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Direct and indirect genetic effects of a social supergene

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Abstract

Indirect genetic effects describe phenotypic variation that results from differences in the genotypic composition of social partners. Such effects represent heritable sources of environmental variation in eusocial organisms because individuals are typically reared by their siblings. In the fire ant *Solenopsis invicta*, a social supergene exhibits striking indirect genetic effects on worker regulation of colony queen number, such that the genotypic composition of workers at the supergene determines whether colonies contain a single or multiple queens. We assessed the direct and indirect genetic effects of this supergene on gene expression in brains and abdominal tissues from laboratory-reared workers and compared these with previously published data from field-collected prereproductive queens. We found that direct genetic effects caused larger gene expression changes and were more consistent across tissue types and castes than indirect genetic effects. Indirect genetic effects influenced the expression of many loci but were generally restricted to the abdominal tissues. Further, indirect genetic effects were only detected when the genotypic composition of social partners differed throughout the development and adult life of focal workers, and were often only significant with relatively lenient statistical cutoffs. Our study provides insight into direct and indirect genetic effects of a social supergene on gene regulatory dynamics across tissues and castes in a complex society.

KEYWORDS
gene expression, greenbeard, indirect genetic effects, polygyny, Solenopsis invicta, supergene

1 | INTRODUCTION

Social interactions among conspecific individuals create a scenario in which interacting phenotypes influence trait variation (Farine et al., 2015; Moore et al., 1997). When the phenotype of an individual is influenced by environmental variation attributable to the genotypes of its social partners, this is termed an indirect genetic effect (Moore et al., 1997). Because individuals rely on social care

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to develop into adults in eusocial species, indirect genetic effects can be a heritable, evolutionarily-responsive, source of trait variation (Linksvayer, 2015; Linksvayer & Wade, 2005; Wolf et al., 1998). Variation in aggression (Avalos et al., 2020), size, and reproductive allocation (Linksvayer, 2006; Vojvodic et al., 2015) have each been attributed to indirect genetic effects in studies of eusocial insects.

Recent studies have uncovered many instances of supergenes (i.e., tight clusters of two or more loci each affecting a different developmental or behavioural characteristic), which provide integrated control of complex adaptive phenotypes segregating within species (Schwander et al., 2014; Thompson & Jiggins, 2014; Wellenreuther & Bernatchez, 2018). In eusocial insects, supergenes are frequently responsible for intraspecific variation in social organization (Kay, Helleu, & Keller, 2022). Understanding the direct and indirect genetic effects of supergenes is of particular interest in social organisms (Kay, Helleu, & Keller, 2022) or in situations where supergenes induce variation in mating systems (Mank, 2022).

In the fire ant *Solenopsis invicta*, indirect genetic effects of a supergene underpin the behavioural regulation of colony queen number by workers (Ross & Keller, 2002; Wang et al., 2013). In monogyne colonies, a single reproductive queen resides with her worker offspring, while in polygyne colonies multiple reproductive queens reside with workers composed of multiple family groups (Keller, 1993). This variation in queen number is associated with a suite of important individual- and colony-level phenotypic differences (DeHeer, 2002; DeHeer et al., 1999; Gotzek & Ross, 2007; Ross & Keller, 1995, 1998). The supergene controlling this fundamental social polymorphism is formed by three overlapping chromosomal inversions that result in high levels of linkage disequilibrium between >500 protein coding genes (Helleu et al., 2022; Huang et al., 2018; Wang et al., 2013; Yan et al., 2020). The fire ant supergene thus provides a system with prominent roles for direct and indirect genetic effects in shaping variation in social organization.

Reproductive queens in monogyne colonies of *S. invicta* are all homozygous for the *Social B* (*SB*) supergene haplotype, which has largely retained ancestral synteny, whereas reproductive queens in polygyne colonies in the U.S. are all heterozygous for *SB* and the inverted supergene haplotype *Social b* (*Sb*) (Ross, 1997; Wang et al., 2013; Yan et al., 2020). Importantly, the presence of workers with the *Sb* haplotype affects the behaviour of *SB/SB* workers. When the queen is removed from colonies containing only *SB/SB* workers, the workers accept only a single replacement queen with the genotype *SB/SB* (*SB/Sb* and supernumerary queens are executed), but *SB/SB* workers that coinhabit colonies with *Sb*-bearing workers will accept multiple *SB/Sb* replacement queens (Gotzek & Ross, 2008; Ross & Keller, 2002). Supergenes mediating polymorphic social organization have now been discovered in at least four additional ant lineages and it is likely that they play an important role underlying variation in social organization in many other eusocial insects (Kay, Helleu, & Keller, 2022).

So far, only a few studies have investigated the consequences of indirect genetic effects on gene expression in social insects. In the honey bee *Apis mellifera*, the mixing of workers with different genotypes has been shown to have indirect effects on individual worker behaviour and transcription of genes in the brain (Gempe et al., 2012). By contrast, no detectable indirect genetic effects were detected in the clonal raider ant *Ooceraea biroi* when gene expression was compared between workers exposed to social partners from different clonal lineages (in this species thousands of genes are differentially expressed between individuals of the different lineages) (Kay, Alciatore, et al., 2022). In *S. invicta*, indirect genetic effects of the social supergene have previously been investigated on several occasions (Arsenault et al., 2020; Dang et al., 2019; Wang et al., 2008). In a first study using microarrays of pooled whole bodies of workers (Wang et al., 2008), a greater number of genes were influenced by indirect effects associated with the *Gp-9* genotypic composition of their colony than by the direct effect of their own *Gp-9* genotype (*Gp-9* is a an odorant binding gene located in the social supergene (Wang et al., 2013)). In another study comparing gene expression of pools of antennae from multiple workers there were similar numbers of genes influenced by direct and indirect genetic effects (Dang et al., 2019). Finally, a comparison of gene expression of two tissues of individual prereproductive queens revealed substantially fewer genes differentially expressed because of indirect genetic effects than direct genetic effects within each tissue type (Arsenault et al., 2020). The contribution of direct and indirect effects of the supergene on transcriptional variation seems to vary according to biological context and scale in *S. invicta*. Thus, an assessment of general trends across castes and tissues is warranted to test for general properties of direct versus indirect genetic effects in the system.

Here, we present the first report of direct and indirect effects of the fire ant supergene in brains and gasters of the worker caste of *S. invicta*. We integrated the analyses of our data with previously reported data for prereproductive queens (Arsenault et al., 2020) to provide a comprehensive view of the direct and indirect genetic effects of the supergene across castes (Figure 1). We examined direct genetic effects using transcriptomes from brains and abdominal tissues of *SB/SB* and *SB/Sb* workers and unmated winged queens (gynes) from polygyne colonies. We examined indirect genetic effects of the supergene using transcriptomes from adult *SB/SB* workers and gynes that developed in a monogyne social environment (where all nestmate workers have the genotype *SB/SB*) and a polygyne social environment (where nestmate workers are mix of genotypes *SB/SB* and *SB/Sb*; Figure 1). Our results suggest that direct genetic effects of the fire ant supergene result in larger gene expression differences than indirect genetic effects and that direct genetic effects are more consistent than indirect genetic effects across tissue types and castes. We also conducted cross-fostering experiments of late-stage pupae to investigate how the social environment affects patterns of gene expression as adults. Previous studies revealed that *SB/SB* workers shift from rejecting all *SB/Sb* queens to accepting multiple *SB/Sb* queens when their colony contains more than 10%–15% *Sb*-bearing adult workers (Gotzek & Ross, 2008; Ross & Keller, 2002). We raised these *SB/SB* individuals until two weeks post-eclosion to investigate the role of social environment during the first weeks of adulthood. These cross-fostering experiments

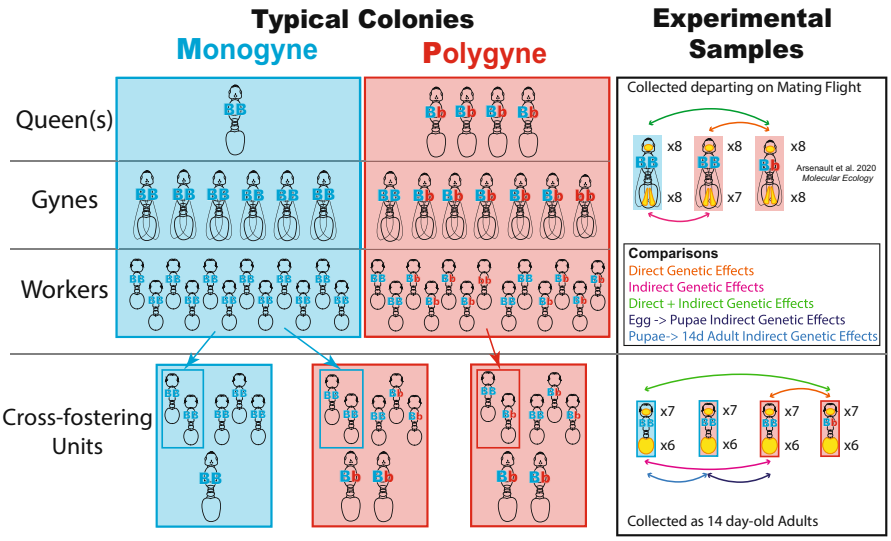

FIGURE 1 Experimental design. Monogyne colonies (large blue box) have a single, unwinged reproductive queen, sterile workers, and, seasonally, winged unmated queens (gynes), all with the *SB/SB* genotype ("BB" in figure). Polygyne colonies (large red box) contain multiple, unwinged reproductive queens with the *SB/Sb* genotype ("Bb" in figure). The gynes in polygyne colonies can have a *SB/SB*, *SB/Sb*, or *Sb/Sb* genotype, although *Sb/Sb* gynes are rare because the *Sb* haplotype recessively induces early mortality of females. Polygyne workers are a mixture of *SB/SB* and *SB/Sb* individuals (*Sb/Sb* workers are also rare). Pupae from monogyne colonies were cross-fostered in both monogyne and polygyne units (blue arrows and small blue boxes) while pupae from polygyne colonies were cross-fostered in polygyne units (red arrows and small red boxes). In the experimental samples, the colour of the box border around the workers indicates whether individuals were raised in monogyne (blue) or polygyne (red) colonies until pupation. The colour of the worker indicates whether individuals were raised in monogyne (blue) or polygyne (red) colonies from pupation until 14–16 days into adulthood. Brains and ovaries of gynes and brains and gasters of workers (marked in gold) were collected for RNA-seq analyses (data for gynes are from (Arsenault et al., 2020)). Sample sizes are included next to each tissue/caste.

revealed no significant indirect effects of the supergene on transcription when the type of social environment experienced by sampled individuals differed only during adulthood.

2 | MATERIALS AND METHODS

2.1 | Worker RNA-seq data

S. invicta fire ant colonies were collected in Florida, United States and reared for one month under standard laboratory conditions (Jouvenaz et al., 1977) before any experimental manipulations. Prior to, and during experiments, ants were fed a diet of crickets, mealworms and a mixture of sugar, fruits, and vegetables. To study the indirect genetic effects (on gene expression in adults) of the social environment (i) from egg to the pupal stage, (ii) during the first 14–16 days as adults (i.e., after eclosion from the pupae), and (iii) for the combined effects of these periods, we transferred 25–40 dark worker pupae (i.e., 0–48 h prior to emergence) between recipient colonies that were formed from 10 stock monogyne and nine stock polygyne colonies. *SB/SB* worker pupae from monogyne donor colonies were transferred to both monogyne and polygyne recipient colonies and *SB/SB* and *SB/Sb* worker pupae from donor polygyne colonies to polygyne recipient colonies. We did not perform the reciprocal introduction of pupae from polygyne to monogyne colonies. Because the *Sb* haplotype recessively induces early mortality in U.S. populations (Hallar et al., 2007; Ross, 1997), *Sb/Sb* adults are rare and were excluded from our study. A given stock colony was never used more than once as a donor or recipient colony for a given treatment and pupae were never transferred between donor and recipient colonies originating from the same stock colony. All recipient colonies contained 250 workers which were marked by cutting the middle leg at the level of the mid-femur, allowing the easy identification of cross-fostered workers. Monogyne recipient colonies contained one monogyne reproductive *SB/SB* queen while polygyne recipient colonies contained two egg-laying dealate *SB/Sb* queens (mating status unknown). More than two queens are typically observed in *S. invicta* polygyne colonies (Ross, 1993; Ross et al., 1996), but the indirect genetic effect of *SB/Sb* workers on *SB/SB* workers to achieve social conversion occurs independently of queen number, genotype, or history (Gotzek & Ross, 2008). Sixteen days after the cross-fostering, we sampled all 0.6–0.9 mg minor workers with six intact legs for the RNA-seq analyses (these individuals were all 14–16 days old). The decision to sample single focal individuals was made for two reasons: first, we planned to correlate gene expression levels with DNA methylation data from the same individuals (but the DNA methylation data were not of sufficient quality to pursue this aim), and, second, this sampling scheme is comparable to the gyne data we reanalysed (Arsenault et al., 2020).

Every sampled worker was genotyped using an allelic discrimination assay (Shoemaker & Ascunce, 2010). We dissected the brains of workers on ice in phosphate-buffered saline (PBS) solution, removing

neighbouring glands and neural elements attaching the brain to the cuticular periphery. Gasters were removed by a single cut. Dissected samples were stored in liquid nitrogen. Total nucleic acids from brains were extracted using an Agencourt FormaPure kit (Beckman Coulter Life Sciences) with volumes scaled down by a factor 4. The first two washing steps (wash buffer and 70% ethanol) were carried out twice. Half of the extracted nucleic acids were eluted with 20 µl of RNase free water and concentrated to 5 µl. The sample was digested by adding 20 µl of DNase I mix (60 µl contains 1.5 µl 1 M Tris–HCl [pH 7.5], 3.75 µl 0.1 M $MgCl_2$, 1.5 µl 2.5 M KCl, 1.5 µl 0.1 M DTT, 3.75 µl RNasin Plus [40 u/µl; Promega], 5 units of DNase I [RNase-free; cat no. 04716728001; Roche] and 47.5 µl RNase-free water) and it was then incubated at 37°C for 15 min. Then, 25 µl of T.E. buffer (pH 7.5) and SDS (0.2% final concentration) were added and RNA was extracted with chloropropane. We added 8 µl of linear acrylamide (5 mg/ml; Ambion) as a carrier to the aqueous phase and nucleic acids were precipitated by adding sodium acetate to 0.3 M and 2.5 volumes of ethanol followed by a wash in 70% ethanol. The pellet was air-dried for 5 min and resuspended in 9 µl of RNase-free water. Sequencing libraries for brains were prepared using SMARTer Ultra Low Input RNA for Sequencing-V3 kit (Takara Bio) with a modified protocol provided by manufacturer for brains. After the fragmentation step (volume 75 µl), 8 µl of acrylamide carrier was added and double-strand cDNA was precipitated with ethanol followed by a wash in 70% ethanol. The pellet was air-dried for 5 min, resuspended in 10 µl 10 mM Tris–HCL (pH 8.5) and processed further with the sequencing kit. Sequencing libraries for gasters were prepared using the KAPA stranded RNA-seq kit (Kapa Biosystems) following the manufacturer's instructions. Paired-end 100-bp reads were generated for all samples using an Illumina HiSeq2500.

2.2 | Gyne RNA-seq data

S. invicta gyne RNA-seq data were generated for a prior study, with detailed sampling and library preparation protocols described therein (Arsenault et al., 2020). Briefly, gynes embarking on mating flights were collected in the field in Georgia, US. Each Illumina sequencing library was prepared from a genotyped individual's brain or ovary tissues following the Smart-seq2 protocol (Picelli et al., 2014), and 75-bp single end reads were sequenced. Eight *SB/SB* gynes were sampled from eight monogyne colonies, and eight pairs of *SB/SB* and *SB/Sb* gynes were sampled from eight polygyne colonies. By sampling gynes (alate prereproductive queen) immediately prior to their mating flights, we were able to compare the indirect genetic effects of the supergene on *SB/SB* individuals raised in monogyne and polygyne colonies while avoiding the large differences in reproductive status among individuals that are associated with age differences.

2.3 | Bioinformatic analyses

RNA-seq reads were trimmed using TrimGalore (https://github.com/FelixKrueger/TrimGalore) and aligned to the "SINVBB1" genome assembly (Yan et al., 2020) using STAR (Dobin et al., 2013) with the 2-pass alignment procedure. In the gyne data, between 10.7 and 28.9 million reads were aligned in each sample while the worker data had higher coverage with all samples having between 33.3 and 158.2 million reads (Supporting Information Data F). Gene counts exported from STAR were used for differential expression analyses with edgeR (Robinson et al., 2009). Differential expression was computed for each caste and tissue separately to minimize batch effects from the separate library preparation and sequencing procedures. Within each caste, differential expression was first computed using a pairwise approach. Low coverage genes were filtered using the default edgeR procedure ("filterByExpr"), where five samples (based on 70% of the minimum sample size of a group) need to have at least 10 reads aligned and the total count across all samples need to be greater than 15. Samples from gyne brains retained 10,150 genes after filtration, gyne ovaries retained 10,260, worker brains retained 12,006, and worker gasters retained 11,574 of the 16,314 total genes in the genome annotation. Library sizes were normalized using edgeR's "calcNormFactors" which computes scale factors using a trimmed mean of M-values approach. Common and tagwise dispersions were computed and Quasi-likelihood F-tests were performed to compute differential expression (Robinson et al., 2009). Generalized linear model comparisons were performed using models with variables designated for caste, tissue, natal colony type, and supergene genotype (cross-fostered samples were not included in this analysis). Gene ontology (GO) functional annotations for genes were assigned with the eggnog-mapper (version 1.0.3) (Huerta-Cepas et al., 2018) and enrichment of functional terms was assessed statistically using the default "weight01" Fisher's exact test in topGO (Alexa & Rahnenfuhrer, 2010). In all differential expression comparisons, a series of FDR-corrected *p*-value thresholds were utilized (FDR < 0.01, 0.05, and 0.1) to give a more complete view of the structure of the results. While FDR < 0.01 minimizes false-positives, it increases the likelihood of false-negatives. These patterns reverse as the FDR threshold is lowered. Consequently, we were able to identify genes with highly significant FDR values while also using less stringent FDR cutoffs for global analyses (transcriptome intersections and GO term enrichment) that are more robust to false positives.

3 | RESULTS

3.1 | Direct genetic effects

Seventy-one genes were differentially expressed between brains of *SB/SB* and *SB/Sb* workers and 23 between brains of *SB/SB* and *SB/Sb* gynes (false discovery rate [FDR] < 0.1; Figure 2a). Eighty-six genes were differentially expressed between gasters (the abdominal segments following the post-petiole) of workers and 215 genes between ovaries of gynes with different supergene genotypes (FDR < 0.1; Figure 2a). In all tissues and castes where the gene expression of *SB/SB* and *SB/Sb* was compared, the differentially expressed genes tended to be upregulated in *SB/Sb* individuals (Figure 2e) and more

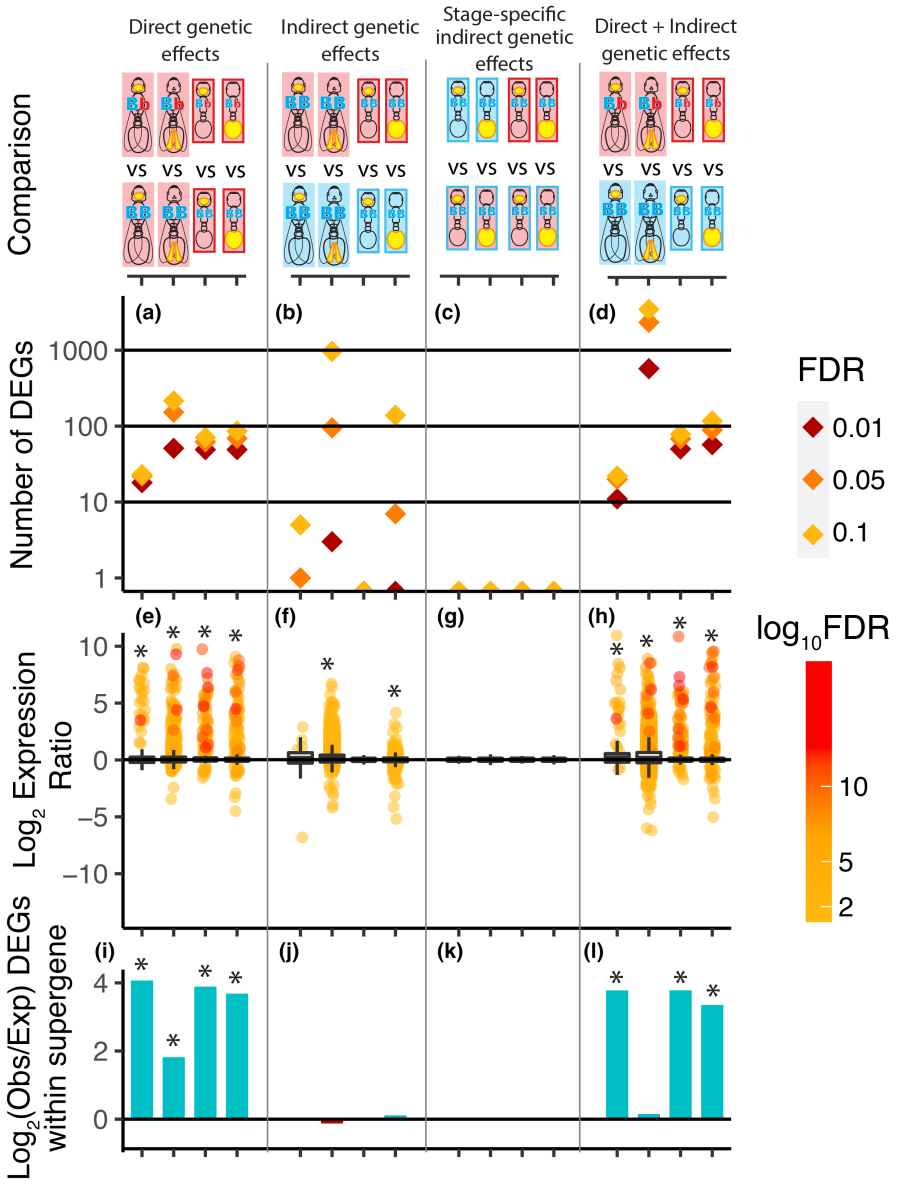

FIGURE 2 Direct and indirect genetic effects on gene expression. For each pairwise comparison, the sample types are illustrated at the top of the figure (as described in Figure 1). (a–d) the numbers of differentially expressed genes (DEGs) in each pairwise comparison are plotted at multiple significance levels. (e–h) Log_2-transformed gene expression ratios. Directionality of each plot mirrors the directionality of the schematic for that comparison at the top of the figure (positive values indicate higher expression in *SB/Sb* individuals in panel (e) and the polygyne social environment in panel f). Each plot includes a box plot showing the ratios for all genes, including those that are not significant (whiskers show observations within 1.5 * IQR of the lower and upper quartiles). Each dot represents a DEG (FDR <0.1). Significant directional bias in differential expression is indicated with * (chi-squared test; $p < .05$). (i–l) Log_2-transformed ratio of overrepresentation within rather than outside the supergene region (observed/expected) for significant DEGs (FDR <0.1). Positive values indicate a higher proportion of DEGs found within the supergene versus the rest of the genome. Statistical significance of enrichment is indicated with * (chi-squared test; $p < .05$).

of the differentially expressed genes were located within the supergene region than expected by chance (Figure 2l).

Genes that were differentially expressed in one tissue or caste because of direct genetic effects were frequently differentially expressed in the other tissue or caste (Figure 3a). A majority of differentially expressed genes in both worker and gyne brains were differentially expressed in other sample types, and eight of these genes were differentially expressed in all four direct genetic effect comparisons (Figure 3a). Four of these eight genes had functionally annotated homologues: *growth arrest specific 8* (*gas-8*), *nose resistant to fluoxetine-6* (*nrf-6*), and two *Harbinger Transposase derived-1* (*harbi1*) genes (Supporting Information Data C). To further characterize the direct genetic effects on transcription, we used a generalized linear model to identify genes that were significantly differentially expressed by genotype when including samples of both castes and tissues and modelling the effects of genotype, social environment, caste, and tissue (McCarthy et al., 2012). This analysis revealed 145 significant genes that were differentially expressed by the direct genetic effects of genotype (FDR <0.1; 115 differentially expressed genes at FDR <0.05, 77 at FDR <0.01; Figure 3c). GO functional enrichment tests (Fisher's exact test, $p < .01$) for these direct genetic effect genes (FDR <0.1) revealed that *locomotion*, *reproduction*, and numerous metabolism terms were overrepresented (Supporting Information Data A).

3.2 | Indirect genetic effects

The number of genes expressed differentially by indirect genetic effects (comparison of *SB/SB* adults reared in different social environments from egg through two weeks of adulthood) was lower than the number of genes differentially expressed by direct genetic effects (comparison of *SB/SB* and *SB/Sb* adults from the same social environment) in all comparisons (worker brain, worker gaster, gyne brain, and gyne ovaries), when using an FDR of 0.05 or 0.01 as a cutoff for significance. At the least stringent statistical cutoff FDR

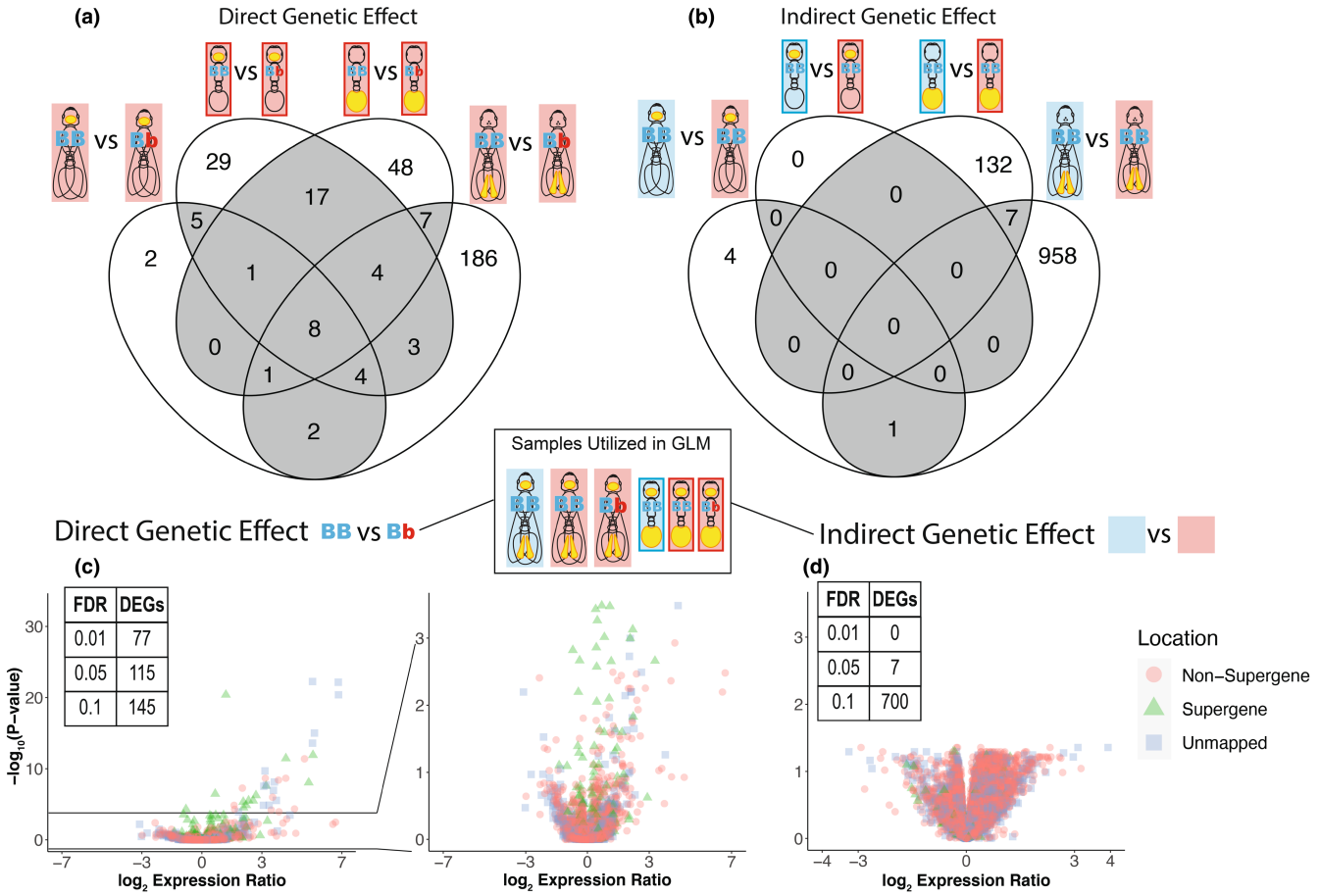

FIGURE 3 Consistency of direct and indirect genetic effects on gene expression across tissues and castes. Venn diagrams show significant (FDR <0.1) differentially expressed genes (DEGs) arising from pairwise comparisons representing (a) direct genetic effects and (b) indirect genetic effects in distinct tissues and castes. Generalized linear models were used to assess the effects of (c) supergene genotype (*SB/SB* [BB] vs. *SB/Sb* [Bb]; direct genetic effect) and (d) social environment (polygyne vs. monogyne; indirect genetic effect) among both tissues and castes, with $\log_2$(expression ratio) and significance values generated from these models shown in volcano plots. Between panels c and d is a portion of panel c shown with significance scaled as in panel d.

of 0.1, this was also true for brains of workers and gynes but not for abdominal tissues (Figure 2a,b). Genes differentially expressed by indirect genetic effects tended to be upregulated in individuals from polygyne colonies as compared with individuals from monogyne colonies in the gasters and ovaries of workers and gynes respectively (Figure 2f, positive $\log_2$ expression ratios). In contrast to direct genetic effects, genes differentially expressed by indirect genetic effects were not located within the supergene region more frequently than expected by chance (Figure 2j). The transcriptional signature of indirect genetic effects was also much less pronounced than that of direct genetic effects across sample types; on average, the absolute value fold-change was significantly lower for genes differentially expressed by indirect genetic effects than for genes differentially expressed by direct genetic effects for three of four caste and tissue combinations (worker brains, worker gasters, and gyne ovaries at FDR <0.1; Welch's two-sample *t*-tests, $p < .01$; $\log_2$fold-change ranges in gyne brains were 1.4 to 8.1 in the direct effects comparison and − 6.8 to 2.9 in the indirect effects comparison, in gyne ovaries were − 3.5 to 9.7 in the direct effects comparison and 1.4 to 6.7 in the indirect effects comparison, in worker brains were − 1.6 to 9.7 in the direct effects comparison with no differentially expressed genes in the indirect effects comparison, and in worker gasters were − 4.5 to 9.0 in the direct effects comparison and − 5.2 to 4.1 in the indirect effects comparison; Figure 2e,f). These findings each mirror the previous findings using solely gyne data (Arsenault et al., 2020). Our analysis of age- and size-matched worker samples helps to rule out the possibility that differences in gene expression could result from age-induced differences (Lucas et al., 2017) because the age of gynes can vary at the time of nuptial flight (Arsenault et al., 2020; Nipitwattanaphon et al., 2013). While there are well-documented behavioural differences between workers in monogyne and polygyne colonies (Gotzek & Ross, 2008; Keller & Ross, 1998; Ross & Keller, 1998, 2002; Trible & Ross, 2016; Zeng et al., 2022), we observed only few genes that were differentially expressed in brains of individuals exposed to these different social environments, with none observed in workers and only five in gynes (FDR <0.1; Figure 2b). Thus, pronounced indirect genetic effects of the fire ant supergene were primarily detectable in abdominal

tissues, in contrast to the direct genetic effects, which were prevalent in abdominal tissues and in the brain.

To quantify the indirect genetic effects that occurred from eclosion until the pupal stage, we compared *SB/SB* workers that always experienced a polygyne social environment and *SB/SB* workers that experienced a monogyne social environment until the pupal stage and then a polygyne social environment through the first 14–16 days as adults. This analysis revealed no significant differences in gene expression (FDR > 0.1; Figure 2c). To quantify the indirect genetic effects that occurred during the first 14–16 days as adults, we compared *SB/SB* workers that always experienced a monogyne social environment and *SB/SB* workers that experienced a monogyne social environment until the pupal stage and then a polygyne social environment during the first 14–16 days as adults. This analysis also revealed no significant differences in gene expression (FDR > 0.1; Figure 2c).

The proportion of differentially expressed genes (FDR < 0.1) that overlapped between castes or tissues was 22 times higher for the set of genes influenced by direct genetic effects (Figure 3a) than those influenced by indirect genetic effects (Figure 3b). To further characterize the indirect genetic effects of monogyne versus polygyne social environments, we utilized a generalized linear model to identify genes that were significantly differentially expressed when including samples of both castes and tissues in a model that considered the effects of genotype, social environment, caste, and tissue (McCarthy et al., 2012). This analysis revealed 700 significant genes that were differentially expressed by the indirect genetic effects of the social environment at a lenient statistical cutoff (FDR < 0.1; 7 differentially expressed genes at FDR < 0.05, 0 at FDR < 0.01; Figure 3d).

The genes differentially expressed in the direct genetic effects generalized linear model (FDR < 0.1) exhibited higher absolute $\log_2$-transformed expression ratios than genes differentially expressed in the indirect genetic effects generalized linear model (Welch's two-sample *t*-test; $p < .01$; Figure 3c,d). GO functional enrichment tests (Fisher's exact test, $p < .01$) revealed an overrepresentation of genes differentially expressed in response to indirect genetic effects (FDR < 0.1) that were annotated with the terms *response to stimulus*, *oogenesis*, and various neural development terms (Supporting Information Data A).

3.3 | Direct and indirect genetic effects

Given that the behaviour of workers depends both on their own genotype and social environment (due to the presence of *SB/Sb* workers only in polygyne colonies), we wanted to test for a combinatorial effect of both colony social environment and supergene genotype on transcription. Analyses of these combined direct and indirect genetic effects were performed by comparing gene expression of *SB/SB* individuals from a monogyne social environment to *SB/Sb* individuals from a polygyne social environment (Figure 2d). The numbers of the differentially expressed genes (FDR < 0.1) in brains due to these combined effects in workers ($n = 78$) and gynes ($n = 22$) were similar to the sum of differentially expressed gene tallies from separate analyses of direct and indirect genetic effects in each caste (workers: $n = 71 + 0 = 71$; gynes: $n = 23 + 5 = 28$). In worker gasters, many fewer genes were differentially expressed (FDR < 0.1) by combined indirect and direct effects ($n = 117$) than the sum of tallies from separate analyses of these effects ($n = 86 + 139 = 225$). This could be explained by overlap between direct and indirect effect gene sets (examined below), opposing directionality of direct and indirect effects on expression, and/or noise in the data. Gyne ovaries offer a stark contrast to the other sample types, in that there were over 2000 more differentially expressed genes (FDR < 0.1) observed in response to the combination of direct and indirect genetic effects ($n = 3451$) than in separate tallies of indirect and direct genetic effects ($n = 215$ and 966, respectively).

A generalized linear model including samples of both castes and tissues revealed that the overlap between direct genetic effects and indirect genetic effects on differential gene expression was higher than expected by chance (Fisher's exact test; $p < .05$; Figure 4a). However, when considering tissues and castes separately, the overlap between direct and indirect genetic effects on gene expression was significantly greater than expected by chance in only one of the four pairwise comparisons (gyne brains), where it was small

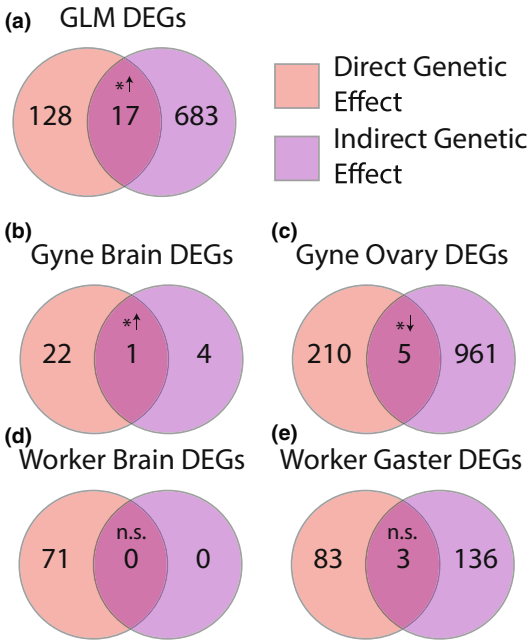

FIGURE 4 Overlap between genes that are significantly differentially expressed by direct and indirect genetic effects. Venn diagrams show the overlap in significantly (FDR < 0.1) differentially expressed genes (DEGs) arising from direct and indirect genetic effects across the (a) generalized linear model (GLM) comparisons, (b) gyne brain pairwise comparisons, (c) gyne ovary pairwise comparisons, (d) worker brain pairwise comparisons, and (e) worker gaster pairwise comparisons. Significant overlaps are indicated by an * (Fisher's exact test: $p < .05$), with upward arrows indicating that the overlap is greater than expected and downward arrows indicating that the overlap is less than expected; n.s., nonsignificant ($p > .05$).

(Fisher's exact test; $p < .05$; Figure 4b–e). In gyne ovaries, the overlap was significantly less than expected by chance (Fisher's exact test; $p < .05$; Figure 4c). The genes differentially expressed by both direct and indirect genetic effects, considered separately, included a zinc-finger containing transcription factor, *limulus clotting factor C*, two transposase-derived *HARBI1* proteins, and three odorant receptor genes (Supporting Information Data D).

4 | DISCUSSION

Our study suggests that direct genetic effects (i.e., *SB/SB* vs. *SB/Sb* genotype) have a greater influence on patterns of gene expression than indirect genetic effects (i.e., being raised in a monogyne social environment containing only *SB/SB* workers or a polygyne social environment containing both *SB/SB and SB/Sb* workers). This contrasts with two earlier studies, which concluded (i) that fire ant worker gene expression profiles (whole body) are more strongly influenced by indirect genetic effects associated with differences in the genetic composition of monogyne and polygyne colonies than by the direct genetic effects of their own supergene genotype (Wang et al., 2008) and (ii) that, in worker antennae, the number of genes differentially expressed in response to direct genetic effects is similar to the number differentially expressed in response to indirect genetic effects (Dang et al., 2019). These contrasting results may stem from differences in experimental design and statistical approach. In our experiments, we used body parts of single individuals (6–8 replicates per tissue/caste/genotype/social environment) (Arsenault et al., 2020), with workers reared in small colonies to facilitate age matching. By contrast, Wang et al. (2008) used pools of 7–10 individuals for each of the 20 replicates and Dang et al. (2019) pools of 36–55 individuals for each of the four replicates. Because our analyses showed that indirect genetic effects have a lower effect on the magnitude of changes in gene expression than direct genetic effects, it is possible that using pools of individuals (i.e., because of lower variance) or more replicates increased the likelihood of detecting genes influenced by indirect genetic effects compared to direct genetic effects. The possibility that the variance in the indirect genetic effects experienced by different individuals is higher than the variance in direct genetic effects experienced by different individuals is an intriguing hypothesis that warrants further study.

Whether direct or indirect genetic effects more greatly impact transcription is a multidimensional question: the degree of difference in expression and the number of affected loci are both relevant. By some metrics we do observe a greater number of genes undergoing expression changes in response to indirect than direct genetic effects (worker gaster and queen ovaries and combined sample GLM, each at FDR <0.1 but not more stringent cutoffs), offering apparent agreement with prior findings (Wang et al., 2008). However, our study suggests general agreement between tissue types and castes on the following: gene expression ratios are generally larger for direct than indirect genetic effects and indirect genetic effects on transcription appear more tissue- and caste-specific than direct effects. Moreover, we detected no significantly differentially expressed genes in the brains of workers in response to indirect genetic effects of the supergene, despite the presence of many differentially expressed genes arising from direct genetic effects with the same statistical power. This stands in contrast to the prediction of pervasive indirect genetic effects stemming from prior results from whole workers in *S. invicta* (Wang et al., 2008) and is more in line with results from a recent study of indirect genetic effects on brains in the clonal raider ant (Kay, Alciatore, et al., 2022).

The overrepresentation in the supergene region of genes differentially expressed due to direct genetic effects, but not indirect genetic effects, is consistent with *cis*-regulatory effects of supergene evolution and/or differences in chromatin accessibility between the *SB* and *Sb* haplotypes. Prior analysis of the gyne data alone also pointed to such effects (Arsenault et al., 2020). Evolutionary divergence of supergene-encoded proteins (Martinez-Ruiz et al., 2020; Pracana, Levantis, et al., 2017; Pracana, Priyam, et al., 2017) probably also make *trans*-regulatory contributions to the observed direct genetic effects, but these are not expected to disproportionately impact expression within the supergene (Signor & Nuzhdin, 2018). Moreover, a large-scale analysis of multiple tissue types in humans revealed that *cis*-regulatory sequence variants often influence transcription in a constitutive fashion across tissues (Ardlie et al., 2015). We found that direct genetic effects on gene expression patterns were often consistent across tissues, offering apparent support to the hypothesized *cis*-regulatory impact of evolution of the fire ant social supergene.

It has previously been noted that differentially expressed genes arising from direct genetic effects (Arsenault et al., 2020) and combined direct and indirect genetic effects (Martinez-Ruiz et al., 2020) of the fire ant supergene are overwhelmingly more highly expressed in *SB/Sb* than *SB/SB* individuals. Remarkably, this holds true for genes residing outside the supergene region, suggesting that *trans*-mediated gene upregulation is caused by the inversion-derived *Sb* haplotype (Arsenault et al., 2020; Martinez-Ruiz et al., 2020). The genetic change(s) responsible for widespread gene upregulation in response to the *Sb* haplotype remains unknown, but effects of *cis*-regulatory evolution and gene duplication (Fontana et al., 2020) on *trans*-regulatory factors could be of relevance. Remarkably, we also observed more gene upregulation than expected by chance due to indirect genetic effects of the *Sb* haplotype – that is, higher expression of differentially expressed genes in *SB/SB* individuals cohabitating with *SB/Sb* individuals than those cohabitating with only *SB/SB* individuals. This suggests the *Sb* haplotype may lead to gene upregulation through both direct and indirect genetic effects, despite a largely nonoverlapping sets of genes being influenced.

The lack of significant gene expression changes associated with cross-fostering *SB/SB* worker pupae from a monogyne to a polygyne social environment until two weeks post-eclosion suggests that *SB/SB* worker tolerance of multiple *SB/Sb* queens may not require a transcriptional response (at least not at a scale detectable with our methods). A possible explanation for this finding is that the presence of *SB/Sb* workers may induce passive habituation of *SB/SB* workers to the *Sb* chemical greenbeard and thereby induce them to accept *SB/Sb* queens. Such a behavioural change without a detected change

in brain gene expression is consistent with findings from a study of variation in aggressive behavioural response to social cues in honey bees (Rittschof, 2017) and a study of indirect genetic effects on brain gene expression in the clonal raider ant (Kay, Alciatore, et al., 2022). Thus, a growing body of evidence suggests that context-specific behavioural variation can arise from variation in the social environment without requiring fundamental changes to gene regulation at a detectable level in whole brains. Nevertheless, our results do suggest that indirect genetic effects accrue through the full course of development in *S. invicta* (when the social environment is consistent from egg to two-weeks as adults), as detected in abdominal tissues. Establishing a more fine-scale time course of the social supergene's effects on development, both direct and indirect, remains an outstanding problem.

Queens of *S. invicta* face a unique selective pressure during reproductive development in polygyne colonies, which probably explains why their ovarian tissues exhibit the greatest observed indirect genetic effects. As they mature in polygyne colonies, *SB/SB* gynes become targets of spiteful greenbeard-mediated elimination by *Sb*-bearing workers (Gotzek & Ross, 2008; Keller & Ross, 1998; Trible & Ross, 2016). The *SB/SB* gynes that survive in polygyne colonies until mating flights have been found to be slightly, but significantly, lighter than *SB/SB* gynes embarking on flights from monogyne colonies (DeHeer et al., 1999; Keller & Ross, 1995), reducing their independent colony-founding potential (DeHeer, 2002). The differential expression between the *SB/SB* gynes of monogyne and polygyne colonies could be associated with morphological and/or physiological differences associated with these weight differences (Arsenault et al., 2020).

Some of the conclusions we reached are similar to those of a genome wide association study (GWAS) of direct and indirect genetic effects conducted in laboratory mice (Baud et al., 2021) despite the very different approach of mapping trait variation as opposed to transcriptional variation. Effect sizes of loci associated with direct genetic effects on trait variation were found to be higher than effect sizes of loci associated with indirect genetic effects on trait variation (Baud et al., 2021), mirroring the larger gene expression ratios we observed for direct genetic effects than for indirect genetic effects. In contrast to this GWAS study (Baud et al., 2021), we observed a modest but significant overlap in the genes with significant indirect and direct genetic effects on transcription in *S. invicta*.

Among the genes that exhibit a significant transcriptional response to both direct and indirect genetic effects are a zinc-finger domain-containing transcription factor, which represents a candidate trans-regulatory protein that may contribute to the widespread direct genetic effects observed outside the supergene region in *S. invicta* (Arsenault et al., 2020; Martinez-Ruiz et al., 2020). Also responsive to direct and indirect effects is *limulus clotting factor C*, which encodes an enzyme linked to antibacterial hemolymph clotting (Iwanaga, 1993; Iwanaga et al., 1992) and, notably, three odorant receptor genes. Odorant receptors play an important role in chemical communication and therefore in social organization. There are also many documented amino acid substitutions between odorant receptor proteins encoded by the *SB* and *Sb* haplotypes of the fire ant supergene (Cohanim et al., 2018).

As in other social animals (Avalos et al., 2020; Baud et al., 2021; Gempe et al., 2012; Linksvayer, 2006; Vojvodic et al., 2015), direct and indirect genetic effects are pronounced in *S. invicta*. By examining direct and indirect genetic effects of the fire ant supergene on gene regulatory dynamics in samples from distinct tissues and castes we show that direct genetic effects on transcription are generally more detectable than indirect genetic effects and that direct genetic effects are more consistent across biological contexts than indirect genetic effects. Our results also add evidence to the notion that behavioural plasticity in response to the social environment need not be accompanied by detectable differences in brain gene expression (Kay, Alciatore, et al., 2022). Comparative study of other supergene systems (Kay, Helleu, & Keller, 2022) will help to test the generality of our findings across taxa.

AUTHOR CONTRIBUTIONS

Designed research: Samuel V. Arsenault, Oksana Riba-Grognuz, DeWayne Shoemaker, Brendan G. Hunt, and Laurent Keller; Performed research: Samuel V. Arsenault and Oksana Riba-Grognuz; Contributed new reagents or analytical tools: DeWayne Shoemaker; Analyzed data: Samuel V. Arsenault; Wrote the paper: Samuel V. Arsenault, Brendan G. Hunt, and Laurent Keller.

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CONFLICT OF INTEREST

None.

OPEN RESEARCH BADGES

This article has earned an Open Data badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available as described in the data availability statement.

DATA AVAILABILITY STATEMENT

Worker RNA-seq reads have been made available from NCBI's Gene Expression Omnibus (GEO), accession GSE205680; gyne RNA-seq reads from GEO accession GSE149726. R markdown document of the analyses performed in this manuscript is available at https://github.com/ArsenaultResearch/Arsenault_RibaGrognuz_Direct_Indirect_Effect.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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