Molecular underpinnings of

plasticity and supergene-mediated polymorphism in fire ant queens

Alex H. Waugh[*,1], Michael A. Catto[2], Samuel V. Arsenault[3], Sasha Kay[2], Kenneth G. Ross[2], Brendan G. Hunt[*,1,2]

1. Department of Genetics, University of Georgia, Athens, GA, USA
2. Department of Entomology, University of Georgia, Athens, GA, USA
3. John Harvard Distinguished Science Fellowship Program, Harvard University, Cambridge, MA, USA

*Corresponding awaugh19@uga.edu, huntbg@uga.edu

Abstract

Characterizing molecular underpinnings of plastic traits and balanced polymorphisms represent two important goals of evolutionary biology. Fire ant gynes (pre-reproductive queens) provide an ideal system to study potential links between these phenomena because they exhibit both supergene-mediated polymorphism and nutritional plasticity in weight and colony-founding behavior. Gynes with the inversion supergene haplotype are lightweight and depend on existing workers to initiate reproduction. Gynes with only the ancestral, non-inverted gene arrangement accumulate more nutrient reserves as adults and, in a distinct colony-founding behavior, initiate reproduction without help from workers. However, when such gynes overwinter in the natal nest they develop an environmentally induced lightweight phenotype and colony-founding behavior, similar to gynes with the inversion haplotype that have not overwintered. To evaluate the extent of shared mechanisms between plasticity and balanced polymorphism in fire ant gyne traits, we assessed whether genes with expression variation linked to overwintering plasticity may be affected by evolutionary divergence between supergene haplotypes. To do so, we first compared transcriptional profiles of brains and ovaries from overwintered and non-overwintered gynes to identify plasticity-associated genes. These genes were enriched for metabolic and behavioral functions. Next, we compared plasticity-associated genes to those differentially expressed by supergene genotype, revealing a significant overlap of the two sets in ovarian tissues. We also identified sequence substitutions between supergene variants of multiple plasticity-associated genes, consistent with a scenario in which an ancestrally plastic phenotype responsive to an environmental condition became increasingly genetically regulated.

Keywords:

inversion polymorphism, molecular evolution, genetic assimilation, phenotypic plasticity, RNA-seq

Introduction

Phenotypic plasticity refers to the capacity of a single genotype to produce a range of phenotypes in response to different environmental conditions (Stearns, 1989; West-Eberhard, 2003). Plasticity is observed in a wide variety of traits in all domains of life, giving rise to evolutionarily significant morphological, physiological, behavioral, and life history variation (Sommer, 2020; Dupont et al., 2023). When environmental conditions fluctuate, plasticity can permit organisms to respond adaptively to the environment by producing either continuous or discrete trait variation, with the latter resulting in alternative phenotypes. In contrast, alternative phenotypes can also be maintained as part of a genetically regulated balanced polymorphism. Two important aims of evolutionary biology are to unravel the molecular basis of plastic traits and to elucidate the genetic processes that sustain balanced polymorphisms. A promising strategy to achieve both objectives involves studying organisms where similar phenotypes arise independently as either plastic responses to environmental stimuli or as genetically regulated outcomes of balanced polymorphisms.

The red imported fire ant (*Solenopsis invicta*) provides an opportunity to study the interplay of plasticity and genetic regulation in the context of a chromosomal inversion-derived supergene, a taxonomically widespread genomic architecture for the long-term maintenance of alternative morphs in natural populations (Wellenreuther & Bernatchez, 2018; Schwander *et al.,* 2014). In fire ants, polygyny (worker toleration of multiple queens) is a secondary characteristic derived from monogyny (worker toleration of only a single queen; Boomsma et al., 2014). *S. invicta* and multiple congeners exhibit either monogyny or polygyny as determined by a social chromosome bearing an inversion polymorphism that arose relative recently (during the last 0.5 million years) and spread via introgression (Wang et al., 2013; Stolle et al., 2019; Yan et al., 2020; Helleu et al., 2022; Stolle et al., 2022). This inversion polymorphism acts as a supergene that regulates a suite of morphological and life history traits associated with colony queen number (Wang et al., 2013; Yan et al., 2020; Kay et al., 2022; Chapuisat, 2023).

As part of the regulation of monogyne and polygyne life histories, the fire ant social chromosome regulates the maintenance of two gyne (pre-reproductive queen) ecotypes whose weight accumulation and corresponding colony founding behavior can be predicted by the presence or absence of the derived inversion-carrying supergene allele (*Sb*; Figure 1A; Keller & Ross, 1993a, 1993b; DeHeer et al., 1999; Keller & Ross, 1999; DeHeer, 2002; Wang et al., 2013). *S. invicta* gynes homozygous for the

ancestral gene arrangement at the supergene (*SB/SB*) embark on mating flights with robust nutrient reserves and proceed to found colonies from scratch in their claustral chamber independently of worker assistance (DeHeer & Tschinkel, 1998; DeHeer et al., 1999; DeHeer, 2002). In contrast, gynes heterozygous for the derived supergene allele (*SB/Sb*) accumulate fewer nutrient reserves before embarking on mating flights and are thus only able to successfully rear brood when initiating egg-laying in the presence of workers (Keller & Ross, 1993a; DeHeer, 2002). Unlike *SB/SB* gynes, *SB/Sb* gynes are accepted by existing polygyne colonies after completing a mating flight. Gynes homozygous for the derived supergene allele (*Sb/Sb*) are also accepted by workers in polygyne colonies but are exceptionally low weight and of low fitness (DeHeer, 2002; Hallar et al., 2007).

The association between gyne weight and mode of colony founding is not unique to fire ants and is observed in many ant species (Keller & Passera, 1989, 1990). Variation in individual gyne weight is substantially influenced by the amount of fat content an individual accrues during development and maturation (Keller & Passera, 1990; Keller & Ross, 1993b, 1993a). Species whose gynes exhibit greater than 40% relative fat content by dry weight typically engage in independent colony founding whereas other species whose gynes accrue less relative fat typically engage in dependent founding (Keller & Passera, 1989). In fire ants, fat content makes up 44% of total dry body weight for independently founding *SB/SB* gynes, roughly 32% for dependently founding *SB/Sb* gynes, and even less for low fitness *Sb/Sb* gynes which seldom succeed in becoming functional queens (Keller & Ross, 1993a; DeHeer et al., 1999; Keller & Ross, 1999; DeHeer, 2002). Thus, fire ant gynes conform to the association between nutrient reserves and mode of colony founding observed across a variety of ant species.

Interestingly, gynes reared in monogyne colonies (all *SB/SB*) that eclose at the end of the mating season typically overwinter in their natal nest and lose weight over the course of the winter (Figure 1B; Fletcher & Blum, 1983; Tschinkel, 1996; DeHeer & Tschinkel, 1998; Helms & Godfrey, 2016). The overwintered *SB/SB* gynes of *S. invicta*, which make up 8-10% of annual gyne biomass produced by a colony (Morrill, 1974), exhibit plasticity in traits that eventually develop to resemble in many ways those of gynes carrying the *Sb* supergene (Tschinkel & Howard, 1978; Fletcher & Blum, 1983; Tschinkel, 1993; Tschinkel, 1996). First, overwintered gynes, like *Sb*-carrying gynes (Keller & Ross, 1993a), exhibit reduced fat content compared to their non-overwintered *SB/SB* counterparts (Tschinkel, 1996; Helms & Godfrey, 2016). Importantly, the fat reserves of overwintered *SB/SB* gynes typically fall below the estimated 40% fat content threshold estimated to be necessary for successful independent founding (Keller & Passera, 1989, Tschinkel, 1996). Moreover, both spring-reared and overwintered gynes exhibit similar head

widths in *S. invicta*, suggesting similar overall body sizes at the time of adult eclosion (Helms & Godfrey, 2016). This indicates the reduced weight phenotype of overwintered *SB/SB* gynes occurs as a consequence of phenotypic plasticity in the adult stage and not because overwintered gynes represent a developmental caste polymorphism.

Second, rather than digging a claustral chamber after mating, both overwintered and *Sb*-carrying gynes engage in dependent founding as they attempt to enter and begin egg-laying in an existing nest where workers are present (DeHeer & Tschinkel, 1998; DeHeer, 2002). Overwintered gynes engage in a specific form of dependent colony founding known as queen replacement (Tschinkel & Howard, 1978; Tschinkel, 1996; DeHeer & Tschinkel, 1998). When successful, queen replacement involves a newly mated overwintered fire ant gyne invading a monogyne nest whose queen recently died. In such a case, orphaned workers will accept and tend to the invading overwintered gyne as their new reproductive queen (Tschinkel & Howard, 1978). It was estimated in one study population that 0.7% of colonies per year adopt replacement queens and based on extrapolation, 3% of mature nests may be expected to be headed by overwintered queens (DeHeer & Tschinkel, 1998). Thus, while the individual success rate via queen replacement for individual overwintered gynes is low, at the population level, this reproductive strategy exists as a viable means of yielding reproductive returns from otherwise underweight, low-fitness gynes. Similar to overwintered *SB/SB* gynes, *Sb*-carrying gynes are ill-equipped for independent nest founding (DeHeer, 2002) and invade other nests after embarking on mating flights, in this case being accepted when the colony is either polygyne or queenless.

Third, overwintered and *Sb*-carrying gynes exhibit maternally incompetent behaviors (Tschinkel, 1996; DeHeer, 2002). When monogyne overwintered gynes (*SB/SB*) have been forced into simulated claustral independent colony founding conditions in the laboratory, they lay eggs, but they do not cluster them as is typical of spring-reared *SB/SB* gynes (Tschinkel, 1996). Overwintered gynes also tend to fail to feed and rear the larvae that hatch from their haphazardly laid eggs (Tschinkel, 1996). Similar maternally incompetent behaviors have been observed among newly mated polygyne *Sb*-carrying queens forced into simulated claustral, independent colony founding conditions in the laboratory (DeHeer, 2002). Maternal incompetence among overwintered *SB/SB* and *Sb*-carrying gynes may occur as a consequence of relaxed selection on the expression of brood tending behavior when queens are in a nutritional state incompatible with independent nest founding.

In this study, we leverage the unique biology of the fire ant system, in which similar alternative phenotypes arise through both plasticity and supergene-mediated balanced polymorphism. We aim to

assess the similarity in the underlying mechanisms of plasticity and a genetic polymorphism by investigating whether the genes associated with plastic responses to overwintering also contribute to supergene-mediated variation. Our first goal is to understand the changes in gene expression associated with overwintering by the fire ant queen caste to better understand the gene regulatory mechanisms associated with plasticity in adult weight and colony founding behavior. Toward this end, we compare gene expression profiles of brain and ovary tissues in overwintered queens to those from non-overwintered, spring-reared queens, with both types collected while embarking on mating flights. We selected these two tissues because they play important roles in the behavioral and reproductive physiology linked to queen phenotypes of interest: independent versus dependent colony founding and metabolism associated with reproductive function (Toth et al., 2020; Arrese & Soulages, 2011). Our second goal is to determine whether some of the genes involved in mediating environmentally induced plasticity in weight and colony founding behavior may also function in the genetic regulation of these phenotypes.

To provide insight into this prospect, we compare the genes that are differentially expressed by overwintering status to those differentially expressed by supergene genotype in brain and ovary tissues at a comparable life history stage (Arsenault et al., 2020). In order to assess whether genes associated with plasticity in nutrient reserves have been perturbed during the course of supergene evolution, we also compare the genes differentially expressed by overwintering status to those with fixed substitutions between *SB* and *Sb* alleles of the supergene (Martinez-Ruiz et al., 2020). Our results indicate that the supergene perturbs a set of genes with enriched overlap with those differentially expressed in association with nutritional plasticity. Plausibly, via genetic assimilation (West-Eberhard, 2003, 2005), the fire ant supergene may act to maintain the balanced queen weight polymorphism by perturbing the molecular underpinnings of ancestral nutritional plasticity. We discuss evidence consistent with this novel evolutionary role for supergenes and call for future work to formally test the hypothesis of supergene-mediated genetic assimilation in fire ants and other organisms.

Materials and methods

Sample collection and processing

Solenopsis invicta alate gynes and workers were aspirated from the tops of nests of the monogyne social form and frozen on dry ice in the field on days of mating flights along roadsides in and around Oconee National Forest in Georgia, USA. Spring-reared *SB/SB* gynes were collected on June 13, 2018, in Greene

County, Georgia (after any overwintering gynes would have departed on mating flights (Fletcher & Blum, 1983); Figure 1A). Monogyne colonies of *S. invicta* in this area do not produce sexuals during the winter months and produce their first and largest pulse of sexuals in early- to mid-spring (Vargo & Fletcher, 1987). Overwintered *SB/SB* gynes were collected on March 18, 2018, in nearby Oglethorpe County, Georgia, prior to the production of non-overwintering adult sexuals in this area (Figure 1B). To confirm monogyne social form of colonies, a *Gp-9* PCR assay on pooled individuals was performed (Valles & Porter, 2003). All samples were stored at -80°C until the addition of RNA*later*-ICE, at which point gynes were stored for at least 24 hours at -20°C prior to dissection.

S. invicta gyne and reproductive queen gasters (abdomens) distend as necessary (e.g., to make room for crop, fat body, and ovarian enlargement). Two-dimensional gaster measurements were taken to confirm size differences between randomly sampled overwintered and spring-reared gynes (Table S1). Sampled overwintered gyne gasters in our study were significantly smaller than those of spring-reared gynes (One-sided Mann-Whitney U; N = 8 SR, 7 OW; $p = 0.0003$; Figure 1C; Table S1), consistent with observations from more extensive sampling efforts of corresponding weight differences (Fletcher & Blum, 1983; Tschinkel, 1996; Helms & Godfrey, 2016), all of which confirm lower nutrient reserves in overwintered gynes.

Dissection and storage of brains and ovaries were performed as described in Arsenault, et al. (Arsenault et al., 2020). RNA was extracted from brains using the RNeasy Micro Kit and from ovaries using the RNeasy Mini Kit with DNase treatment (Qiagen, Valencia, CA). Extracted total RNA integrity and concentration were evaluated on an Agilent 2100 bioanalyzer (Figure S1). Dissected tissue from a single gyne from each colony were used to prepare libraries following the Smart-seq2 protocol developed for low input RNA sequencing applications (Picelli et al., 2014). Based on bioanalyzer readings, approximately 1.6 ng of total RNA was used to make each brain library and approximately 8.0 ng of total RNA was used to make each ovary library. Samples were barcoded and pooled for sequencing at the Georgia Genomics and Bioinformatics Core (Athens, GA) on an Illumina NextSeq sequencer to produce 75 bp, single-end reads. In total, samples from 16 unrelated *SB/SB* gynes, 8 overwintered and 8 spring-reared, from 16 different monogyne nests, yielded 16 brain and 16 ovary RNA-seq libraries for our study.

RNA-seq quality control and alignment

We trimmed reads and performed quality control using *Trim Galore!* v0.6.5. We then used *STAR* v2.7.3a (Dobin et al., 2013) to align reads to the "SINVBB1" genome assembly and associated annotation (GCA_009650705.1; Yan et al., 2020) using the 2-pass alignment procedure. Following alignment, brain libraries contained between 11 and 23 million and ovary libraries between 12 and 27 million uniquely mapped reads. We loaded quantified, gene-level read counts into *edgeR* (Robinson et al., 2009) for subsequent analyses.

For each tissue, we matched the expression cutoff used by Arsenault *et al.* (2020) by removing genes with fewer than 1.11 counts per million (CPM) in all 15 libraries from subsequent analyses. Of the 16,314 annotated genes in the fire ant genome (GCA_009650705.1), 47% (7,675/16,314) and 49% (8,048/16,314) of genes passed our low count filter in the brain and ovary comparisons respectively. PCA and HCA were performed for each tissue comparison separately using the CPM value for all genes that passed our low count filter. To visualize expression variance and relationships between libraries, HCA was performed with the R package *pheatmap* using the "ward D2" clustering method (Figure S2). Brain and ovary libraries from one individual, OW1, were removed due to outlier behavior in our principal component analysis (PCA) and hierarchical clustering analysis (HCA; Figure S2) that suggested potential low complexity of sequencing libraries.

Differential gene expression

For each tissue, we used *glmQLFTest* in *edgeR* (Robinson et al., 2009) to perform a separate pairwise design to test for differentially expressed genes between sample types. To assess similarity in the transcriptomic effects of overwintering and supergene genotype, we re-analyzed the RNA-seq data from Arsenault *et al.* (Arsenault et al., 2020) that were also generated from brains and ovaries of *S. invicta* gynes that were aspirated from the top of mounds on days of mating flights in Northeast Georgia, USA; single *SB/SB*, *SB/Sb*, and *Sb/Sb* gynes were collected from eight separate nests to comprise their biological replicates. Within tissue-type pairwise differential expression analyses of these data and the overwintered versus spring-reared data were performed with a false discovery rate (FDR) significance cutoff < 0.05 (Table S8).

Functional enrichment

We performed gene ontology (GO) enrichment analyses using the "elim" method from *topGO* (Alexa & Rahnenfuhrer, 2010). We first used *biomaRt* (Kinsella et al., 2011) to obtain the *Drosophila melanogaster* ortholog from the Ensembl metazoan database. The background set for these tests consisted of all genes passing the low count threshold for each respective tissue. The foreground sets consisted of differentially expressed genes (DEGs) found in each tissue. Not all brain and ovary DEGs had a *Drosophila* ortholog listed in the Ensembl database and thus were not included in these analyses. When *S. invicta* genes had multiple *Drosophila* orthologs, we used the *D. melanogaster* ortholog with the highest sequence identity to the target *S. invicta* gene. We present significantly enriched biological process GO terms called at *elimKS* < 0.05 (Tables S10-S11).

Nucleotide substitutions between supergene alleles

A prior comparison of 20 *SB* and 20 *Sb S. invicta* haploid male genomes from the US and South America identified a set of 96 genes (Table S12) for which the position of a fixed nucleotide substitution in the *Sb* allele either affected a regulatory region (3' and/or 5' untranslated region, UTR) or changed the amino acid sequence of the expressed protein (nonsynonymous substitution) (Martinez-Ruiz et al., 2020). We compared this aggregated set of genes with fixed substitutions to the genes differentially expressed by overwintering status to identify genes of interest in the hypothesized genetic assimilation of lightweight and dependently founding gynes via molecular evolution. *InterProScan* v5.61 (Jones et al., 2014) with all available applications was run on protein sequences for these genes of interest. To test whether any such substitutions overlap with functional protein domains, genomic coordinates reported by Martinez-Ruiz *et al.* (Martinez-Ruiz et al., 2020) were converted to gene coordinates using a custom R script (Table S13).

Results

Transcriptomic effects of overwintering

Our analysis revealed major effects of overwintering status on the transcriptomes of *S. invicta* gynes. In principal components analyses (PCA) conducted separately for each tissue type, PC1 explained around 20% of the total transcriptomic variance (Figures 2A & 2B), with clear separation of samples by overwintering status on PC1. We observed 667 differentially expressed genes (DEGs; FDR < 0.05) by

overwintering status in brain tissues (Figure 2C) and 1122 DEGs by overwintering status in ovarian tissues (Figure 2D). We observed a significant bias toward upregulation among DEGs in the brains of spring-reared relative to overwintered gynes (65%; 431/667; X^2 =57.0; $p = 4.3\times10^{-14}$) but no such bias in ovaries (51%; 573/1122; $X^2 = 0.51$; $p = 0.47$). The overlap among DEGs by overwintering status between tissues was significantly greater than expected by chance, with 144 DEGs common to both tissue types (Fisher's exact test; odds ratio = 1.48; $p = 0.0001$; Figure 2E). We found that 83% (120/144; odds ratio = 26.2; $p = 1.3\times10^{-12}$; Figure 2F) of overlapping DEGs were consistently up- or down-regulated by overwintering status in each tissue.

To identify candidate functional pathways associated with overwintering status of gynes, we performed Gene Ontology (GO) term enrichment analyses for DEGs (Tables 1 & S2-S7). DEGs by overwintering status in each tissue were enriched for metabolic processes involving three major types of macromolecules, lipids, amino acids, and carbohydrates, with a general pattern of down-regulation in the overwintered sample type (Figure 2). DEGs by overwintering status in brains were enriched for several biological processes with relevance to behavior (Tables 1 & S2). DEGs by overwintering status in ovaries were enriched for several biological processes directly related to female reproductive physiology (Tables 1 & S5) and 'aging' (Table S5). GO enrichment for molecular function (Tables S3 & S6) and cellular component (Tables S4 & S7) among brain and ovary overwintering DEGs can be found in the supplementary tables.

Transcriptomic effects of overwintering relative to supergene genotype

We next compared DEGs by overwintering status in *SB/SB* gynes to DEGs by supergene genotype of spring-reared gynes (eight trios of *SB/SB*, *SB/Sb,* & *Sb/Sb* gynes, each sampled from one of eight polygyne nests; (Arsenault et al., 2020). Of the 1,122 DEGs by overwintering status in ovaries that passed quality control for analysis of genotypic effects, 16% exhibited differential expression between polygyne spring-reared *SB/SB* and *Sb/Sb* gyne ovaries, representing a significantly greater overlap than expected by chance (177/1122; Fisher's exact test; odds ratio = 1.28; $p = 0.004$; Figure 3B). There was no greater overlap than expected by chance between DEGs in brains by overwintering and supergene genotype (Figure 3A) or between DEGs in ovaries by overwintering and *SB/SB* versus *SB/Sb* gynes (Figure 3B). A full gene expression compendium can be found in Table S8.

Among the 177 ovarian DEGs common to overwintering status and *SB/SB* versus *Sb/Sb* supergene homozygotes, 66% (117/177; Fisher's exact test; odds ratio = 3.17; $p = 5.5\times10^{-4}$; Figure 3C)

showed a discordant pattern of expression. This means they were up-regulated in the lighter-weight sample type in one comparison and down-regulated in the lighter-weight sample type in the other comparison, or *vice versa*. The observation that around two-thirds of the loci influenced by both overwintering and supergene genotype show directional discordance in relation to gyne weight indicates that most of these genes are not solely differentially expressed due to a shared state of nutrient reserve depletion in *Sb/Sb* spring-reared gynes and *SB/SB* overwintered gynes.

To assess whether the directional discordance in the overlap of ovarian DEGs by gyne weight is part of a larger trend of transcriptome-wide discordance, we tested for a correlation between expression $\log_2$ fold-change values for overwintering and *SB/SB* versus *Sb/Sb* when setting positive values to indicate higher expression level in the heavier gyne type. This revealed significant negative correlations between the expression fold-change contrasts of heavy and light gynes stemming from overwintering status versus alternate supergene allele homozygosity (*SB/SB* versus *Sb/Sb*) in both the brain (n = 7,657; Spearman's rho = -0.32; $p = 9.7 \times 10^{-180}$; Figure S3A) and ovarian tissues (n = 8,040; Spearman's rho = -0.12; $p = 6.2 \times 10^{-27}$; Figure S3B). A similar comparison with *SB/SB* versus *SB/Sb* heterozygotes was much weaker but still significant in the brain (n = 7,657; Spearman's rho = -0.04; $p = 1.4 \times 10^{-4}$; Figure S3C) and not significant in the ovarian tissues (n = 8,040; Spearman's rho = -0.01; $p = 0.22$; Figure S3D). The significant anticorrelations between fold-change contrasts of heavy and light gynes associated with overwintering status and supergene homozygosity (*SB/SB* vs. *Sb/Sb*) suggests the enrichment for discordance we observed in overlapping ovarian DEGs does coincide with global differentiation of the transcriptome with respect to the two different sources of gyne weight variation (environmental and genetic).

Of the 552 genes mapped to the supergene homologous region (*SB*), 273 and 288 had expression data in the brain and ovary in our study, respectively. We found that 3.6% (24/667; Fisher's exact test; odds ratio = 1.01; $p = 0.91$) of DEGs by overwintering status in brains and 3.8% (43/1122; Fisher's Exact; odds ratio = 1.09; $p = 0.60$) of DEGs by overwintering status in ovaries were mapped to the supergene-homologous region. These genes represent candidates for driving the genetic assimilation of reduced gyne weight gain in *Sb*-carrying gynes, as other genomic regions are freely recombining between *SB* and *Sb* genomes (Yan et al., 2020).

Overwintering DEGs with nucleotide substitutions between *SB* and *Sb*

To further characterize overwintering DEGs with respect to the fire ant supergene-mediated balanced polymorphism, we integrated data into our study from a prior analysis of fixed differences between *SB* and *Sb* alleles of the supergene (Martinez-Ruiz et al., 2020). We used these data to identify overwintering DEGs in each tissue with nucleotide substitutions that either alter amino acid sequences of encoded proteins or fall within the 3' UTR of transcripts, possibly affecting their expression (Mayr, 2017). In the brain, among the 273 genes in the supergene region with gene expression data in our study, 68 had fixed differences identified between *SB* and *Sb* alleles (Martinez-Ruiz et al., 2020). Six percent (4/68) also exhibited differential expression by overwintering status (Fisher's exact test; odds ratio = 0.65; p = 0.85; S9). Among these genes, *carbohydrate sulfotransferase 11-like* stood out as an interesting candidate gene underlying plasticity and supergene-mediated polymorphism in fire ant queens because of its pattern of down-regulation in overwintered gynes and its non-synonymous substitution that could yield similar phenotypic effects in *Sb*-carrying gynes. In the ovary, among the 288 genes in the *Sb* supergene region with gene expression data in our study, 69 had fixed differences identified between *SB* and *Sb* alleles. Sixteen percent of these exhibited differential expression by overwintering (11/69, Fisher's exact test; odds ratio = 1.17; p = 0.37; Table S9). One of these 11 genes, *SEC23-interacting protein*-like, emerged as an interesting candidate. Its *Drosophila* ortholog, *Phosphatidic Acid Phospholipase A1* (*PAPLA1*), is known to produce fly phenotypes similar to overwintered and *Sb*-carrying queens, including lower egg production, reduced metabolic rates, less fat storage, and decreased glycogen reserves (Galikova et al., 2017; see Discussion). Among the combined 15 overwintering DEGs with fixed differences between their *SB* and *Sb* alleles (Martinez-Ruiz et al., 2020), twelve exhibited these substitutions in 3' UTRs and five (including two with 3'UTR substitutions) exhibited one or more amino acid altering missense substitutions (Tables 2 & S9).

Discussion

The purposes of our study were two-fold. First, we aimed to understand the changes in gene expression associated with overwintering in monogyne *S. invicta* to better understand the gene regulatory mechanisms of plasticity in fire ant gyne nutrient reserves and affiliated colony founding behavior. Second, given the resemblance in weight and colony founding behavior between monogyne overwintered gynes (*SB/SB*) and polygyne *Sb*-carrying gynes, we investigated whether the genes associated with overwintering-induced plasticity might also be associated with the genetic regulation of these traits. Although we lack causal evidence linking genotype to phenotype for either overwintering or

Sb-linked trait variation, several results from our study provide insight into the prospect of genetic assimilation by the fire ant supergene.

Genetic assimilation is the evolutionary process by which a phenotype that initially arises as a response to an environmental condition becomes increasingly genetically regulated over time, such that it is expressed even in the absence of the environmental stimulus (West-Eberhard, 2003, 2005). In other words, through selection on environmentally induced trait variation, a phenotype originally induced by environmental factors can become buffered against environmental variation and thus subject to increased genetic control (Waddington, 1953; West-Eberhard, 2003, 2005; Pigliucci et al., 2006; Moczek et al., 2011; Pfennig & Ehrenreich, 2014; Ehrenreich & Pfennig, 2015; Jones & Robinson, 2018; Nijhout et al., 2021; Wood et al., 2023).

Our finding that ovarian overwintering DEGs overlap more than expected by chance with those observed between *SB/SB* and *Sb/Sb* genotypes appears consistent with a scenario in which the regulatory and structural effects of supergene evolution left the nutritional plasticity of adult gynes intact, perhaps by operating instead on the indirect genetic effects experienced by Sb-carrying gynes as they interact with nurse workers in the polygyne social environment (Arsenault et al., 2023; Majidifar et al., 2024). Contrary to our expectations for this scenario, however, most of the genes differentially expressed by both overwintering status and supergene genotype exhibit directionally discordant expression by gyne weight. Thus, although the supergene and overwintering affect many of the same genes, they affect the expression of these genes differently, consistent with evolutionary changes in the molecular machinery regulating nutrient accumulation and/or food seeking behavior in adult gynes. In either scenario, local adaptation is likely to have played an important role in shaping the genetic variation captured and maintained by the supergene (e.g., Feder et al., 2011).

Three fundamental components of fire ant genetics and life history provide further support to genetic assimilation as the appropriate interpretative framework for our study. First, the reliably lightweight phenotype of *Sb*-carrying gynes stands in contrast to the heavyweight phenotype of spring-reared *SB/SB* gynes produced by both monogyne and polygyne colonies (Keller & Ross, 1993a; DeHeer et al., 1999; Keller & Ross, 1999; DeHeer, 2002) but is consistent with the lightweight phenotype of *SB/SB* gynes after overwintering. This could arise from an increase in the genetic regulation (canalization) of adult gyne nutrient reserve accumulation via effects of *Sb* on nutritional plasticity.

Second, prior support for genetic assimilation to decrease plasticity has come from ancestral trait reconstruction to identify lineages where trait plasticity preceded fixation (e.g., (Heil et al., 2004; Levis & Pfennig, 2016; Jones et al., 2017; Wood et al., 2023). In fire ants, phylogenetic analyses support the monogyne social form as ancestral to the polygyne (Ross & Carpenter, 1991; Boomsma et al., 2014). Thus, it is most parsimonious to assume plasticity in gyne weight evident among monogyne overwintered gynes is ancestral to the emergence of the relatively young supergene in this species (Helleu et al., 2022).

Third, chromosome structural rearrangements, such as chromosomal inversions, commonly underpin ecologically relevant polymorphisms in complex traits (Wellenreuther & Bernatchez, 2018; Harringmeyer & Hoekstra, 2022; Chapuisat, 2023) and represent a genomic architecture primed to facilitate genetic assimilation. Genetic assimilation can occur via regulatory and coding sequence evolution since either has the potential to alter or disrupt molecular machinery underlying plasticity to buffer against environmental variation (Scoville & Pfrender, 2010; Ehrenreich & Pfennig, 2015; Levis et al., 2017; Wood et al., 2023). Chromosomal inversions suppress meiotic crossover events and thus can promote the accumulation and fixation of mutations that affect copy numbers and sequences of non-coding, regulatory, and protein coding elements (Hill & Robertson, 1966; Feder et al., 2011; Bachtrog, 2013; Pracana et al., 2017; Wellenreuther & Bernatchez, 2018; Faria et al., 2019; Stolle et al., 2019; Fontana et al., 2020; Martinez-Ruiz et al., 2020). Meiotic crossover suppression also acts to maintain allelic combinations in tight linkage disequilibrium (Wellenreuther & Bernatchez, 2018). Since the loss of plasticity during genetic assimilation can be deleterious if environmental conditions continue to fluctuate, maintenance of high linkage disequilibrium among alleles involved in genetic assimilation can provide a means by which genetically assimilated phenotypes persist in a population amidst environmental fluctuation. Thus, immediate and long-term effects of inversion polymorphisms offer opportunities for selection to act on the genetic machinery underpinning phenotypic plasticity in favor of the stable production of alternative phenotypes and life histories under increased genetic regulation.

We identified some genes that are differentially expressed between spring-reared and overwintered gynes that exhibit fixed differences between *SB* and *Sb* supergene alleles (Martinez-Ruiz et al., 2020). One manner in which genetic assimilation could produce a pattern of discordant expression with respect to gyne weight is through substitutions in 3' UTRs that influence gene expression levels by altering *cis*-regulatory element sequences or mRNA stability (Mayr, 2017). We identified six genes in the supergene region with 3' UTR substitutions and differential expression by both supergene genotype and

overwintering status, three of which exhibit directional discordance in expression by gyne weight. These genes could contribute to genetically assimilated phenotypes of *Sb*-carriers if the substitution in the 3' UTR impacts the activity of transcriptional regulators, disrupts machinery underlying phenotypic plasticity, and results in a phenotype less responsive to variable environmental stimuli.

One gene showing directional discordance by gyne weight in ovarian tissues and a 3'-UTR substitution between *SB* and *Sb* is the G protein coupled receptor (GPCR) *Dopamine receptor 1* (Dop1R1), which exhibited reduced expression in lightweight *SB/SB* overwintered gynes and elevated expression in spring-reared *Sb*-carrying gynes in their respective comparisons to heavyweight *SB/SB* spring-reared gynes. Many GPCRs, including several dopamine receptors, are differentially expressed in response to starvation in *Drosophila* (Ko et al., 2015). Dopaminergic neurons in mushroom bodies of the brain have also been shown to mediate food seeking behavior in *Drosophila* (Landayan et al., 2018; Tsao et al., 2018) and Dop1R1 in particular has been shown to mediate ethanol and methamphetamine intake preference of flies (Kanno et al., 2021). Fire ant queens actively seek out fecundity amplifying excretions from late stage larvae (Cassill & Vinson, 2007), which makes this an interesting candidate pathway by which food seeking behavior could come to differ by supergene genotype in fire ants. If the 3' UTR substitution in *Sb* of this gene causes the observed relative increase in its expression, it could contribute causally to genetic assimilation

Genes displaying concordant expression levels by gyne weight according to overwintering and supergene genotype could also contribute to genetic assimilation but via a different mechanism. These genes could contribute to assimilated phenotypes of *Sb*-carriers if a nonsynonymous substitution directly impacts an encoded protein's structure and function. Like the mechanism proposed for discordant DEGs with 3' UTRs, changes to protein structure and function could also disrupt physiological machinery for plasticity. One gene that fits this pattern, *calcium-independent phospholipase A2-gamma-like,* may play a direct role in the metabolism of fire ants based on its phospholipid metabolic activity in mammals (Kita et al., 2019), and this gene exhibits a nonsynonymous substitution in an annotated functional domain. A second gene with concordant expression by gyne weight and a nonsynonymous substitution in an annotated functional domain is *carbohydrate sulfotransferase 11-like*, which is predicted to be involved in carbohydrate synthetic processes.

In our study, the gene *SEC23-interacting protein-like* is particularly notable for exhibiting differential expression by overwintering status and having three nonsynonymous substitutions in its protein coding region (the greatest number we observed for an overwintering DEG). This gene is the

ortholog of the gene encoding *Phosphatidic Acid Phospholipase A1* (*PAPLA1*) in *Drosophila melanogaster* (Gáliková et al., 2017). Two of the nonsynonymous substitutions in this gene occur within an intrinsically disordered region of the protein, which may be involved in its role in cell signaling (Wright & Dyson, 2015). The pattern of down-regulation in lightweight overwintered gynes and amino-acid changing substitutions in *Sb*-carrying gynes that we observe logically positions this gene as a candidate for genetic assimilation via molecular evolution. Remarkably, PAPLA1 deficiency through genetic perturbation in flies has been shown to cause reduced rates of egg production, lower metabolic rates, reduced fat storage, and reduced glycogen reserves (Gáliková et al., 2017). All of these phenotypic states bear striking similarity to those of *Sb*-carrying *S. invicta* gynes (DeHeer, 2002). Further, the reduced fecundity of *PAPLA1* mutants (Gáliková et al., 2017) is driven in part by egg chamber degeneration, a phenotype that also occurs in response to nutritional shortage (McCall, 2004), thus demonstrating the gene's direct potential to canalize a plasticity-induced phenotype through mutation. Intriguingly, *PAPLA1* fly mutants develop into normally sized adults but have both lower food intake and energy expenditure than normal flies (Gáliková et al., 2017). Although the effects of the supergene substitutions in the primary protein sequence of this gene in *S. invicta* remain unknown, such a gene would seem to be an ideal candidate for optimizing colony energy investment in adult fire ant gynes.

Several limitations of our study design should be considered when interpreting our results. First, sampled overwintered gynes are older than their non-overwintered counterparts. Thus, overwintering DEGs are likely to be shaped in part by differences in the ages of *SB/SB* spring-reared and overwintered gynes (Lucas et al., 2017), consistent with enrichment of the GO term 'aging' we observed. Second, overwintered gynes may have experienced greater environmental stress than spring-reared gynes, which could have long-term consequences on gene expression. Third, *SB/SB* overwintered gynes have accumulated and then depleted nutrient reserves while *Sb*-carrying spring-reared gynes have accumulated fewer nutrient reserves, which may result in physiological differences that directly influence variation in gene activity. Finally, it is possible our samples harbor genetic variation associated with variation in nutrient metabolism. Though we cannot completely rule out the effects of such variation, we did attempt to capture genetic diversity in a balanced manner by sampling only one gyne of each sample type from a given nest. In the future, these limitations could be overcome by testing for associations between genetic variants of *SB* and rates of individual nutrient accumulation in a laboratory experiment and using this information as a point of comparison for genetic differentiation of *SB* and *Sb*. This would provide further insight into the prospect for assimilation by the fire ant supergene and the mechanisms underlying assimilation.

Chromosome structural rearrangements have garnered recent interest as a widespread genomic architecture facilitating the evolution of complex multigenic trait polymorphisms (Wellenreuther & Bernatchez, 2018; Rubenstein et al., 2019; Harringmeyer & Hoekstra, 2022; Kay et al., 2022). However, genetic assimilation as a potential means by which supergenes come to regulate production of a discrete supergene-carrying alternative morph has received little attention. The initial chromosome structural rearrangement and subsequent sequence divergence in its non-recombining region create opportunities for mechanisms underlying plasticity in a trait to be disrupted, resulting in a less plastic phenotype. Furthermore, linkage disequilibrium between supergene loci creates an opportunity to maintain assimilated phenotypes as part of a suite of traits that make up complex polymorphisms within species. Our study highlights the utility of profiling genes with plasticity-associated expression to gain insight into potential assimilation by inversion polymorphisms. Future research should formally test the hypothesis of supergene-mediated genetic assimilation in fire ants and other organisms.

Author Contributions

Conceptualization: AW, SA, KR, BH; Formal analysis: AW, MC; Funding acquisition: BH, KR; Investigation: AW, SK, KR, BH; Visualization: AW; Writing – original draft: AW, BH; Writing – review & editing: all authors.

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Conflict of Interest

None.

Data Availability Statement

Overwintered and spring-reared gyne RNA-seq reads (brain and ovary) have been made available on NCBI SRA (PRJNA1062748).

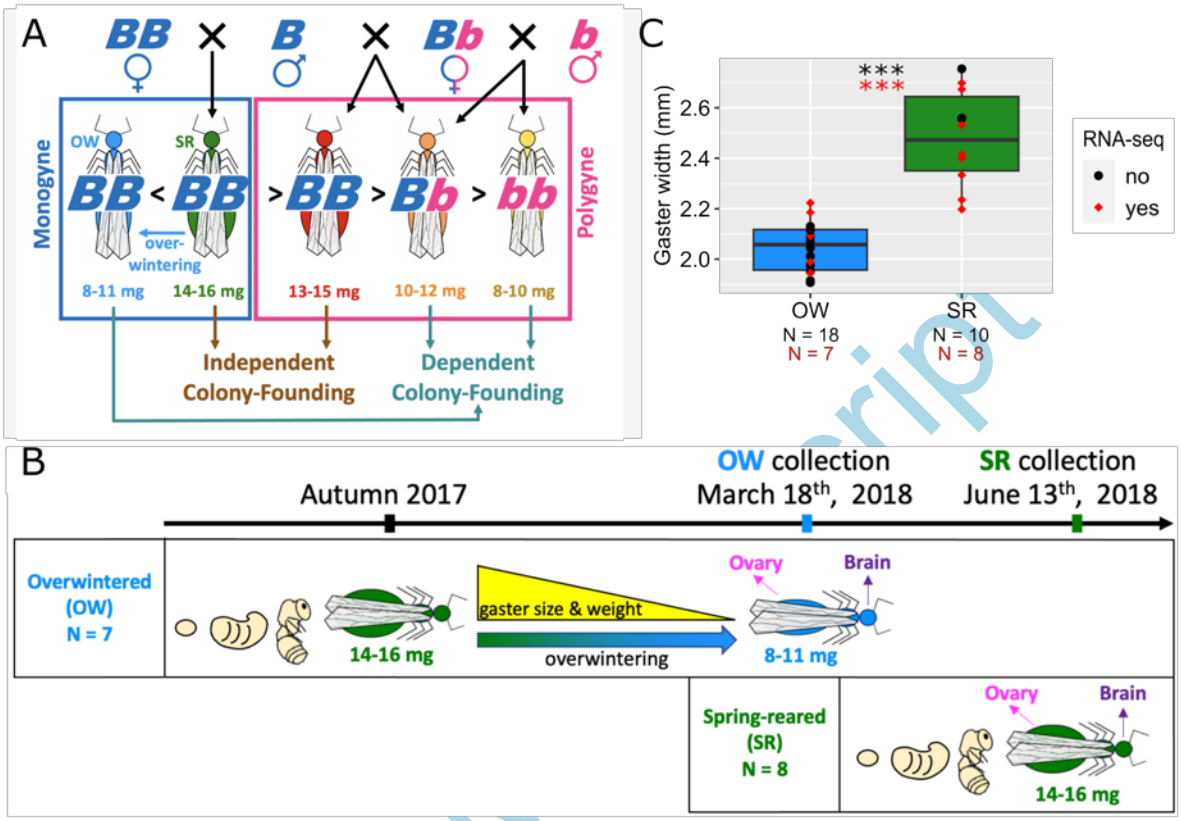

Figure 1. Gyne collection timeline, colony founding behavior, and size differences between overwintered (OW) and spring-reared (SR) gynes. (A) The relationship between gyne weight (spring-reared unless otherwise specified), supergene genotype, and colony founding behavior. Parental supergene genotypes of haploid males and diploid mother queens are shown at top. Typical weight ranges for each type of gyne are shown in milligrams with relationships between pairs of gyne types emphasized using greater than and less than symbols (Keller & Ross, 1993b, 1993a; Tschinkel, 1996; DeHeer et al., 1999; Keller & Ross, 1999; DeHeer, 2002). Supergene genotype is shown in simplified form as *B* = *SB* and *b* = *Sb*. Arrows lead to each gyne type's colony founding mode (independent or dependent). (B) Overwintered gynes (light blue) eclosed in Autumn, 2017 and overwintered in their natal nest before collection in March 2018, when they emerged for their nuptial flight. Spring-reared gynes (green) eclosed in the Spring of 2018 and were collected in June 2018 when they emerged for their nuptial flight. Brain and ovary tissues were extracted from each harvested gyne and used as material for RNA extraction. Sample sizes reflect the final number of biological replicates used for differential gene expression testing following removal of sample OW1 (see methods for details). (C) Boxplots of gyne gaster width for samples measured for this study. Data from gynes used for gene

expression analysis are shown with red diamonds as opposed to black circles for gynes measured but not used for gene expression analysis.

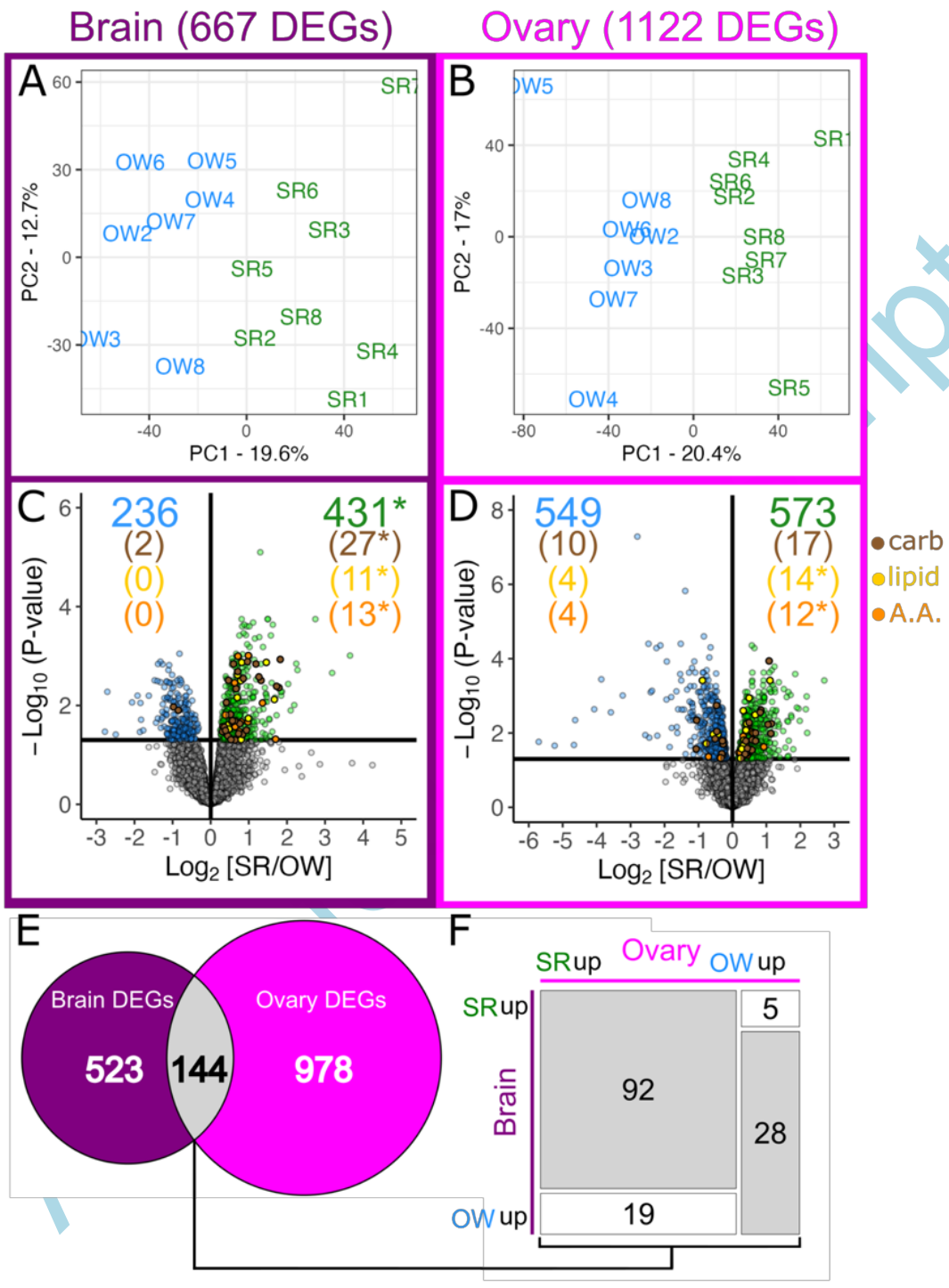

Figure 2. Effects of overwintering on gene expression. PCA plots based on normalized gene expression (CPM) for all genes passing the respective expression cutoff for each tissue: brain (A) and ovary (B). Volcano plots of pairwise gene expression differences between overwintered (OW) and spring-reared (SR) fire ant gyne brains (C) and ovaries (D). For each tissue, differentially expressed genes (DEGs; FDR <

0.05) upregulated in SR (green) and OW (blue) gynes are shown. *S. invicta* DEGs whose *D. melanogaster* ortholog (see methods) is annotated to the GO term 'carbohydrate metabolic processes' (GO:0005975, BP) are shown in brown, 'cellular lipid catabolic process' (GO:0044242, BP) in yellow, and 'alpha-amino acid metabolic process' (GO:1901605) in orange. The total number of DEGs upregulated in OW and SR gynes are shown in blue and green text respectively. Asterisks (*) on the volcano plot signify significant bias toward down-regulation in the overwintered sample type for each set of genes DEGs annotated to 'cellular lipid catabolic process' in the brain (100% (11/11); $X^2 = 11.0$; $p = 0.001$) and ovary (81% (12/14); $X^2 = 5.6$; $p = 0.018$), 'alpha-amino acid metabolic process' in the brain (100% (13/13); $X^2 = 13.0$; $p = 0.0003$) and ovary (75% (12/16); $X^2 = 4$; $p = 0.046$), and 'carbohydrate metabolic process' in the brain (93% (27/29); $X^2 = 21.6$; $p = 3.4\times10^{-6}$) but not the ovary (63% (17/27); $X^2 = 1.8$; $p = 0.178$). Euler plot Overlap of DEGs found in each tissue (Fisher's exact test, $p < 0.001$) (E). Mosaic plot showing the directional concordance in DEGs common to the brain and ovary comparisons (F).

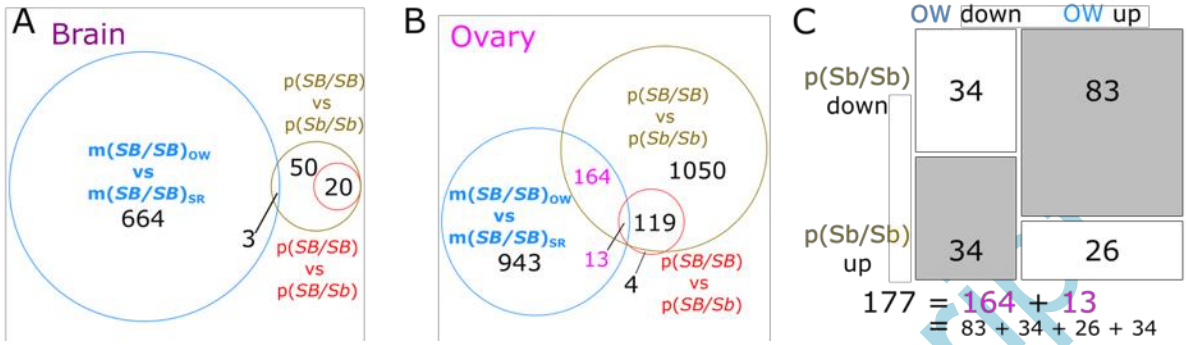

Figure 3. DEGs associated with overwintering and supergene genotype. Euler diagram showing overlaps in sets of differentially expressed genes (FDR < 0.05) found in the brain (A) and ovary (B) comparisons of monogyne *SB/SB* overwintered (OW) vs. monogyne *SB/SB* spring-reared (SR) (blue), polygyne *SB/SB* vs. polygyne *SB/Sb* (red), and polygyne *SB/SB* vs polygyne *Sb/Sb* (gold) gynes. (C) Mosaic plot showing enrichment of directionally discordant (gray) and concordant (white) DEGs among the 177 shared ovary DEGs in the overlap of monogyne *SB/SB* OW vs. monogyne *SB/SB* SR and polygyne *SB/SB* vs. polygyne *Sb/Sb* DEGs. Discordant genes were oppositely up- and down-regulated in the smaller-gaster, lightweight sample type (*e.g.,* up in OW & down in *Sb/Sb* gynes) in both comparisons. The designations “m” and “p” denote gyne colony social form of origin (monogyne and polygyne, respectively).

Table 1. Top 10 significantly enriched gene ontology biological process terms among DEGs according to overwintering status in the brain (Br) and ovary (Ov).

Tissue	GO term ID	GO Term description	Expressed genes	DEGs	Expected DEGs	*p*-value (elimKS)
Br	GO:0055085	transmembrane transport	345	49	29.51	0.00006
Br	GO:0046835	carbohydrate phosphorylation	9	5	0.77	0.00034
Br	GO:0006006	glucose metabolic process	27	8	2.31	0.00102
Br	GO:0043171	peptide catabolic process	7	4	0.6	0.00126
Br	GO:0019563	glycerol catabolic process	4	3	0.34	0.00203
Br	GO:0006166	purine ribonucleoside salvage	4	3	0.34	0.00203
Br	GO:0000381	regulation of alternative mRNA splicing, via spliceosome	59	12	5.05	0.00243
Br	GO:0006812	monoatomic cation transport	170	25	14.54	0.00254
Br	GO:0051606	detection of stimulus	45	10	3.85	0.00279
Br	GO:0007611	learning or memory	84	15	7.19	0.00288
Ov	GO:0009154	purine ribonucleotide catabolic process	5	4	0.7	0.0015

Ov	GO:0016266	O-glycan processing	5	4	0.7	0.0015
Ov	GO:0035337	fatty-acyl-CoA metabolic process	11	6	1.55	0.0015
Ov	GO:0006633	fatty acid biosynthetic process	37	12	5.2	0.0025
Ov	GO:0045823	positive regulation of heart contraction	3	3	0.42	0.0025
Ov	GO:0050906	detection of stimulus involved in sensory perception	12	6	1.69	0.0027
Ov	GO:0015718	monocarboxylic acid transport	16	7	2.25	0.003
Ov	GO:0030720	oocyte localization involved in germarium-derived egg	9	5	1.27	0.0035
Ov	GO:0044242	cellular lipid catabolic process	62	16	8.72	0.0068
Ov	GO:0009064	glutamine family amino acid metabolic process	27	9	3.8	0.0069

Table 2. DEGs by overwintering status with *Sb* substitutions in 3' untranslated regions and/or that affect primary protein sequence.

Gene ID[1]	Gene name	3' UTR subs.[2]	NS subs.[2]	Upreg. OW vs SR[3]	Upreg. *SB/SB* vs *Sb/Sb*	*D. melanogaster* ortholog[4] and FlyBase notes[5]
LOC105203065	*calcium-independent phospholipase A2-gamma-like*	1	2	SR_{ovary}	SB/SB_{ovary}	No fly ortholog; *PNPLA8* in mouse; involved in fatty acid hydrolysis
LOC105193134	*carbohydrate sulfotransferase 11-like*	1	1	SR_{brain}	SB/SB_{ovary}	*CG13937*; Predicted to be involved in carbohydrate biosynthetic process; expressed in fat body
LOC105194585	*SEC23-interacting protein-like*	0	3	SR_{ovary}	Not significant	*PAPLA1*; enables phospholipase activity; required for the

						endoplasmic reticulum to Golgi trafficking of a family of G-protein coupled receptors
LOC105194453	*charged multivesicular body protein 7*	0	2	OW_{ovary}	Sb/Sb_{brain}	*CG5498*; predicted to be involved in late endosome to vacuole transport
LOC105207412	*peroxisomal membrane protein 11C*	0	1	SR_{brain}	SB/SB_{ovary}	*CG33474*; Predicted to be involved in peroxisome fission
LOC105194672	*heat shock 70 kDa protein cognate 5*	1	0	SR_{ovary}	Sb/Sb_{ovary}	*Hsc70-5*; Predicted to enable several functions, including ATP hydrolysis activity; mitochond

						rial protein-transporting ATPase activity; and protein folding chaperone
LOC105199797	*ubiquitin-like domain-containing CTD phosphatase 1*	1	0	OW_{ovary}	SB/SB_{ovary}	*Ublcp1*; binds and dephosphorylates the nuclear 26S proteasome; inhibits proteasome activity
LOC105203081	*semaphorin-5B*	1	0	SR_{ovary}	SB/SB_{brain}	No fly ortholog; *Sema5b* in mouse; involved in neurogenesis during development
LOC105206526	*dopamine receptor 1*	1	0	OW_{ovary}	SB/SB_{ovary}, SB/SB_{brain}	*Dop1R1*; Receptor for dopamine; activity mediated by G

					proteins; involved in memory and learning

[1] Uncharacterized genes and overwintering DEGs with 3' UTR substitutions but no supergene differential expression were excluded (listed in Table S9); [2] (Martinez-Ruiz et al., 2020); [3] OW: overwintered *SB/SB,* SR: spring-reared *SB/SB*; [4] Orthologs are from OrthoDB v11 (Kuznetsov et al., 2022); [5] (Thurmond et al., 2019)

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