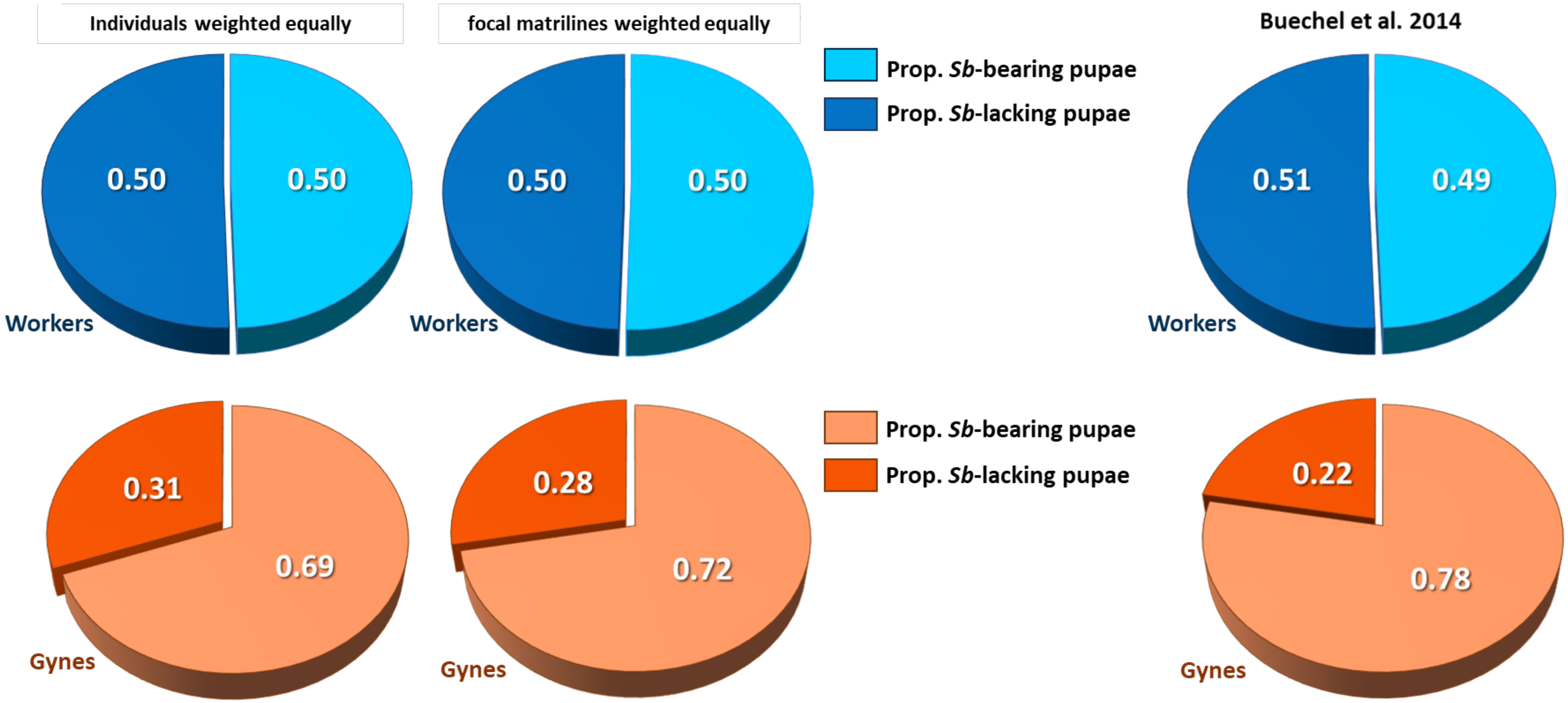


Figure 13. Variables significantly associated with pulses of gyne production by queens. Boxplots depict distributions of values for four variables that differed significantly between queens displaying bouts of gyne production and those that displayed sustained high levels of worker production instead. The boxplot in the orange rectangle shows results for the single variable that significantly distinguished the groups when all gyne producers were considered; the remaining graphs show data for this and two other variables that distinguished the groups when the gyne-producers were restricted to queens exhibiting the ‘weight loss-death’ syndrome.


(a)
Sb deficit
Sb excess
N=37 progenies (mother queens);
n=4174 worker and 2888 gyne pupae
Col. P18-01
queen_A
queen_D
queen_C
Col. P18-03
queen_F
queen_G
queen_A
Col. P18-04
queen_Z
queen_A
queen_E
queen_C
Col. P18-06
queen_C
queen_G
queen_D
Col. P18-07
queen_D
queen_C
queen_A
queen_B
Col. P18-09
queen_H
queen_F
queen_B
queen_E
queen_A
Col. P18-10
queen_D
queen_B
queen_E
queen_A
queen_C
Col. P18-11
queen_B
Col. P18-12
queen_E
queen_G
queen_A
Col. P18-13
queen_F
queen_C
queen_B
queen_D
queen_E
queen_H
workers
gynes
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
Deviation from 0.5 Sb-bearing genotypes
(b)
N=37 progenies; n=5006 worker pupae and 2941 gyne pupae
1.00
0.75
0.50
0.25
0.00
Proportion Sb-bearing genotypes
workers
gynes
expected segregation proportions
P18-01
P18-03
P18-04
P18-06
P18-07
P18-09
P18-10
P18-11
P18-12
P18-13
Colony

(c)



Individuals weighted equally

focal matrilines weighted equally

Buechel et al. 2014

N=47 progenies; *n*=5006 worker pupae and 2941 gyne pupae

N=8 progenies; *n*=331 worker pupae and 402 gyne pupae

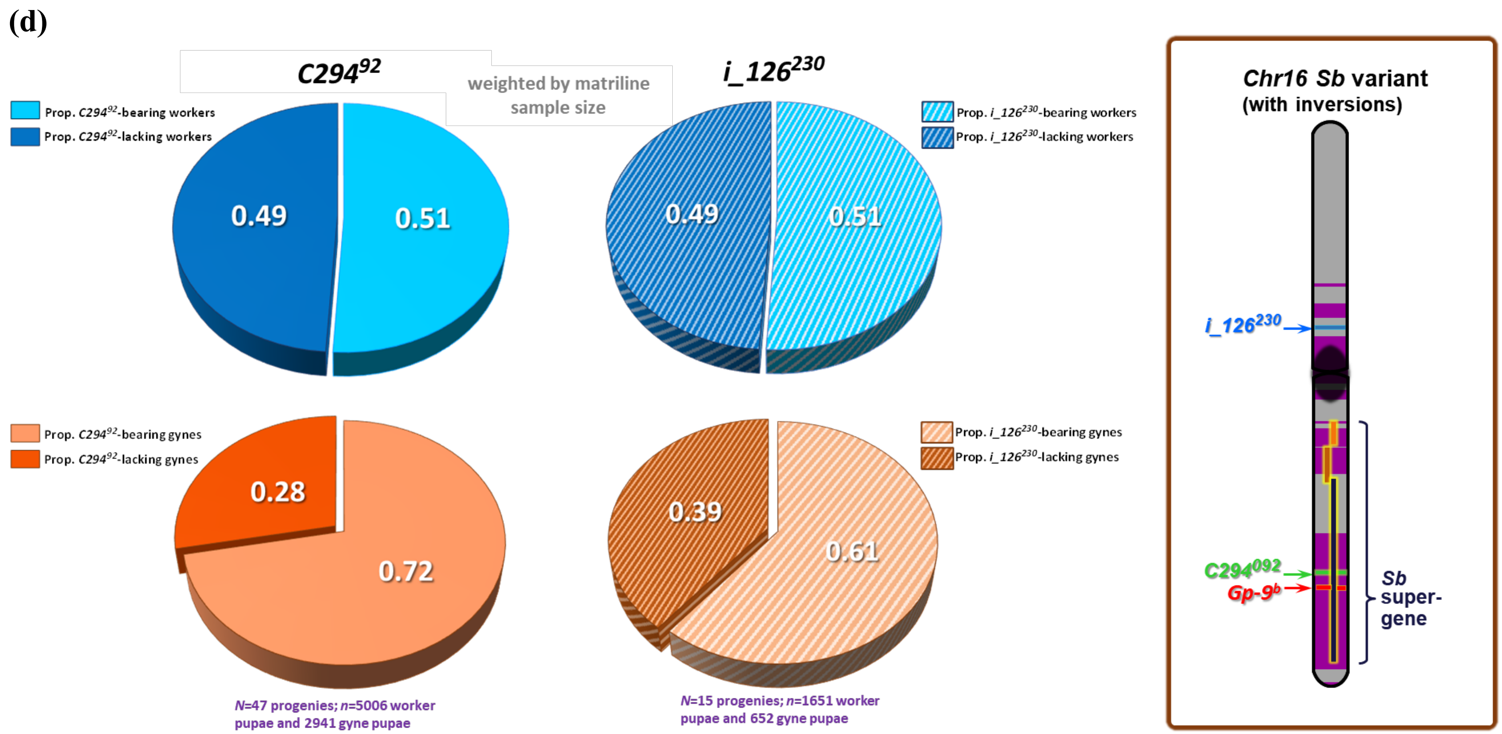
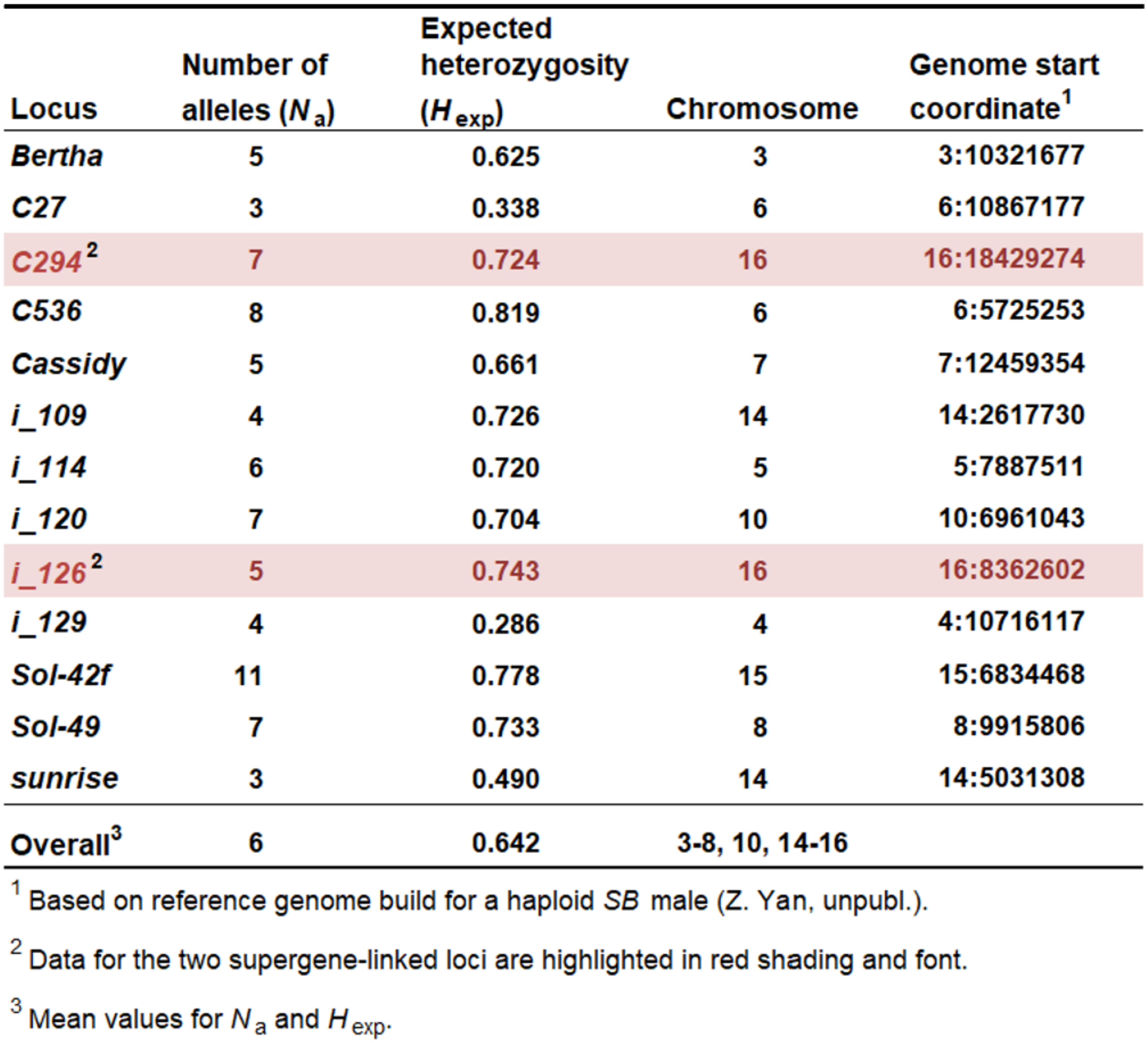


Figure 14. **Segregation of *Sb* supergene markers in gyne and worker pupae. (a)** Deviations of daughter genotypes from 0.5 segregation proportions expected for *Sb*-bearing genotypes in 37 progenies (matrilines/patrilines) produced by heterozygous queens mated to *SB* males, as inferred using the completely linked marker locus *C294*. Only progenies with >10 gynes are included. **(b)** Frequencies of daughter pupa bearing the *Sb* supergene in each of the ten study colonies, as inferred using *C294*. **(c)** Overall population frequencies of daughter pupae bearing the *Sb* supergene, as inferred using *C294*. Results from the study of Buechel et al. (2014) are shown for comparison. **(d)** Comparison of population frequencies of daughter pupae bearing the *Sb*-linked alleles $C294^{92}$ and i_126^{230}. The *Sb* variant of the social chromosome is shown for reference, and includes the location of *Gp-9*, a gene used as a marker for *Sb* by Buechel et al. (2014).

Table 1. Characteristics of microsatellite loci. Thirteen microsatellite loci were genotyped in this study in order to assign offspring to matrilines within the study colonies. Shown here are the number of alleles, expected heterozygosity, and chromosomal location for each marker. Two loci, *C294* and *i_126*, are located on the social chromosome, chr16.



Locus	Number of alleles (N_a)	Expected heterozygosity (H_{exp})	Chromosome	Genome start coordinate[1]
Bertha	5	0.625	3	3:10321677
C27	3	0.338	6	6:10867177
C294[2]	7	0.724	16	16:18429274
C536	8	0.819	6	6:5725253
Cassidy	5	0.661	7	7:12459354
i_109	4	0.726	14	14:2617730
i_114	6	0.720	5	5:7887511
i_120	7	0.704	10	10:6961043
i_126[2]	5	0.743	16	16:8362602
i_129	4	0.286	4	4:10716117
Sol-42f	11	0.778	15	15:6834468
Sol-49	7	0.733	8	8:9915806
sunrise	3	0.490	14	14:5031308
Overall[3]	6	0.642	3-8, 10, 14-16	

[1] Based on reference genome build for a haploid *SB* male (Z. Yan, unpubl.).

[2] Data for the two supergene-linked loci are highlighted in red shading and font.

[3] Mean values for N_a and H_{exp}.

Table 2. Features of the ten experimental colonies surveyed in this study. Summary data for the ten experimental colonies include colony ID, number of queens alive at the beginning and end of the experiment, number of unmated and multiply-mated queens, and total numbers of offspring of each caste successfully genotyped.

Colony	Number of queens at beginning of experiment	Number of unmated queens	Number of mutiply-mated queens[1]	Number of queens alive at time of final (fourth) sample[2]	Total number of worker offspring assigned to matriline (prop. of total workers successfully genotyped)[3]	Total number of gyne offspring assigned to matriline (prop. of total gynes successfully genotyped)[3]
P18-01	5	1	1 (4)	**4**	370 (1.0)	361 (0.994)
P18-03	8	1	1 (7)	6	725 (0.972)	390 (0.997)
P18-04	5	0	2 (5)	5	372 (0.997)	222 (0.953)
P18-06	8	0	0 (8)	5	693 (0.997)	146 (0.960)
P18-07	5	1	0 (4)	5	378 (1.0)	170 (0.994)
P18-09	8	1	0 (7)	7	742 (0.995)	466 (0.987)
P18-10	5	0	1 (5)	3	371 (0.995)	391 (1.0)
P18-11	5	3	0 (2)	3	365 (0.992)	138 (0.926)
P18-12	8	1	0 (7)	7	742 (1.0)	203 (0.995)
P18-13	8	0	2 (8)	2	692 (0.989)	518 (0.988)
Overall	65	8 (0.125)[4]	7 (0.1235)[4]	2-7 (0.723)[5]	5450 (0.993)[4]	3005 (0.961)[4]

[1] Total numbers of mated queens in colony are in parentheses. Two polyandrous queens, from Colony P18-03 and P18-04, evidently mated with three males, the remainder with two.

[2] Data from the first four of up to seven sampling points are analyzed in this study.

[3] The proportion of offspring that were confidently assigned to a matriline, after exclusion of individuals with missing data at five or more loci, is in parentheses

[4] Overall frequency is in parentheses.

[5] Overall proportion of queens surviving through final (fourth) sample.

Table 3. Potential predictor variables for queen reproductive success. Characteristics of queens were examined to identify variables asociated with large and sudden increases in the production of gynes. The 15 variables listed here were used in a preliminary assessment; only four were studied further using the complete data set (see Figure 13).

Variable	Code	Measurement[*]
Survivorship	S	Days alive following t_1
Fecundity	f_1	Number of eggs laid in oviposition test at 21 or 25 days before focal sampling point[†]
Weight	wt	Wet weight at focal sampling point
Relative (in colony) worker maternity apportionment at sampling point preceding focal point	$rwma_0$	Proportion of total colony worker production at t_0
Relative (in nest) fecundity at focal sampling point	rf_1	$f_1/\overline{x}_{\mathrm{f1}}$
Relative (in nest) fecundity at preceding sampling point	rf_0	$f_0/\overline{x}_{\mathrm{f0}}$
Change in fecundity from preceding sampling point, relative to nestmates' fecundity	Δrf_{0-1}	rf_1/rf_0
Proportional change in fecundity from preceding sampling point	Δf_{0-1}	f_1/f_0
Proportional change in weight over preceding half interval	$\Delta wt_{0.5-1}$	$wt_1/wt_{0.5}$
Proportional change in weight from preceding sampling point	Δwt_{0-1}	wt_1/wt_0
Proportional change in fecundity to subsequent sampling point	Δf_{1-2}	f_2/f_1
Proportional change in weight over subsequent half interval	$\Delta wt_{1-1.5}$	$wt_{1.5}/wt_1$
Proportional change in weight at subsequent sampling point	Δwt_{1-2}	wt_2/wt_1
Change in weight, relative to nestmates, at subsequent sampling point	Δrwt_{1-2}	$(rwt_2/\overline{x}_{\mathrm{rwt2}})$ $(rwt_1/\overline{x}_{\mathrm{rwt1}})$
Change in fecundity relative to change in weight from preceding sampling point	Δfwt_{0-1}	$\Delta f_{0-1}/\Delta wt_{0-1}$

[*] subscripts refer to sampling points and half intervals as follows: 0 = sampling point immediately preceding focal point; 1 = focal sampling point; 2 = sampling point immediately subsequent to focal point; 0.5 = half interval point between focal and preceding sampling points (queen weigh-ins only); 1.5 = half interval point between focal and subsequent sampling points (queen weigh-ins only)

[†] developmental periods from laying of egg to formation of pupa for workers and queens, respectively

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