Sexually antagonistic selection promotes genetic divergence between males and females in an ant

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Genetic diversity acts as a reservoir for potential adaptations, yet selection tends to reduce this diversity over generations. However, sexually antagonistic selection (SAS) may promote diversity by selecting different alleles in each sex. SAS arises when an allele is beneficial to one sex but harmful to the other. Usually, the evolution of sex chromosomes allows each sex to independently reach different optima, thereby circumventing the constraint of a shared autosomal genome. Because the X chromosome is found twice as often in females than males, it represents a hot spot for SAS, offering a refuge for recessive male-beneficial but female-costly alleles. Hymenopteran species do not have sex chromosomes; females are diploid and males are haploid, with sex usually determined by heterozygosity at the complementary sex-determining locus. For this reason, their entire genomes display an X-linked pattern, as every chromosome is found twice as often in females than in males, which theoretically predisposes them to SAS in large parts of their genome. Here we report an instance of sexual divergence in the Hymenoptera, a sexually reproducing group that lacks sex chromosomes. In the invasive ant *Nylanderia fulva*, a postzygotic SAS leads daughters to preferentially carry alleles from their mothers and sons to preferentially carry alleles from their grandfathers for a substantial region (~3%) of the genome. This mechanism results in nearly all females being heterozygous at these regions and maintains diversity throughout the population, which may mitigate the effects of a genetic bottleneck following introduction to an exotic area and enhance the invasion success of this ant.

intralocus sexual conflict | reproductive system | invasive species | social insects

The preservation of genetic variation within populations is a complex puzzle in evolutionary biology. Genetic diversity acts as a reservoir for potential adaptations, whereas selection tends to reduce it over generations, fixing advantageous alleles while discarding deleterious ones (1, 2). The maintenance of abundant genetic diversity in natural populations results from the continuous occurrence of mutations across the genome, creating allele polymorphism at low frequencies (mutation-selection balance) (3–5). Allele polymorphism may also be preserved when distinct alleles are selected for under different conditions, such as different environments, seasons, ages, or sexes (diversifying selection) (6–8).

Sexually antagonistic selection (SAS) may promote genetic diversity in natural populations. SAS arises when an allele is beneficial to one sex but harmful to the other; that is, when a phenotypic trait has different optima in each sex but a shared genetic basis (9–14). In this case, distinct alleles are differentially selected in the 2 sexes. Usually, the constraint of a shared gene pool prevents males and females from reaching their optima independently by fixing beneficial alleles in each sex (15), as selection on one sex results in a correlated response in the other (16, 17). Sexual divergence can still evolve without sequence differences between the sexes through environmental sex determination (18) or sex-biased expression via genomic imprinting (19, 20), sex-specific dominance (21–23), or sex-specific gene regulation (24, 25). Genetic differences between the sexes often occur through the evolution of sex chromosomes, releasing the sexes from the constraint of a shared autosomal genome, while the absence of recombination between sex chromosomes prevents the production of detrimental intersexual phenotypes (26–28). Therefore, sex chromosomes allow a sex-determining locus to cosegregate via linkage with sexually antagonistic (SA) genes that are now no longer expressed in the harmed sex. Ironically, this creates an opportunity for SA variation to accumulate on the shared sex chromosome due to haplodiploid expression (26). For example, in species with heterogametic males (i.e., males are XY and females are XX), the X chromosome is expected to be a hot spot for sexually antagonistic selection, as it is found twice as often in females than in males (29) (vice versa for species with heterogametic ZW females). A recessive allele that provides a small benefit to males but a substantial cost to females may be initially shielded from natural selection in females, whereas it has no such opportunity in hemizygous males. This allele may readily increase in frequency until the costs to the harmed sex match the benefits to the helped sex (29, 30). Therefore, a locus located on the X chromosome can stably segregate recessive female-detrimental and male-beneficial SA alleles as protected polymorphisms (31–33).

In the insect order Hymenoptera, in which males are haploid and females are diploid, the sex of an individual in many species is determined by its heterozygosity at the complementary sex-determining (CSD) locus (34), not by distinct sex chromosomes. Fertilized diploid eggs develop into females when heterozygous at this locus, while unfertilized eggs, haploid and hemizygous, grow into males (35). Although it is generally assumed that males and females of hymenopteran species do not differ in their genome sequences, their entire genomes display an X-linked pattern,

Significance

In Hymenoptera, males and females display striking phenotypic differences despite lacking sex chromosomes. The constraint of a shared gene pool has been thought to prevent them from separately reaching sex-specific fitness optima by fixing different alleles in each sex. However, their entire haplodiploid genomes display an X-linked pattern that predisposes them to sexually antagonistic selection (SAS), whereby an allele is beneficial to one sex but harmful to the other. Here we report the first instance of SAS in a haplodiploid organism, the invasive ant *Nylanderia fulva*, with distinct alleles across a large portion of the genome differentially selected in each sex. Our findings shed light on a newly described genetic pattern and provide further insight into sexual conflicts in haplodiploids.

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as every chromosome is found twice as often in females than in males. This feature theoretically predisposes these species to extensive SAS, as a recessive male-benefit/female-detriment allele may accumulate to greater frequencies through protected polymorphism in diploid females, whereas a dominant female-benefit/male-detriment allele may be promoted through numeric imbalance, since females have double the number of chromosomes (36).

Here we report marked genetic differences maintained between males and females of a single, sexually reproducing species that lacks sex chromosomes. These genetic differences between the sexes despite sexual reproduction represent an example of SAS in a hymenopteran species. In the invasive tawny crazy ant *Nylanderia fulva*, a set of alleles is differentially selected in each sex, while the remainder of the genome is randomly transmitted. Therefore, females and males exhibit strongly divergent genotypes. We show that alleles at these loci are randomly inherited at the egg stage and then differentially selected for later in each sex. As previously predicted, the haplodiploid system of Hymenoptera can promote SAS (36). This study also reveals a unique system in which SAS occurs in an invasive species. SAS may preserve genetic diversity through diversifying selection within an introduced population while preserving nearly 100% heterozygosity in female offspring in a large portion (~3%) of the genome. Although this system is costly, resulting in the death of approximately one-half of female offspring, it may enhance the invasion success of this species by mitigating the loss of heterozygosity resulting from a founder event, reducing the expression of recessive deleterious mutations in both the reproductive and worker castes.

Results

Daughters Carry Alleles from Their Mothers and Sons Carry Alleles from Their Grandfathers for Some Genomic Regions while Randomly Possessing the Rest of the Genome. Our genetic study revealed drastic differences in allele frequencies between male and female individuals at 2 microsatellite markers, *L06* and *L07* (Fig. 1*A*). For these markers, all males were haploid and carried a single allele (arbitrarily termed *A* for both loci; $n = 43$), while almost all females (98.7%; $n = 361$) were heterozygous with allele *A* and another allele (*B* or *C*), giving either *A*/*B* or *A*/*C* genotypes (Fig. 1*A*). As a consequence, the allelic distribution observed in markers *L06* and *L07* is significantly different from that expected under random mating, with more heterozygous individuals than expected, resulting in strong departure from Hardy–Weinberg equilibrium (HWE) in female castes ($P < 0.001$). Remarkably, none of the females investigated exhibited a *B*/*C* genotype at either locus, and only 0.5% of workers and 0.5% of queens carried a *B*/*B* or *C*/*C* genotype; still, *B* and *C* alleles represent 50% of the allelic diversity within the population (expected *B*/*C* + *B*/*B* + *C*/*C* = 25%) (Fig. 1*A*). Likewise, only 1.8% of workers and 0.26% of gynes and queens exhibited an *A*/*A* genotype (expected *A*/*A* = 25%) at 1 of these 2 loci. In contrast, all males analyzed ($n = 43$) were haploid and carried the allele *A* at both loci. The occurrence of the single allele *A* in the male caste is unlikely to stem from nonsampling of the 2 other alleles (*B* and *C*), as these probabilities are low ($P = 1.13 \times 10^{-13}$ for both *L06* and *L07*). For these markers, a difference in the distribution of allelic frequencies was observed between male and female individuals (χ^2 test, $P < 0.001$), but no difference was observed between the female worker and queen castes ($P = 0.469$ and 0.328 for *L06* and *L07,* respectively). Overall, these results suggest that all males consistently carry the same allele, while sexually produced females reliably inherit the male allele and a different allele from their mother and thus are nearly 100% heterozygous. Marker *L02* also exhibited a strong departure from HWE among female castes ($P < 0.001$), as well as a difference in the distribution of allelic frequencies between male and female castes ($P < 0.001$). These results may suggest a pattern similar to that of markers *L06* and *L07*, even though *L02* has only 2 alleles (*A* and *B*) (*SI Appendix*, Fig. S1).

In contrast to *L02*, *L06*, and *L07*, in the remaining 9 microsatellite markers studied, the distribution of allelic frequencies did not differ between male and female individuals (χ^2 test, $P > 0.05$ for all) (Fig. 1*A* and *SI Appendix*, Fig. S2). Moreover, the heterozygosity observed in female castes did not differ from that expected under random mating, as no departure from HWE was observed ($P > 0.05$ for all except for *L18* in the queen caste [$P = 0.039$] and *L10* in the worker caste [$P = 0.040$]).

Alleles Are Randomly Inherited but Differentially Selected in Each Sex Later in Development. To assess allele inheritance throughout brood development, we set up single queen colonies under laboratory conditions. Queens are usually mated with a single male (37). Nine of the 10 mother queens isolated produced diploid broods (mean ± SD, 13.9 ± 6.9). All (100%) of the brood at the later stages of development (larval stage 2 to nymph; $n = 72$) was heterozygous at *L06* and *L07*, and 100% and 95% of individuals at the first larval stage were heterozygous at *L06* and *L07*, respectively (Fig. 1*B*). Unexpectedly, almost 40% of the eggs genotyped (6 of 17) were homozygous at the *L06* and *L07* markers, as well as heterozygous at 1 or more other markers (i.e., diploid). All these individuals were homozygous *A*/*A* at loci *L06* and *L07*. At the other 9 loci, brood heterozygosity did not differ between the distinct stages of development ($P = 0.388$) and was not different from that expected under random mating ($P = 0.960$) (Fig. 1*B* and *SI Appendix*, Fig. S3).

The brood produced by the remaining mother queen all carried a single allele at each marker ($n = 11$; 2 eggs + 9 larvae to nymph) and were likely haploid males. The mother queen was heterozygous at 4 loci: *L06*, *L07*, *L03*, and *L13*. Both alleles of the mother queen were present in the males produced at loci *L03* and *L13*, but only allele *A* was found at loci *L06* and *L07*, even though the mother queen was heterozygous *A*/*B* at both loci.

Females Exhibit a Highly Outbred Genome. To assess the extent of the genomic region under sexually antagonistic selection, we analyzed a subset of 10 female individuals using double-digest restriction site-associated DNA sequencing (ddRADseq). After filtering, a total of 2,364 SNPs were found in all samples analyzed. These SNPs were spread across 362 of the 2,800 scaffolds of *N. fulva*'s genome. Eighty-eight of these scaffolds showed a significant deviation from HWE. Of these 88 scaffolds, 82 of them showed significant negative values of the inbreeding coefficient, F_{IS}, with 68 of them 100% heterozygous, representing 2.92% of the *N. fulva* genome (Fig. 2 and *SI Appendix*, Fig. S4). Basic Local Alignment Search Tool (BLAST) searches revealed that the microsatellite markers *L06*, *L07*, and *L02* were located on 3 different scaffolds exhibiting significant negative values of F_{IS} (*L06*: scaffold 111, $F_{IS} = -0.576$; *L07*: scaffold 120, $F_{IS} = -0.538$; and *L02*: scaffold 156, $F_{IS} = -0.568$). Interestingly, these markers were not located in scaffolds containing loci involved in sex determination in Hymenoptera (doublesex: scaffolds 27, 42, and 45, $F_{IS} = -0.009$, 0.001, and 0.002; transformer: scaffolds 3 and 185, $F_{IS} = 0.500$ and −0.062). In contrast, only 6 of the 362 scaffolds covered by the SNP analysis showed significant positive F_{IS}, values, with 5 of them 100% homozygous. As a consequence, the *N. fulva* genome was biased toward outbreeding, with most of the scaffolds significantly more heterozygous than expected under random mating. This outbreeding outcome shows that female offspring inherit alternate alleles from their mother while males inherit alleles from their grandfather (males in the Hymenoptera have no father). This also indicates that the divergent selection between the sexes is not merely an artifact found on 3 microsatellite markers but most likely is spread across a substantial genomic region.

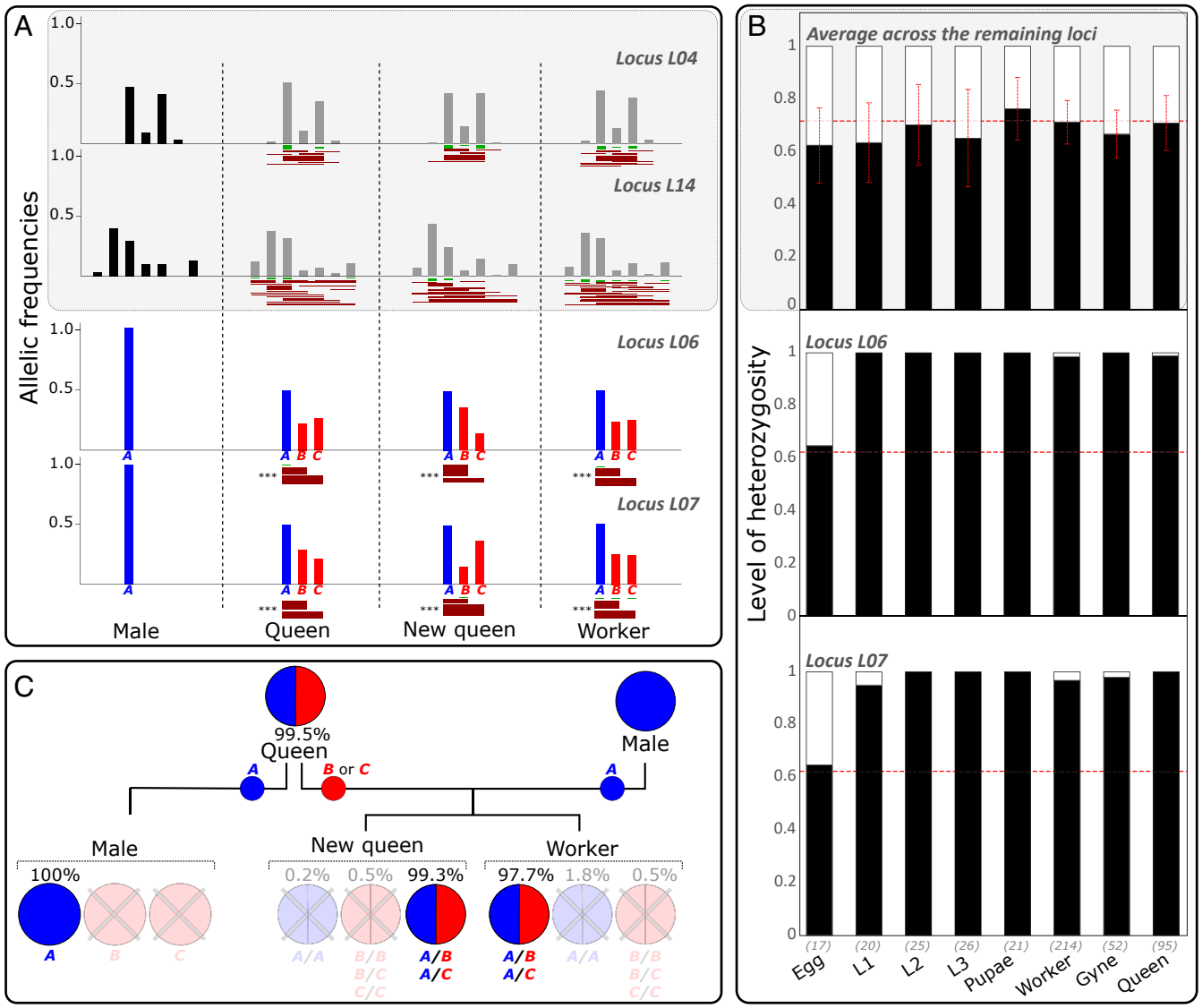

Fig. 1. (*A*) Allelic distribution observed in males, queens, new queens, and workers for selected loci (*L06* and *L07*) and nonselected loci. Here only *L04* and *L14* are shown, the other loci are shown in *SI Appendix*, Fig. S2. For each female group (i.e., queens, new queens, and workers), the horizontal bars below each distribution represent the proportions of homozygosity and heterozygosity for each allelic combination. The thickness of the bar is proportional to the frequency of the observed genotype. Asterisks indicate whether the proportion of genotypes significantly differs from that expected under HWE. ***$P <$ 0.0001. (*B*) Levels of heterozygosity observed for queens, new queens (gynes), and workers at several stages of development. The dotted line represents the level of heterozygosity expected for each marker. The levels of heterozygosity are given for markers *L06* and *L07*, and an average is given for the 9 remaining loci. (All markers analyzed separately are shown in *SI Appendix*, Fig. S3.) The error bars define the SD across the 9 loci. The numbers in brackets indicate the number of samples analyzed in each category. (*C*) Schematic representation of the SAS described in the tawny crazy ant. The proportion of observed genotypes is given under each caste.

Discussion

Our genetic analyses shed light on a unique pattern of sexual divergence in an introduced population of the tawny crazy ant *N. fulva*. We report an occurrence of marked genetic differences maintained between males and females within a single, sexually reproducing species that lacks sex chromosomes. In this species, strong postzygotic selection leads daughters to preferentially carry alleles from their mothers, and sons to preferentially carry alleles from their grandfathers, for large regions of the genome. As a consequence, the sexually produced female castes are highly heterozygous, as they inherit different alleles from their mother and father (Fig. 1*C*). This sexually antagonistic selection may represent a serious cost for colonies, however, as it results in the death of approximately one-half of the female offspring. It may yet preserve genetic diversity in the population and heterozygosity within the female offspring, which may enhance the invasion success of this species, potentially reducing the inbreeding depression that usually follows introduction events (38).

Except in the case of sex chromosomes, genetic differences between males and females are not expected in a sexually reproducing species. This is even more unlikely to occur in Hymenoptera because there are no sex chromosomes, with the sex of an individual determined by its heterozygosity at the CSD locus (34). We can rule out the possibility that the genetic divergence between males and females in *N. fulva* is due exclusively to the physical location of the 3 diverging markers near the regions including the CSD locus or other key sex-determining loci, for several reasons. First, one would expect to find both *B*/*C* heterozygous females and males carrying *B* and *C* alleles. In

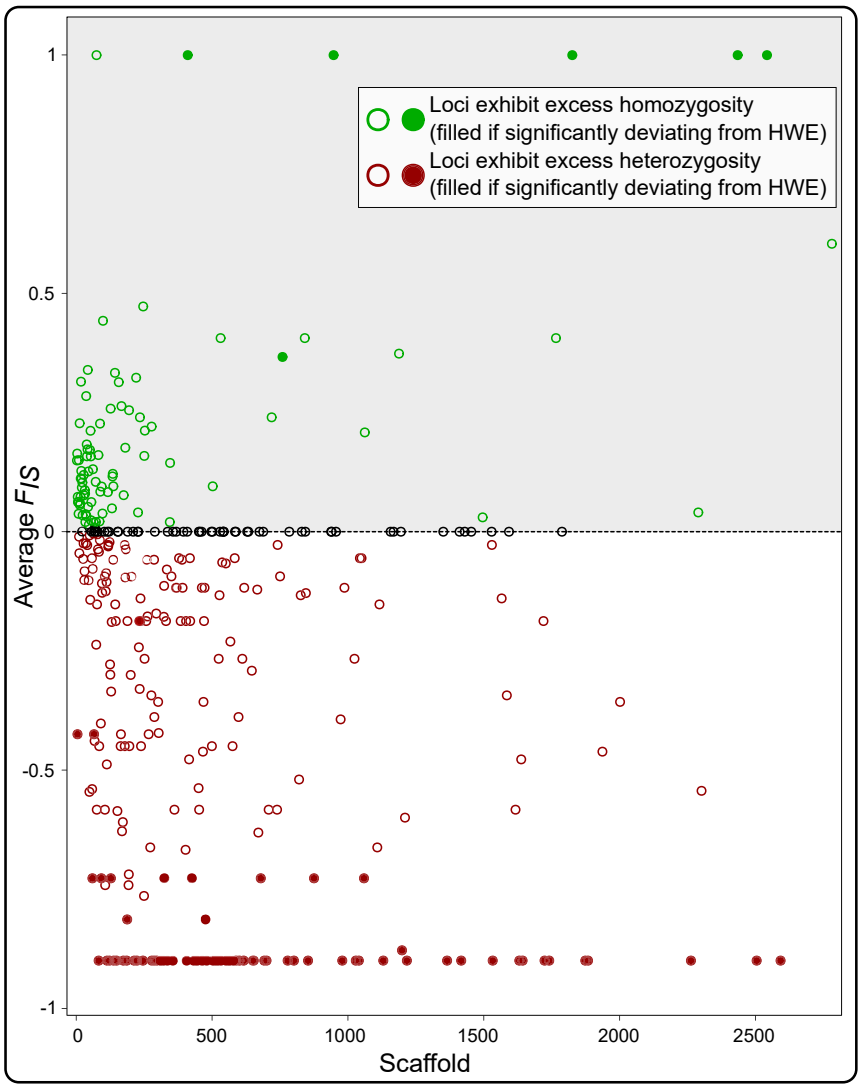

Fig. 2. Average inbreeding coefficient, F_{IS}, across loci for each of the 362 scaffolds spread across the *N. fulva* genome (2,800 scaffolds). Filled scaffolds show a significant deviation from HWE. Only SNPs present in 100% of the samples were used. Results obtained with SNPs present in at least 50% of the samples are shown in *SI Appendix*, Fig. S4.

addition, we observed the presence of some, albeit rare, homozygous females. Finally, our BLAST searches revealed that none of the sex-determining loci were located on any of the same scaffolds as the diverging microsatellites, and none of them exhibited a significant departure from HWE. Instead, the sexual divergence observed in *N. fulva* may result from an intralocus sexual conflict hampering the development of particular sex-specific genotypes and thus selecting for distinct genotypes in males and females (i.e., SAS). This conflict leads to a pattern of differential survival whereby females preferentially carry alleles from their mothers and males preferentially carry alleles from their grandfathers, while sexually produced females consistently inherit both sets of alleles. This pattern is not restricted to the 3 diverging microsatellite markers; rather, it appears to occur over a substantial genomic region, as a high level of outbreeding was detected among workers using genome-scale analyses. Unfortunately, the genome of *N. fulva* is not yet assembled into chromosomes; therefore, we were unable to test whether the selection observed in this species matches specific regions on 1 or more chromosomes. Further studies are needed to investigate whether SAS occurs primarily in a small set of genes and has expanded to include a larger genomic region through a selective sweep or whether this genomic region encompasses a set of SAS genes inherited together without recombination (i.e., a supergene). The recruitment of SAS loci into this region, and their linked inheritance, may act as a "sexual chromosome."

To date, SAS has been reported in a broad variety of taxa including red deer (39), a moth (40), a seed beetle (41), salmon (21), the fruit fly (42), and a dioecious plant (43), which suggests this phenomenon is widespread. However, our study is the first reported case of SAS in a hymenopteran species, despite their haplodiploid system potentially predisposing them to SAS (36). As mentioned above, hymenopteran females are diploid, while males are haploids, and thus their entire genomes should display an X-linked pattern and potentially harbor extensive SA alleles. The haplodiploid expression may also radically increase the degree of SAS. On the one hand, a nonbeneficial allele for males is exposed to stronger selection in the hemizygous state (44), and thus its frequency is expected to be lower than in species with diploid males (45, 46) due to its genetic purging in haploid males. On the other hand, the exposure of a male-beneficial allele in the hemizygous state can lead to its increased frequency in the population when recessive in females, even if this allele is associated with a significant cost to females. It thus creates a protected

polymorphism when the cost to females counterbalances the benefit to males (29, 30). For these reasons, sexually antagonistic polymorphisms are expected to accumulate in haplodiploid genomes despite being more harmful to one sex than they are beneficial to the other (31–33).

This study provides evidence of SAS in an invasive species, where it may play a role in invasion success. The maintenance of genetic diversity is crucial for Hymenoptera, as they suffer from an additional cost of inbreeding (47). When a female mates with a male carrying the same allele as one of hers at the CSD locus, one-half of the fertilized brood will develop into diploid homozygous males. These males are generally sterile or unviable; therefore, their production represents a serious cost (48, 49). As inbreeding increases the probability that a female will mate with a related male carrying a similar CSD allele, it likewise increases the proportion of females producing sterile diploid males. Consequently, they are particularly prone to the loss of genetic diversity that inevitably results from a founder event following introduction (47). Our study highlights a unique system in which SAS may preserve genetic diversity through diversifying selection within an introduced population despite representing a drastic load for colonies.

Finally, our results demonstrate the occurrence of SAS in a social insect with caste determination, where the division of reproductive tasks may buffer the evolution of differential optima between queens and males. Yet social insects are particularly likely candidates for taxa in which SAS may occur, as phenotypic differences between males and females arise at numerous scales (50). Among them, males and queens exhibit pronounced morphological dimorphism, strong divergence in life span, and distinctly different reproductive roles. Queens can be several orders of magnitude larger than males, and they engage in reproduction for up to several years, whereas the males usually die after the reproductive period when they are only a couple of weeks old. These multiple phenotypic differences between the sexes probably require substantial genotype–sex interactions to enable distinct developmental and behavioral outcomes, suggesting that SAS might be more widespread among social insects.

Genetic differences between males and females have been previously reported in the *Formica aquilona-Formica polyctena* hybrid ant complex These 2 species interbreed and produce hybrid females (51). Queens transmit some alleles exclusively to their daughters and other alleles exclusively to their sons, making male-pure lineages within each species. This segregation distortion results in differences between male and female genomes, with females carrying alleles not present in conspecific adult males and lacking homozygous alleles expected under random mating (51). This transmission ratio distortion depending on the sex of the offspring is likely to have evolved in conjunction with strong selection against hybrid males (52). The reproductive system uncovered in our study results in similar genetic outcomes, as females carry alleles not found in males and lack alleles in the homozygous state as is expected under random mating. Likewise, all males of *N. fulva* analyzed are pure lineage, whereas all females are "hybrids" exhibiting heterozygosity at the affected genomic regions. However, the results for *N. fulva* are slightly different from those reported in the *F. aquilona-F. polyctena* complex, given that a single lineage occurs in the male population. The *Formica* complex seems to have originated from an ancient hybridization event between 2 species (51), whereas the system reported here in *N. fulva* likely evolved from a single lineage (i.e., one species; ref. 37).

It has not been determined whether the segregation distortion in the *Formica* complex occurs during the prezygotic stage or the postzygotic stage. However, the sexual divergence observed in *N. fulva* does not stem from differential transmission of the queen's alleles (i.e., at the prezygotic stage), but rather arises in large part from distinct alleles being differentially selected in females at later stages of development; whether postzygotic or prezygotic selection maintains the occurrence of males possessing only *A* alleles remains unknown. In this study, a fair proportion of diploid eggs were homozygous at the 3 diverging markers, while almost all female offspring were heterozygous at later stages of development. This suggests that postzygotic genetic incompatibilities may hamper the development of homozygous females, with sexually antagonistic loci potentially influencing key sex-specific processes early in development. This result is somewhat unforeseen, given that SAS is usually expected only when sex roles become meaningfully defined later in development, as the juvenile stages of the 2 sexes share common fitness objectives (53). These diploid homozygous eggs could possibly be either unviable or killed by workers and thus fail to develop beyond the first larval stage. In the honeybee *Apis mellifera*, workers detect diploid male larvae and kill them before they reach adulthood, thereby limiting the cost of their production and development into adult drones (54).

The genetic consequences *of N. fulva*'s reproductive system resemble those reported in several hybridogenetic ants in the genera *Pogonomyrmex, Solenopsis*, and *Cataglyphis* (55–57). In hybridogenetic species of these genera, 2 lineages occur within populations. In each lineage, interlineage hybrids become workers, pure lineage diploid eggs develop into queens, and unfertilized (i.e., pure lineage) haploid eggs become males. In the *Pogonomyrmex barabatus-Pogonomyrmex rugosus* complex, the colony's single queen mates multiple times, both with males of their own lineage and males of the alternative lineage, to produce new queens and workers (55). In the *Solenopsis geminata-Solenopsis xyloni* complex, colonies of *S. xyloni* contain multiple singly-mated queens. Queens that mate with a male of their own species produce only new queens, while queens mated with an *S. geminata* male produce only workers (56). In hybridogenetic species of the genus *Cataglyphis*, queens mate with males of the alternative lineage to produce hybrid workers and use asexual reproduction or intralineage matings for the production of new queens (57–59). In contrast to the reproductive system observed in *N. fulva*, genetic differences in these hybridogenetic species have been observed between lineages but not between males and females. Furthermore, in the *Pogonomyrmex* and *Solenopsis* complexes, genetic differences result from a hybridization of 2 species, while the mating system of *Cataglyphis* ants involves asexual reproduction. Therefore, their reproductive systems stand in stark contrast with the single-species sexually reproducing system of *N. fulva*. However, like *N. fulva,* these species provide examples of unorthodox reproductive systems that have evolved and persisted despite being exceptionally wasteful. In these species, the development of viable and fertile colonies relies on the appropriate ratio of intralineage and interlineage matings, as colonies headed only by interlineage-mated queens would produce workers to establish a colony but not new queens, and vice versa (59–61).

The sex-specific difference seen in *N. fulva* also has similarities to that reported in 3 other invasive ant species: *Paratrechina longicornis*, *Wasmannia auropunctata*, and *Vollenhovia emeryi*. In these species, male and female differences are maintained through asexual reproduction, whereby queens are clones of their mothers and males are clones of the queens' mates (62–64). Since workers arise from classical sexual reproduction between male and female gene pools, they are 100% heterozygous. This strategy may act as a preadaptation to invasion by helping them cope with the reduced genetic diversity inevitably faced by introduced populations (62). The genetic difference observed in these species is present not only between males and females, but also among female castes. Thus, the outcomes of this reproductive system differ from those observed in *N. fulva*. In the tawny crazy ant, the sex differences are maintained despite sexual reproduction, and there is no difference in the mode of production between workers and queens (37). Consequently, nearly all females of both castes are heterozygous in the affected genomic region(s).

Conclusion

Our study reveals an unusual pattern of inheritance in the introduced population of the tawny crazy ant *N. fulva*, with drastic genetic differences between the sexes despite sexual reproduction. In this species, strong postzygotic selection maintains a distorted segregation inheritance whereby daughters preferentially carry a maternally inherited allele set while sons carry a paternally inherited allele set. This allelic pattern provides evidence of sexually antagonistic selection in a haplodiploid organism, supporting the prediction that these species might potentially be prone to SAS due to their X-linked genomes (36). In *N. fulva*, the magnitude of SAS is surprisingly large, with male-beneficial alleles being lethal to females and vice versa. This results in highly heterozygous females, as they always inherit alternate alleles from their mother and father. This sexually antagonistic selection preserves genetic diversity within the introduced population of *N. fulva* and heterozygosity in a large genomic region among the female offspring, thus potentially mitigating bottleneck effects.

Whether this strategy represents a preadaptation to invasion or whether this species remains stuck in a maladapted equilibrium remains unclear. A fair proportion of homozygous eggs never reach adulthood, with females consistently wasting resources on zygotes that are destined to die. This represents a serious cost for colonies and may severely limit the reproductive potential of this species. Yet this strategy seems to be widespread across the entirety of *N. fulva's* introduced range, as a high proportion of heterozygous workers have been reported in sampled localities from Texas to Georgia (98.4% and 97.4% for the markers *L06* and *L07*, respectively; $n = 867$) (37). Further studies are needed to investigate whether this strategy is already present in the native population of the invasive tawny crazy ant or whether it represents an incomplete postintroduction shift in reproductive strategy that has emerged to mitigate the effects of genetic depletion following the ant's introduction. Similarly, the determination of the size of the genomic region(s) and the different genes involved await further investigation. These results could potentially lead to the discovery of a "sexual chromosome" whereby a specific combination of alleles impedes the development of offspring into a particular sex.

Materials and Methods

Sixteen nests of *N. fulva*, including workers, queens, virgin queens, and males, were collected from 4 localities in Texas. In almost all workers sampled ($n = 214$), we found an extremely high level of heterozygosity at 2 of the 12 microsatellite loci analyzed (*Results* and Fig. 1), indicating that alleles transmitted by the queens and their mates were almost never identical at these markers and/or that individual homozygotes at these loci were unviable. To investigate allele inheritance in different castes, we genotyped a sample of workers, queens, virgin queens, and males for each nest. To assess the level of heterozygosity throughout brood development, the remaining colonies from sampled nests were brought to the laboratory and maintained under laboratory conditions (12:12 light cycle, constant 26 °C, cockroach and sugar water diet). Ten queens from different polygynous colonies were isolated with at least 100 nestmate workers to establish single-queen laboratory colonies. Care was taken to remove all brood before the experiment to ensure that the single queen within each laboratory colony laid newly produced brood. Brood was sampled in each colony at various stages of development: egg, larval instar 1 to 3, and nymph. At the end of the experiment, the queen and all sampled brood from the laboratory colonies were extracted and genotyped.

Total genomic DNA was extracted from each individual using a modified Gentra Puregene extraction method (Gentra Systems). Each individual was genotyped at 12 microsatellite markers previously developed for *N. fulva* (37). Amplicons were amplified using a Bio-Rad thermocycler T100 and visualized on an ABI 3500 capillary sequencer against an LIZ500 internal standard (Applied Biosystems). Microsatellite alleles were scored using Geneious v.9.1 (65).

The number of alleles, allele frequencies, measures of observed and expected heterozygosity, and *F* statistics were estimated using FSTAT (66) and Genepop on the Web (67). These parameters were assessed and compared within and between all castes and brood at the different stages of development. Brood was considered diploid when at least 1 out of the 12 markers was heterozygous, and considered haploid when all markers were homozygous, but sexing the egg at the first stage of development was not feasible. All statistical analyses were run using the R software environment (R Foundation for Statistical Computing).

Genomic DNA from 1 queen and 9 workers from the US-introduced population were used to construct ddRAD libraries. These individuals were randomly sampled across 8 localities in Texas (San Antonio, La Marque, Iowa Colony, Smithville, Buda, Austin, Silsbee, and Bryan) and 2 localities in Georgia (Chatham and Camden). The double digestion was completed with SphI and EcoRI restriction enzymes. A unique indexed barcode was assigned to each sample. Paired-end libraries were constructed on the Illumina platform, with each sample run on 2 flowcell lanes. Stacks v.2.3b (68) was used to demultiplex the paired-end reads by their unique barcodes. Reads were aligned to the *N. fulva* reference genome currently made of 2,800 scaffolds using the Burrows–Wheeler aligner (69). Aligned reads were then run through the reference-based pipeline of Stacks, which built and genotyped the paired-end data and also called SNPs using the population-wide data per locus. Only SNPs present in all of the samples ($n = 2{,}364$) were kept for downstream analyses. The 2,364 SNPs analyzed were located in 362 different scaffolds, representing 57.60% of the *N. fulva* genome. Population genetic statistics were generated in Stacks for each SNP locus, and the resulting output was exported to R. The inbreeding coefficient, F_{IS}, was calculated for each locus and averaged for each scaffold. A significant negative value of F_{IS} (outbreeding) indicates a high level of heterozygosity, suggesting that distinct alleles were inherited from each parent. The analysis was replicated with SNPs present in at least 50% of the samples ($n = 9{,}779$). These SNPs were located in 736 scaffolds, representing 71.24% of the *N. fulva* genome. The results are provided in *SI Appendix*, Fig. S4. BLAST searches were performed to locate the microsatellite markers *L06*, *L07*, and *L02* on the different scaffolds of the *N. fulva* genome. Additional BLAST searches were performed to locate different key loci involved in sex differentiation in hymenopteran species: doublesex (including mab-3–related transcription factors *A1*-like, *A2*-like, and *A2*) and transformer (including transformer-2 protein homolog beta-like and pre-mRNA splicing factor *CWC25*-like).

Data Availability. The data reported in this study have been deposited in the Open Science Framework database, https://osf.io (DOI: 10.17605/OSF.IO/C5FQZ).

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