ORIGINAL ARTICLE

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Experimental disruption of social structure reveals totipotency in the orchid bee, *Euglossa dilemma*

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Eusociality has evolved multiple times across the insect phylogeny. Social insects with greater levels of social complexity tend to exhibit specialized castes with low levels of individual phenotypic plasticity. In contrast, species with simple social groups may consist of totipotent individuals that transition among behavioral and reproductive states. However, recent work has shown that in simple social groups, there can still be constraint on individual plasticity, caused by differences in maternal nourishment or social interaction. It is not well understood how these constraints arise, ultimately leading to the evolution of nonreproductive workers. Some species of orchid bees form social groups of a dominant and—one to two subordinate helpers where all individuals are reproductive. Females can also disperse to start their own nest as a solitary foundress, which includes a nonreproductive phase characterized by ovary inactivation, not typically expressed by subordinates. Little is known about individual flexibility across these trajectories. Here, using the orchid bee *Euglossa dilemma*, we assess the plasticity of subordinate helpers, finding that they are capable of the same behavioral, physiological, transcriptomic, and chemical changes seen in foundresses. Our results suggest that the lack of nonreproductive workers in *E. dilemma* is not due to a lack of subordinate plasticity.

KEY WORDS: **phenotypic plasticity, orchid bees, social evolution, *Euglossa*.**

The evolution of obligate eusociality, such as seen in ants, honey bees, and termites, is expected to result in a transfer of reproductive plasticity from the individual level to the colony level (Taylor et al. 2019). Species with obligate eusocial behavior may exhibit irreversible reproductive and nonreproductive castes with traits that are adapted to specific tasks within the colony, with these traits being determined during the development (Rehan and Toth 2015). In contrast, individuals of species forming small, cooperatively breeding groups are often totipotent as adults, with any member of the social group exhibiting the flexibility to serve as the primary reproductive, with dominant and subordinate roles defined after eclosion (Strassmann et al. 2002; Johnson and Linksvayer 2010). While there is substantial debate about the life-history features present in the ancestors of obligate eusocial species (Linksvayer and Johnson 2019), extant species with small social groups are frequently used as model systems to evaluate hypotheses about solitary to social life-history transitions (Kronauer and Libbrecht 2018).

Empirical study has shown that, while these species do show higher adult reproductive flexibility than obligately eusocial species, they may still experience constraints on their adult plasticity, either through alternative developmental trajectories or social interactions (Lawson et al. 2017; Awde and Richards 2018). Understanding how and when changes in plasticity first arise is important in identifying the mechanisms leading to the evolution of fixed, nonreproductive worker castes that are developmentally determined (Linksvayer et al. 2011; Jones et al. 2017). In the small carpenter bee *Ceratina calcarata*, for instance,

mothers may undernourish their first female offspring, creating a small-bodied helper who does not reproduce on her own but provisions her siblings, which will disperse to start their own nests (Lawson et al. 2016). These helper individuals have been suggested to represent "caste-antecedents," showing extensive overlap in gene expression patterns with eusocial workers (Shell and Rehan 2019). Similarly, the facultatively eusocial Halictid bee *Megalopta genalis* appears to rely on maternal manipulation of offspring provisions to create small-bodied females that become nonreproductive workers (Kapheim et al. 2011), though these workers can still assume a vacant queen position and reactivate their ovaries if given the opportunity (Jones et al. 2017). In contrast, adults of some species forming small social groups show no apparent signs of constraint on adult plasticity. Some primitively eusocial hover wasps and some allodapine bees, for example, have little to no consistent body size differences that correlate with social hierarchy, with all adults capable of any social or reproductive role (Schwarz and Woods 1994; Field et al. 1999; Sumner et al. 2002). Ultimately, to understand how the specific life-history features of species with simple social groups relate to the evolution of eusociality requires evaluation of these features in a phylogenetic context (Linksvayer and Johnson 2019; Shell et al. 2021).

Uncovering the evolutionary history of sociality in the corbiculate bees (honey bees, bumblebees, stingless bees, and orchid bees), most of which are well-known for their complex obligately eusocial colonies, has been hampered both by phylogenetic uncertainty regarding the relationships among lineages (Engel and Rasmussen 2020) and by the apparent lack of closely related species showing small or intermediate social group sizes (Danforth 2002). While recent work has reduced much of the phylogenetic uncertainty among the corbiculate bee lineages (Romiguier et al. 2016; Bossert et al. 2017), the lack of data to inform the seemingly abrupt evolution of eusocial behavior remains a challenge. However, part of this difficulty arises due to the lack of information about life-history variation among the orchid bees, the earliest branching lineage of the corbiculate bees, which have primarily been considered to be solitary (Cameron 2004; Fischman et al. 2017).

Indeed, the state of orchid bee social behavior has been a puzzlement for biologists, who have, until somewhat recently, relied on rare observations of nesting to characterize behavior across the 200+ orchid bee species (O'Toole and Raw 1991; Ramírez et al. 2002). After observing that some orchid bees had multiple preconditions favoring eusociality, such as overlapping generations, long-lived individuals, and semipermanent nests, Roberts and Dodson (1967) posed the question, "why, then, has there been no evolution of distinct worker and reproductive castes among these bees?" As new data emerge, however, it is increasingly clear that numerous orchid bee species do show diverse social behaviors, though there is still no evidence for true nonreproductive castes among individuals in a social group, which have always been found to have activated ovaries. Social behaviors documented among orchid bee species include communal nesting, multifemale nest founding, overlapping generations, and the division of labor between dominant nest guards and subordinate foragers (Augusto and Garófalo 2004; Capaldi et al. 2007; Cocom Pech et al. 2008; Solano-Brenes et al. 2018).

In the orchid bee *Euglossa dilemma*, the focal species of this study, nests are started by a solitary foundress that constructs a nest of plant resin and provisions an initial brood batch with pollen and nectar. After completing these brood cells, the foundress ceases foraging and reproduction and transitions into a "guard" phase to protect her developing brood. When a foundress enters this nonreproductive guard phase, her ovaries inactivate and reduce in size. This shift to guard behavior is associated with changes in gene expression across the brains and the ovaries including genes associated with social behavior in eusocial species (Saleh and Ramírez 2019). After spending up to 2 months in the guard phase, offspring emerge, and the nest enters the social phase. During this transition to social behavior, the foundresses' ovaries reactivate and she then becomes the dominant bee, while one to two of her female offspring may remain as subordinate helpers. Other female offspring disperse to begin their own nests. Between individuals in a social nest, there is a division of labor, with the dominant bee remaining in the nest with the brood while the subordinate bee forages for offspring. Both the dominant and subordinate bees are reproductive and mated; however, the dominant bee eats and replaces all subordinate laid eggs, indirectly resulting in a functional reproductive division of labor.

Like several other orchid bee species, *E. dilemma* shows behavioral plasticity among social roles. Subordinates can transition to a dominant position when the dominant is removed in a multifemale nest (Andrade-Silva et al. 2016; Séguret et al. 2021; N. Saleh, personal observation). Although this plasticity is notable, the transition is between two reproductive behaviors that show relatively slight physiological differences (Saleh and Ramírez 2019). In contrast, the transition from the foundress phase (reproductive) to the guard phase (nonreproductive) in the solitary portion of the lifecycle is pronounced and involves behavioral and physiological changes (Saleh and Ramírez 2019). However, it is unclear if subordinates, who remain in their natal nest as foragers, can express the full range of plasticity shown by dispersing foundresses. This is unclear because the guard phase, which occurs after the provisioning of the first brood, can be entirely absent in orchid bee social nests, due to continuous generations as nest size grows (Augusto and Garófalo 2009; Boff et al. 2017). Alternatively, when a social nest does show an interval of reproductive inactivity between broods, it may be short, or the subordinate bee may abandon the nest early (Augusto and Garófalo

2011). Consequently, this plasticity is highly variable in subordinate helpers relative to the predictable, prolonged changes seen in solitary foundresses.

In this study, we seek to assess the individual plasticity of *E. dilemma* subordinate helpers, testing the hypothesis that they can regulate their reproductive physiology dynamically, expressing both reproductive and nonreproductive phenotypes, despite these nonreproductive phenotypes being absent in typical social interactions. This study aims to provide insight into whether the lack of true nonreproductive castes in *E. dilemma* is, in part, due to a lack of reproductive plasticity in subordinates. We assess this by isolating individual subordinates and disrupting their social behavior to simulate conditions experienced by solitary foundresses starting their own nest. We then collect behavioral, physiological, chemical, and transcriptomic data from these isolated subordinates to determine the degree to which phenotypic changes mirror those of solitary foundresses.

Methods

NEST OBSERVATION

Nest observations were conducted in Ft. Lauderdale, FL, where a naturalized *E. dilemma* population has been present for around 15–20 years (Skov and Wiley 2005). Wooden nest boxes were placed on the eaves of buildings in Ft. Lauderdale in which *E. dilemma* females naturally founded nests. Transparent red plexiglass lids were placed on top of these wooden boxes to facilitate video recording and behavioral observation. In some nests, infrared CCTV cameras were used to record 24 h continuous video through the lid on top of the nest boxes. We also surveyed nests daily, checking nest occupancy. In the evening, following the return of all bees to the nest, individual bees were tagged with numbered, plastic discs superglued to the thorax.

NEST MANIPULATION

To test the hypothesis that subordinate helpers will express the plasticity exhibited by foundresses, we first identified nests containing subordinate individuals and then we experimentally removed the interaction with other dominant or subordinate nestmates to determine how their behavior progressed in isolation. Our approach is illustrated in Figure 1 and possible outcomes are illustrated in Supporting information Figure S1. First, in the summers of 2018 and 2019, we identified nests in the guard phase, where offspring from the first brood had not yet emerged. Following offspring emergence, we waited until individuals remaining in the nest showed dominant or subordinate relationships before manipulation. We define an individual as subordinate if it has provisioned at least one brood cell with egg replacement by the dominant (the original female in the nest, typically the mother). After this is confirmed, we removed all individuals from the nest except the first bee that showed subordinate behavior. Removal of individuals occurred after dark, to confirm that all bees were present. The subordinate bee left behind was not handled in this process. The time elapsed before nestmate removal varied among nests, to ensure that all offspring in the brood cells had emerged. If additional females emerged after nestmate removal, the remaining subordinate could transition to dominant behavior and there would be no opportunity for that subordinate to express the nonreproductive changes seen in foundresses. In three of 14 nests where manipulation was performed, several offspring from the first generation failed to emerge (due to disease, parasitism, or unknown causes) and these brood cells were carefully cut from the nest using a sterilized razor when nestmate removal occurred.

After nestmate removal, we observed the behavior of the remaining subordinate to monitor changes in foraging behavior and/or the start of guarding the second batch of brood cells. We classify an individual as in the "guard" phase if it has discontinued all pollen foraging trips and remains inside the nest with a resin seal over the nest entrance during normal foraging hours (sunrise to sunset) on a day where foraging is seen in other nests. Individuals that became guards were collected after showing 14–15 days of guarding behavior. In total, we performed 14 removals. Individuals collected from these treatments are hereafter referred to as "isolated subordinates."

GUARD AND REPRODUCTIVE INDIVIDUALS FOR COMPARISON

To compare the changes in isolated subordinates to those occurring naturally in dispersing foundresses, we collected a set of control individuals, which constructed nests as solitary foundresses before they naturally transitioned to guarding behavior, for 14–15 days ($n = 9$). We hereafter refer to these individuals as "natural guards." We also recorded brood size for several additional natural guard individuals not collected or disturbed ($n = 4$). In addition, we collected dominant ($n = 5$) and subordinate ($n = 5$) individuals, to compare reproductive phenotypes (dominants and subordinates) to nonreproductive phenotypes (isolated subordinates and natural guards). This allows us to assess whether isolated subordinate phenotypes more closely resemble undisturbed bees at the reproductive stage (dominant and subordinate) or undisturbed bees at the guard stage.

SAMPLE COLLECTION

To collect individuals, entire nest boxes were placed on dry ice to incapacitate bees, which were then removed from the nest and immediately frozen in liquid nitrogen for RNA extraction and sequencing. The entire collection process was completed within minutes of the nest's box removal from the field. Collection of

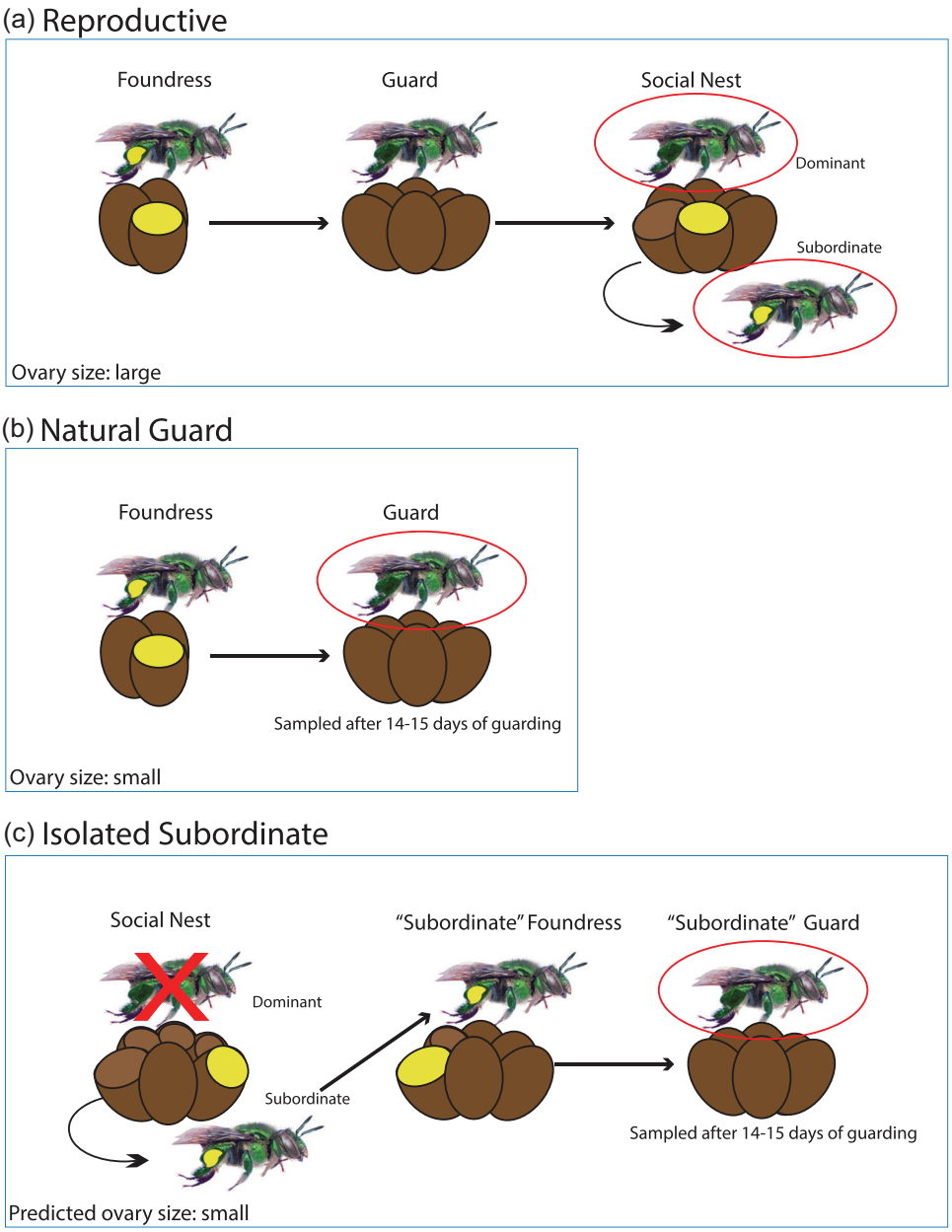

Figure 1. **Design for subordinate isolation experiment, illustrating behavioral progression of individuals and nests from the three different sampled groups (A-C). The blue boxes encompass the entire behavioral sequence of each group and red ellipses show which specific behaviors were sampled from these groups. The ovary sizes or predicted ovary sizes of the sampled individuals are listed in the blue boxes. Yellow on the hindleg (corbicula) or in the brood cell represents pollen and ongoing provisioning. A light brown ellipse on top of a brood cell indicates that offspring have emerged and that the brood cell is empty. (A) Natural nest progression, where individuals are sampled performing reproductive behaviors (dominant and subordinate). (B) Naturally guarding individuals sampled 14–15 days after showing guard behavior. (C) Isolation treatment where the dominant individual was removed (indicated by the red "X"). Isolated subordinates that transitioned to guarding behavior were collected after 14–15 days.**

individuals occurred between 12 and 4 PM during normal afternoon
foraging. After storage in liquid nitrogen for 1–3 weeks, samples were transferred to a −80°C freezer until further phenotypic analysis. Three isolated subordinates were collected in the summer of 2018, with all other isolated subordinates, natural guards, and dominants and subordinates collected in the summer of 2019. An extreme weather event resulted in a truncated collection season in the summer of 2019, requiring collection of all dominant and subordinate samples as well as one isolated subordinate on a single day. This isolated subordinate individual was collected after 10 days of guarding, in contrast

to all other natural guards and isolated subordinates, which were collected after 14–15 days of guarding. We assess the possible impact of these collection irregularities in Supporting information Appendix 1.

OVARY SIZE MEASUREMENT

An ovary size index was calculated using the sum of the longest basal oocyte in each ovary (two measurements), divided by the intertegular distance, to account for body size. We refer to this measurement when "ovary size" is mentioned. Oocyte length and intertegular distance were measured without knowledge of treatment or behavior to avoid possible bias in measurement. We also compare individuals in this study to *E. dilemma* guarding individuals from Saleh and Ramírez (2019). The ovary size index from individuals in Saleh and Ramírez (2019) is available only with measurements from the longest basal oocyte (as compared with the longest basal oocyte of each ovary, measured in this study). Consequently, we adjust our ovary size index to this slightly different approach only when comparing samples between the two studies.

GENERAL STATISTICAL ANALYSIS

Statistical analysis was conducted in R version 3.6 (R Core Team 2020). For assessing differences among group means one-way ANOVAs were used, with Tukey's HSD tests to assess pairwise relationships. We used a Levene's test and a Shapiro-Wilk test to verify ANOVA assumptions. If either assumption was violated, we proceeded instead with Kruskal-Wallis tests using Steel-Dwass tests for pairwise comparisons (Douglas and Michael 1991). All statistical tests were done with reproductive individuals (subordinates and dominants) considered together as one group.

CHC EXTRACTION, DATA GENERATION, AND ANALYSIS

Cuticular hydrocarbon (CHC) differences, which are associated with behavior in *E. dilemma* (Saleh et al. 2021), were extracted from one pair of fore and hindwings, as in Martin et al. (2009), by placing them in 100 μL of hexane for 10 min. Following this, hexane was transferred to a new GCMS vial and left overnight in a fume hood to evaporate. The next day, 30 μL of hexane was transferred to the vials, which were then run on the GCMS, using a 1 μL splitless injection on a GC-MS (Agilent 7890B GC, 5977A MS), with modifications to the protocol from Choe et al. (2012), which started at 100°C for 1 min, increasing 15°C per minute until 300°C was reached, after which the program held at 300°C for 3 min. Helium was used as the carrier gas. Wing extracts have been shown to accurately reflect CHCs on the abdominal surface of *E. dilemma* females (Saleh et al. 2021).

Chromatograms from the GC-MS were integrated to include peaks with an area corresponding to at least 0.1% of the largest peak. Chemical identification was accomplished by comparing to available data for *E. dilemma* (Pokorny et al. 2014; Pokorny et al. 2015; Saleh et al. 2021). We excluded peaks that were not identified as CHCs (linear and branched alkenes and alkanes). After removing non-CHC peaks, the relative abundance of each CHC peak per sample was calculated, generating proportional data. In addition to the individuals used in the rest of the study, CHCs from several additional dominants (additional $n = 3$, total $n = 8$) and subordinates (additional $n = 3$, total $n = 8$) collected from the same field seasons, were available and included in the analysis (Supporting information Table S4). *Euglossa dilemma* has a well-characterized CHC polymorphism segregating in Florida populations that complicates chemical comparison among samples but does not appear to be related to social behavior (Saleh et al. 2021). Consequently, we exclude samples from the rarer CHC morph ($n = 4$) from downstream analysis for clarity (remaining $n = 31$). We show an NMDS plot based on Bray-Curtis dissimilarity as implemented in the Vegan R package (Oksanen et al. 2019) with all samples in Supporting information Figure S2 ($n = 35$) and data from all individuals are included in Supporting information (Table S4).

For the remaining samples (total $n = 31$, dominants $n = 7$, subordinates $= 8$, isolated guards $= 7$, natural guards $= 9$), we used random forest analysis (number of trees $= 10{,}000$) to determine if the four sampled behaviors could be classified based on their CHCs and to identify compounds that might contribute to any identified differences (Jansen et al. 2016; Oliveira et al. 2016; Bruckner and Heethoff 2017). For visualization, we constructed a multidimensional scaling plot based on the proximity matrix from the random forest analysis (Monin et al. 2018). Furthermore, we generated a confusion matrix based on the out of bag (OOB) sample, which provides an error rate for classifying samples into our four behavioral groups. Random forest analysis was run using the randomForest R package (Liaw and Wiener 2002).

RNA EXTRACTIONS, SEQUENCING, AND QUALITY CONTROL

For brain dissections, we removed the cuticle around the head while samples were on dry ice. Next, frozen heads with the cuticle removed were placed in RNAlater ICE for at least 16 h at −20°C. After RNAlater ICE thaw, brains were dissected from the heads on dry ice and immediately transferred to trizol for RNA extraction. We dissected the ovaries by first removing sections of abdominal cuticle from frozen samples on dry ice. The abdomens were then thawed in RNAlater ICE for at least 16 h at −20°C before being dissected on dry ice. Ovaries were photographed with a scale bar and then immediately placed in trizol.

We followed the standard RNA extraction trizol protocol, with glycogen added to the brain samples but not the ovary samples to help increase yield. After extraction, RNA was cleaned using an Invitrogen Turbo DNA-free kit and then quantified using a Qubit. Next, RNA quality was checked on a Bioanalyzer (Agilent) and library construction commenced on samples with high-quality RNA. These samples consisted of 26 brains (dominant = 5, subordinate = 5, natural guard = 7, isolated subordinate = 9) and 29 ovaries (dominant = 5, subordinate = 5, natural guard = 9, isolated subordinate = 10).

RNA samples were submitted for library preparation and sequencing to the Vincent J. Coates Genomic Sequencing Laboratory at UC Berkeley. Libraries consisting of 150 bp paired-end reads were sequenced on a Novaseq 6000, generating approximately 30 million reads per library (mean = 30.40 million, SD = 4.89 million, range = 23.47–47.90 million, N = 55). After sequencing, we evaluated the quality of reads with FastQC (version 0.11.7, Andrews 2010). Initial sample clustering using MDS in EdgeR suggested one brain sample from a natural guard individual to be an outlier with no obvious biological explanation. Furthermore, inspection of the FastQC reports for this individual showed quality score drops in sequencing not shown in the other samples, so this individual (SRNS33) was dropped from all gene expression analysis (Supporting information Fig. S3). Two other brain samples clustered separately from the other samples along one axis in the MDS plot of gene expression data (SRNS11 and SRNS53; Supporting information Fig. S3); however, these were a dominant and subordinate gathered from the same nest. No technical reason could be identified that drove this pattern (they were collected at the same day/time as other samples and showed no obvious sequencing anomalies). Consequently, it appeared most likely that biological variation associated with shared nesting may be responsible and so these samples were included in our analysis, which aims to capture realistic levels of biological variation found in field established nests. The data for all sequenced samples can be found at NCBI under the Bioproject Accession PRJNA750777.

DIFFERENTIAL GENE EXPRESSION ANALYSIS

We followed the analytical approach of Saleh and Ramírez (2019), to facilitate comparison of results (as shown in Supporting information Appendix 1). Briefly, we used Kallisto (Bray et al. 2016) for producing transcript counts based on genes from the *E. dilemma* genome (Brand et al. 2017). After transcript quantification, we filtered genes in the ovary and brain data set separately, so that each of the two data sets consisted of genes with at least one count per million (CPM) in at least five of the libraries, which represented the smallest behavioral group sample size. For the brain data, this resulted in 11,041 genes and 10,132 genes for the ovary data filtered from the total gene set of 16,127 genes. We used edgeR-robust (Zhou et al. 2014) with default settings and the glmLRT function with FDR <0.05 to identify differentially expressed genes (DEGs) among the four sampled behavioral groups. We used TMM normalization to account for differences in the amount of reads among libraries. Hierarchical clustering and heatmap construction were conducted using Euclidean distance and Ward.D2 clustering using gplots version 3.0.1 (Warnes et al. 2020).

GENE NETWORK ANALYSIS

We conducted gene network analysis to identify co-expressed networks of genes underlying ovary size differences among individuals. This analysis may provide additional insight into functional connections between sets of genes underlying phenotypes of interest that may not be captured during standard differential expression analysis (Faragalla et al. 2018). To do this, we used the WGCNA (weighted gene co-expression network analysis) package in R (Langfelder and Horvath 2008) to identify modules of genes showing co-expression. We then used the module eigengenes, which summarize the expression of each module, to assess correlation between ovary size measures and gene expression. WGCNA parameters largely followed recommended values from published tutorials and R code used for the analysis can be found in the Dryad data file (Saleh 2022). Briefly, using the filtered and normalized gene sets from differential expression analysis, gene modules were detected using a soft thresholding power for which the scale-free topology index value was greater than 0.85. For ovaries, this soft thresholding power was four and for the brains it was five. The minimum module size was 30 genes and the module merging cut-off value was 0.25.

GENE LIST COMPARISONS

We compared genes identified through differential expression analysis and WGCNA to genes previously identified as associated with reproductive plasticity in other bee species. Specifically, we compared the results of this study to data from the abdomens of *M. genalis* queens and workers (Jones et al. 2017) and the abdomens of *Apis mellifera* egg laying or nonegg laying workers (Galbraith et al. 2016). These comparisons represent two origins of eusociality and different levels of eusocial organization. *Apis mellifera* has a complex eusocial organization with large colonies but it is more closely related to *E. dilemma* than *M. genalis* and, thus, may share features associated with behavior in the mostly eusocial corbiculate bees. In contrast, *M. genalis* forms small, facultatively eusocial groups that more resemble the social structure of *E. dilemma*, though sociality has arisen independently in these groups. In addition, we compared our results to genes identified as differentially expressed between natural guards and subordinates in Saleh and Ramirez (2019), to assess the degree to which our results overlap with this study also

done on *E. dilemma*. For comparisons to *M. genalis*, we identified orthologous genes between the *E. dilemma* peptide set and the predicted peptides from Jones et al. (2017) using a reciprocal best hit blastp search (e-value <1E-5). For *A. mellifera* comparisons, we converted our *E. dilemma* gene lists into honey bee gene IDs (OGSv 3.2; Elsik et al. 2014) with a conversion list from Brand et al. (2017). We used DAVID 6.8 to perform GO term analysis with Benjamini-Hochberg corrected *p*-values using honey bee OGSv 3.2 gene IDs. To identify significant overlaps between two compared gene sets, we performed hypergeometric tests to identify overlaps greater than expected by chance when compared to the shared universe of analyzed genes.

Results

BEHAVIORAL RESPONSE OF ISOLATED SUBORDINATES

We conducted 14 manipulations on naturally colonized nest boxes in the field, removing all individuals except for a single subordinate bee (Supporting information Tables S1, S4). In these 14 manipulated nests, three isolated subordinates disappeared before transitioning to guarding behavior. Two of these three disappeared after completing one additional brood cell, following isolation and one disappeared in the morning after isolation. One isolated subordinate disappeared from the nest after guarding began, but before collection. The remaining 10 bees successfully transitioned to guarding behavior and were collected and processed for subsequent analysis. Four of these 10 females did not provision additional brood cells after isolation before transitioning to guard behavior, ceasing foraging, and brood cell construction upon isolation. Six of 10 females provisioned at least one brood cell after isolation before guarding behavior began. We also compared the final brood size of isolated subordinates and naturally guarding individuals, finding that isolated subordinates began guarding a smaller number of brood cells on average compared to naturally guarding bees (5.3 vs. 7.8, $F_{1,22} = 7.62$, $p = 0.011$, Supporting information Fig. S4).

ISOLATED SUBORDINATES EXHIBIT REDUCTION IN OVARY SIZE

We examined the ovary size of isolated subordinates collected after showing guarding behavior, comparing them to naturally guarding bees and reproductive individuals (dominants and subordinates). We find that isolated subordinates and natural guards show a reduction in ovary size relative to reproductive individuals, though ovaries of isolated subordinates and natural guards are statistically indistinguishable ($F_{2,26} = 33.08$, $p < 0.001$; Fig. 2). We find no difference in body size among these three groups (Kruskal-Wallis $\chi^2 = 2.7$, df = 2, $p = 0.26$, Supporting information Fig. S5). Given the lack of ovary size index differences between the isolated subordinates and natural guards, we combined these groups (n = 19) and compared their ovary size index measurements to ovary size index measurements from naturally guarding individuals measured in Saleh and Ramírez (2019) (n = 15). The individuals in Saleh and Ramírez (2019) were collected after performing guarding behavior for a longer time range (minimum 2 weeks with some samples likely up to 6 weeks). Consequently, comparison to those samples can indicate whether the reproductive transition measured in this study is complete or if the reduction in ovary size would continue beyond 2 weeks into the guarding phase. We find that our sampled individuals, which guarded for 14–15 days, had larger ovaries on average than natural guards from Saleh and Ramírez (2019), which guarded for longer periods on average ($F_{1,32} = 10.25$, $p < 0.01$; Supporting information Fig. S6).

ISOLATED SUBORDINATES SHOW SHIFT IN CHCs

We examined variation in the CHC profiles, finding 17 previously characterized alkanes and alkenes (Saleh et al. 2021), and identified changes that may be associated with behavior and reproduction. In contrast to the ovary size data, individuals do not separate strictly based on reproductive state, with dominants, isolated subordinates, and natural guards mostly clustering separately from subordinates (Fig. 3). The random forest OOB error rate, which indicates the percent of individuals not correctly assigned to their behavioral category, was 58.06% indicating a high rate of error. Examination of the confusion matrix (Supporting information Table S2) shows that the greatest source of error was in assigning natural guards (error rate 88.9%) and isolated subordinates (error rate 85.7%). The model struggled to differentiate between the two categories and incorrectly categorized six of nine natural guard samples as isolated subordinates, and five of seven isolated subordinates as natural guards. Subordinates had the lowest classification error rate (12.5%) with only one subordinate incorrectly classified as a dominant, while dominant bees showed an elevated error rate (42.9%). Longer carbon length alkenes, particular carbon length 31 and 27 were most important in distinguishing among behaviors (Supporting information Fig. S7), with subordinates having a lower relative abundance of these compounds than the other behaviors.

ISOLATED SUBORDINATES SHOW GENE EXPRESSION PATTERNS CONSISTENT WITH GUARDING BEHAVIOR

Our gene expression analysis had two aims: (1) identify gene expression patterns associated with the sampled behaviors and (2) determine whether isolated subordinates resemble natural guards or other behavioral phases based on their expression profiles. To this end, we first found all unique DEGs among pairwise comparisons of the four sampled behavioral groups. We identified 412

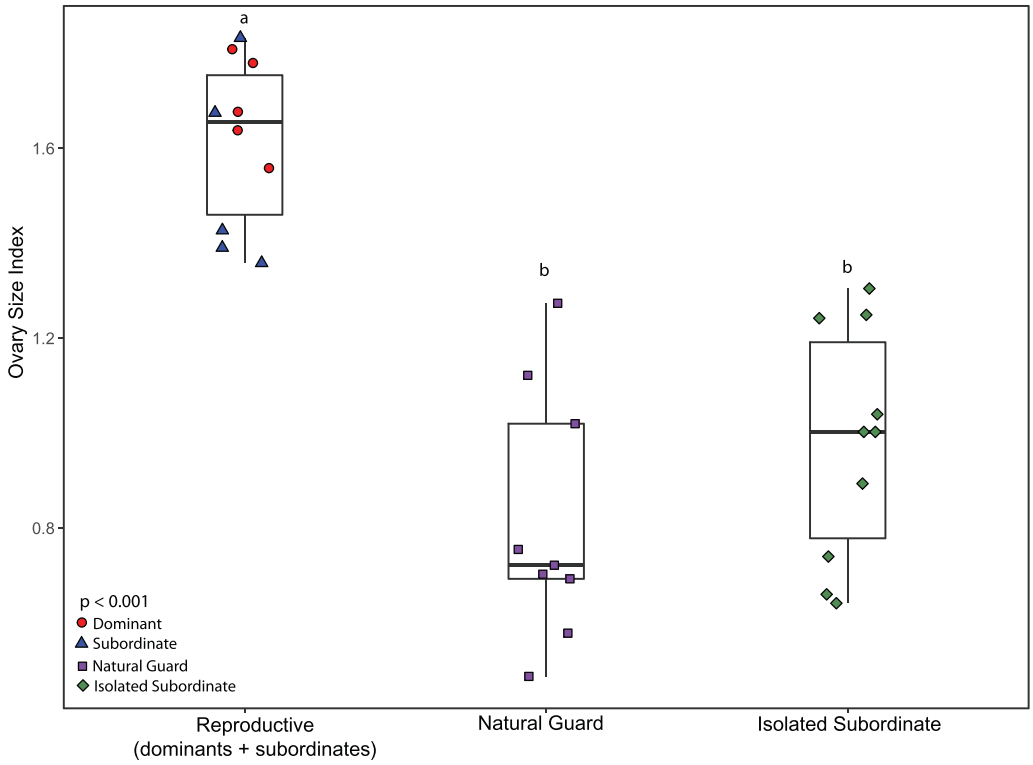

Figure 2. **Ovary size index among reproductive individuals, natural guard individuals, and isolated subordinate individuals. Letters indicate statistical groupings determined by a Tukey HSD test with the *p*-value <0.001 calculated using a one-way ANOVA. The box plots show the median value in each group.**

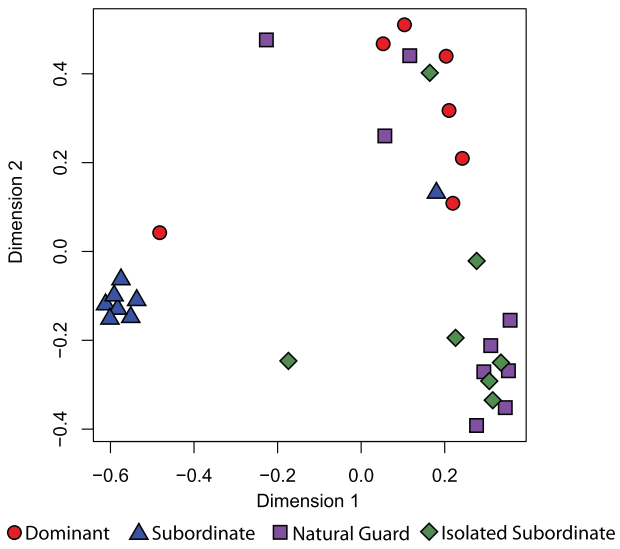

Figure 3. **Multidimensional scaling plot based on the proximity matrix of individuals generated in random forest analysis. Unique color or symbol combinations represent the different behavioral groups.**

DEGs in the ovaries and 132 DEGs in the brains among the four groups. The number of DEGs between each comparison is found in Supporting information Table S3. The full differential expression results are found in Supporting information File 3. Overall, the comparison of isolated subordinates and natural guards revealed few differences in gene expression (1 DEG in the ovaries and 0 DEGs in the brain), in contrast to isolated subordinates versus subordinates from an active social nest (73 DEGs in the ovaries and 94 DEGs in the brain). These brain and ovary DEGs showed significant overlap with DEGs independently identified between natural guard and subordinate individuals in the brains (49/88 shared DEGs, $p < 0.001$) and ovaries (41/68 shared DEGs, $p < 0.001$) from Saleh and Ramírez (2019). Hierarchical clustering based on expression patterns of the 412 ovary DEGs revealed two clusters mostly corresponding to reproductive (dominants and subordinates) and nonreproductive (isolated guards and natural guards) phenotypes (Fig. 4). This gene set is enriched for multiple GO-terms, including "signal" and "transmembrane" (Supporting information Table S5). Furthermore, this gene set includes genes known to be associated with reproductive and social behavior in insects; for example, DNMT3, broad-complex, corazonin receptor, yellow-g, yellow-g2 (Drapeau et al. 2006; Paul et al. 2006; Okada et al. 2016; Gospocic et al. 2017). In the brains, hierarchical clustering of the 132 DEGs clustered subordinates separately from the three other behaviors (Fig. 5). This gene set was enriched for multiple GO-terms including "signal" and "vision." This gene set also includes genes known to be involved

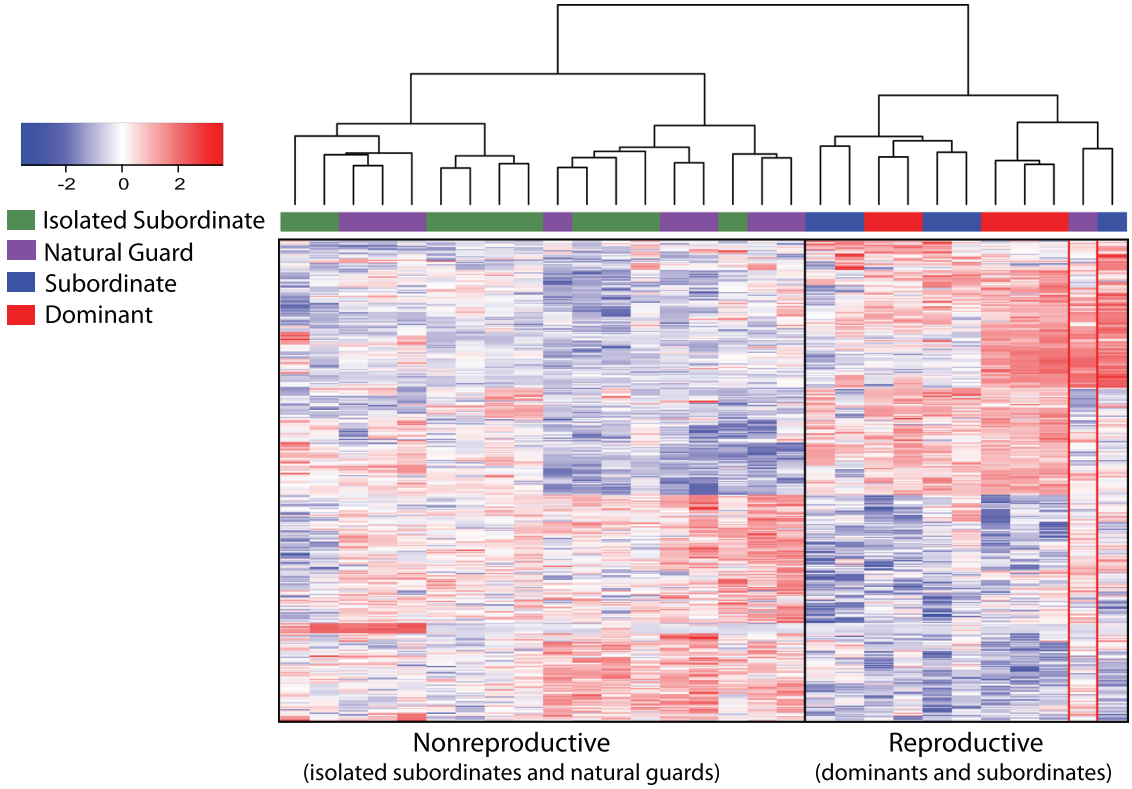

Figure 4. **Hierarchical clustering of samples based on expression of 412 pairwise DEGs identified among behaviors in the ovaries. Color key shows the $\log_2$ scaled expression relative to the mean value for each gene. Nonreproductive and reproductive clusters are highlighted with black boxes, with one natural guard that does not cluster according to reproductive state is highlighted in red. Sample clustering was based on using Euclidean distance with the Ward.D2 clustering method.**

in insect social and reproductive behavior, such as hexamerin 70c, hormone receptor-like 38, prohormone 2, and prohormone 3 (Okada et al. 2016; Shpigler et al. 2019).

We also performed specific contrasts in the EdgeR model to compare sampled behaviors based on reproductive state and initial dispersal strategy. For reproductive state, we performed a contrast between the two reproductive groups (dominants/subordinates) and the two nonreproductive groups (isolated subordinates/natural guards). For initial dispersal strategy, we compared females that emerged in the nest and stayed as helpers (subordinate/isolated subordinate) and females that dispersed to begin their own nest on emergence (dominants/natural guards). Considering the reproductive state contrast, we find 514 DEGs in the ovaries and 109 DEGs in the brains. In the ovaries, 318 genes are shared between this reproductive contrast and the 412 DEGs identified among all pairwise behavioral comparisons above ($p < 0.001$). This is in line with hierarchical clustering supporting reproductive state as the major factor driving differential expression patterns among behavioral groups in the ovaries. In the brains, 64 of the 109 genes are shared with the 132 pairwise DEGs identified across behaviors, a greater overlap than expected by chance ($p < 0.001$). However, clustering in the brain by behavioral DEGs does not correspond primarily to reproductive state, as opposed to the ovaries (Figs. 4 and 5). Considering differing dispersal strategies (subordinates and isolated subordinates vs. dominants and natural guards), we find less genes overall, with nine DEGs in the brain and three DEGs in the ovaries.

In summary, our differential expression analysis finds that isolated subordinates and natural guards show similar expression profiles at identified DEGs (as seen in hierarchical clustering) and we find only one DEG across tissues between the two behaviors. In addition, reproductive changes rather than initial dispersal strategy appear to be associated with more expression differences in both the brains and ovaries.

GENE NETWORK ANALYSIS IDENTIFIES MODULES OF GENES THAT ARE HIGHLY CORRELATED WITH OVARY SIZE

Using WGCNA, we identified 13 modules of co-expressed genes in the brains and 13 modules in the ovaries and examined correlations between these modules and ovary size. Multiple module eigengenes, which represent the first principal component summarizing the expression of genes within each module (Langfelder and Horvath 2008), were correlated with ovary size changes

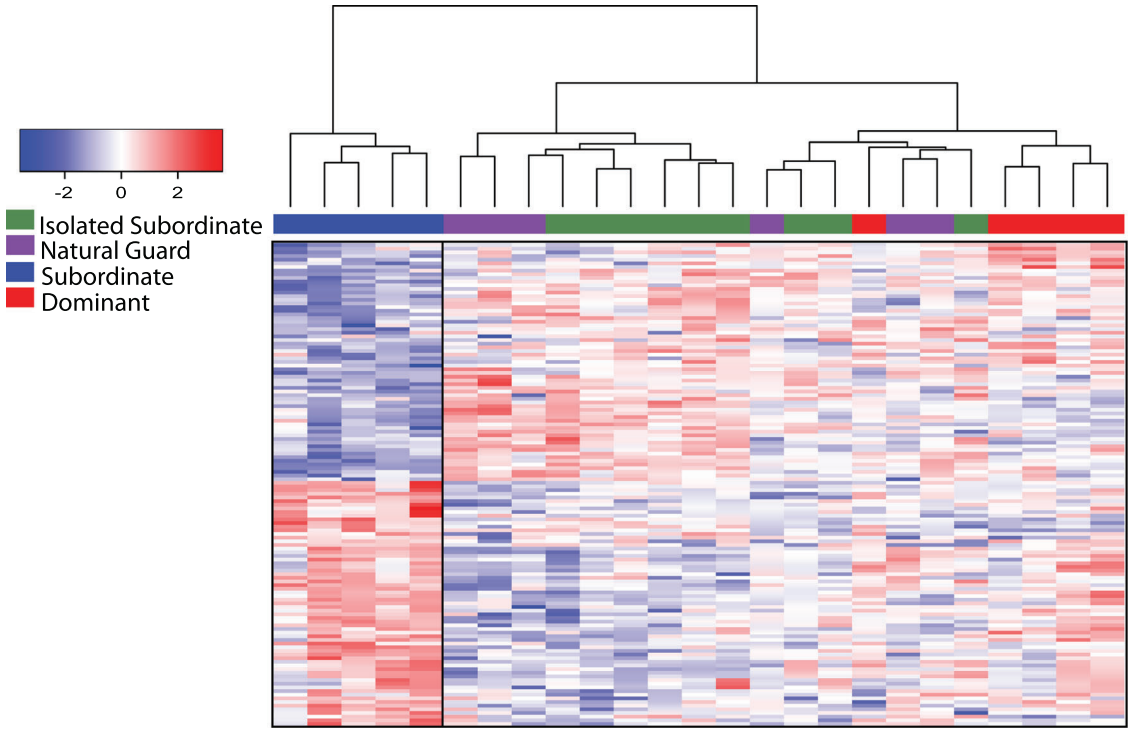

Figure 5. **Hierarchical clustering of samples based on expression of the 132 pairwise DEGs identified among behaviors in the brain. Black boxes highlight the subordinate cluster and the cluster containing the other three behaviors. Color key shows the $\log_2$ scaled expression relative to the mean value for each gene. Sample clustering was based on using Euclidean distance with the Ward.D2 clustering method.**

across the sampled brain and ovary tissues. The full results and analysis are detailed in Supporting information tables and the accompanying R code (found in the Dryad data file), including identities of the genes present in each module (Supporting information Table S6), connectedness values (kME) for genes in these modules (Supporting information Tables S7 and S8), gene enrichment analysis for the focal modules discussed below (Supporting information Table S5), and a correlation matrix comparing all the modules in both tissues with ovary size index (Supporting information Fig. S8). Several especially strong connections between co-expression modules and ovary size are highlighted here. Following FDR correction for 26 comparisons to ovary size, eigengenes from eight ovary modules and two brain modules were significantly correlated with ovary size variation. Two ovary module eigengenes showed especially strong correlation with ovary size. Ovary module six, which consisted of 371 genes, showed a strong negative correlation with ovary size ($r = -0.89$, $p < 0.001$, Fig. 6) and ovary module 10, consisting of 116 genes, showed a strong positive correlation with ovary size ($r = 0.89$, $p < 0.001$, Supporting information Fig. S9). The genes in ovary module six were significantly enriched for the "DNA replication" KEGG pathway, while there were no significantly enriched terms for module 10. In the brain, module three, a large module of 1256 genes, showed the highest correlation ($r = -0.54$, $p = 0.028$, Supporting information Fig. S10) with ovary size variation. This module was enriched for several terms, including "coiled coil," "transducer," and "nucleic acid binding," and included multiple genes with known associations with social behavior such as syntaxin-1A and dopamine receptor D1 (Sasaki 2010; Kocher et al. 2018). This module was also correlated with ovary module 6 ($r = 0.62$, $p < 0.01$), suggesting co-expression across tissues. In line with this, 71 of the 350 possible overlapping genes in ovary module six are shared with brain module three, a larger overlap than expected by chance ($p < 0.001$).

CROSS-STUDY GENE LIST COMPARISONS

We compared genes identified through differential expression and WGCNA to those associated with worker-related reproductive plasticity in *A. mellifera* and *M. genalis*, two species showing complex and simple eusocial organization, respectively. We focus these comparisons on genes related to the reproductive differences we identify, to test the hypothesis that genes involved in reproductive plasticity identified here overlap with genes associated with reproductive plasticity in other species. In the ovaries, genes upregulated in natural guards and isolated subordinates (nonreproductive) versus dominants and subordinates (reproductive), significantly overlapped with genes upregulated in both *M. genalis* worker versus queen abdomens (64/126 shared

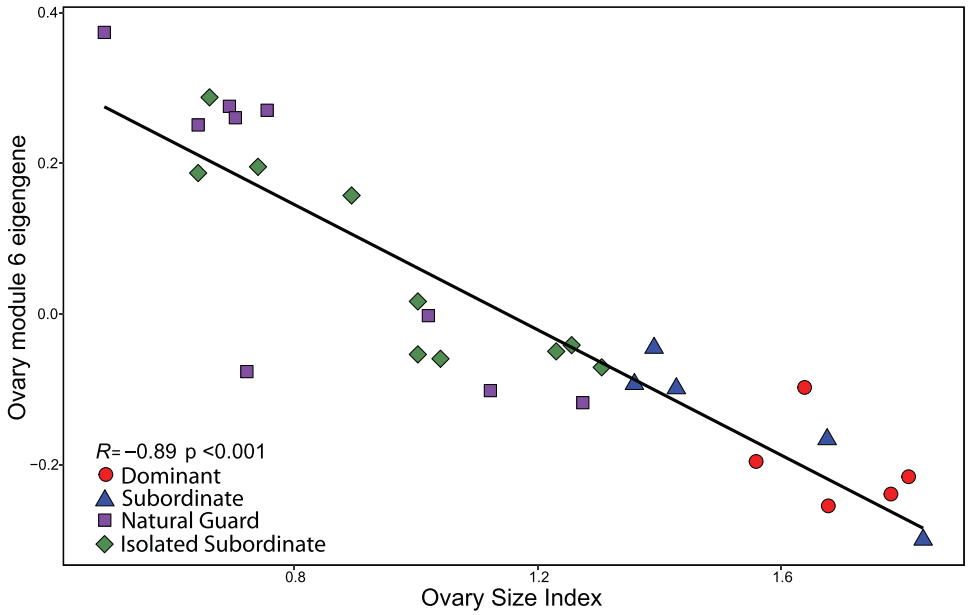

Figure 6. Correlation between eigengene values and ovary size index measurements from module 6, containing 371 genes detected with WGCNA of ovary transcriptome data. Spearman correlation coefficient is shown. The *p*-value <0.001 was FDR adjusted for 26 ovary size comparisons.

genes, $p < 0.001$) and in the abdomens of nonegg-laying versus egg-laying honey bee workers (35/196 shared genes, $p < 0.001$). Considering the opposite contrast, genes upregulated in reproductive versus nonreproductive *E. dilemma* individuals, these genes significantly overlap with genes upregulated in *M. genalis* queens versus workers (65/163 shared genes, $p = 0.044$) but not with egg-laying versus nonegg-laying workers (36/227 shared genes, $p = 0.24$).

We also compared the gene lists from *M. genalis* and *A. mellifera* to the genes identified in the two WGCNA modules with the strongest associations with ovary size (ovary modules six and 10). Genes present in module six significantly overlapped with genes differentially expressed between *M. genalis* queens and worker abdomens (159/227 shared genes, $p < 0.01$) and *A. mellifera* nonegg-laying versus egg-laying workers (89/266 shared genes, $p < 0.001$). In contrast, module 10 did not significantly overlap with *M. genalis* queen versus worker abdomen DEGS (49/80 shared genes, $p = 0.5$) or *A. mellifera* nonegg-laying versus egg-laying honey bee workers (26/92 shared genes, $p = 0.06$).

Discussion

In this study, we disrupt the social structure of small colonies to assess the plasticity of *E. dilemma* subordinate females, finding that socially isolated subordinate bees are highly flexible, capable of expressing largely the same behavioral, physiological, chemical, and gene expression changes that dispersing foundress bees show naturally across solitary behavioral phases. In addition, we identify genes strongly associated with reproductive plasticity that overlap with genes associated with worker physiology in eusocial bees. This suggests that the lack of nonreproductive workers in *E. dilemma* is not due to a lack of plasticity in subordinates, which show high behavioral and physiological flexibility.

THE INITIAL EFFECT OF SOCIAL DISRUPTION ON *E. DILEMMA* SUBORDINATES

Our experimental manipulation of 14 individuals resulted in 10 subordinate individuals that transitioned to guard behavior and were collected, although there was variation in the timing of this transition (Supporting information Table S1). Three of the 14 individuals disappeared or died before transitioning to guarding behavior and one individual died after transitioning to guarding behavior before collection. Four of the 10 individuals that transitioned to guarding behavior stopped provisioning additional brood cells after isolation and two of 10 provisioned only one additional brood cell before guarding. This suggests that some individuals may be responding to nest disruption by beginning to guard their brood early, relative to foundresses that have transitioned to guarding. This may be what is driving the slightly smaller average brood size of isolated subordinates (Supporting information Fig. S4). This response could be due to stress imposed by the treatment itself. However, of the four of 14 bees that disappeared after isolation but before collection, three finished provisioning at least one brood cell after isolation. Only one bee disappeared the day following isolation without continuing to forage, which suggests that the isolation treatment may

not have been an extreme source of stress to most individuals. Alternatively, it could be that the changing social environment affects decisions about optimal nest defense and brood size, though the data presented here cannot address this. Although some individuals appeared to respond directly to isolation by quickly transitioning to guard behavior, others continued provisioning long after isolation. One individual, for instance, provisioned an additional seven brood cells following isolation, ultimately guarding a relatively large brood of nine. In general, it is unclear what mix of environmental and genetic factors influence the size of the first brood. Additional work to disentangle sources of variation on brood size is necessary to provide greater insight into behavioral mechanisms underlying the transition from foraging to guarding behavior.

REPRODUCTIVE PHYSIOLOGY AND BEHAVIOR MAY DIFFERENTIALLY INFLUENCE TRANSCRIPTOMIC AND CHEMICAL VARIATION

Although the transition to guarding behavior is accompanied by a clear reduction in ovary size in isolated subordinates (Fig. 2), reproduction is not necessarily the only influence on the phenotypes we examined. In the brain, for instance, hierarchical clustering of samples based on DEGs from pairwise comparisons of behaviors revealed two primary clusters that were not correlated with reproductive state (Fig. 5). Instead, samples grouped subordinates separately from the other behavioral groups (dominants, natural guards, isolated subordinates). This is largely in line with findings from Saleh and Ramírez (2019), where brain variation associated with social hierarchy clustered individuals according to foraging or nonforaging behavior rather than reproductive state, such that dominants and natural guards clustered together and subordinates and foraging foundresses (not sampled in this study) clustered together. Considering this, clustering of the isolated subordinates with dominants and guards is consistent with these individuals making the same behavioral changes associated with a transition from foraging outside the nest to remaining inside the nest during the day. The "vision" GO term was enriched in the gene set, and it may relate to a transition from foraging to remaining in the dark nesting environment. In addition, analysis of CHC profiles revealed mostly the same clustering pattern as seen in brain DEGs, with subordinates clustering separately from the other non-foraging behaviors. This pattern could be explained by differential light exposure among individuals, along with behavioral and physiological variation, which can have a strong impact on CHC profiles due to UV exposure and degradation (Hatano et al. 2020).

In our samples, subordinate bees were younger on average than the other sampled groups (typically true in natural nests), which may additionally affect the transcriptomes and CHC profiles. However, the absolute difference in age between isolated subordinates or natural guards and dominants is likely greater than the difference in age between isolated subordinates/natural guards and subordinates. Dominants sampled in this study have, for example, completed the guard phase and then participated in social nesting for multiple weeks. Consequently, if age itself is primarily driving the phenotypic variation, the effect would not be strictly linear. If the patterns that we observe were driven primarily by age and not foraging or nonforaging status, for example, we might expect to see natural guards, isolated subordinates, and subordinates grouped together, as these three groups should be closer together in age on average than any group is to dominant bees. Furthermore, analysis of independently collected *E. dilemma* transcriptomes from Saleh and Ramírez (2019) (Supporting information Appendix 1, Figs. A7 and A8), shows a signal of behavioral clustering with the DEGs identified in this study, suggesting that these DEGs are likely associated with behavior independent of any sampling biases that could be present in this dataset.

The clustering pattern that we identified in CHC profiles and pairwise brain DEGs contrasts with the sample clustering that we identified based on the pairwise ovary DEGs, which revealed two clusters mostly corresponding to the reproductive or nonreproductive phenotypes, also seen clearly in the ovary size index measurements (Fig. 2). Although clustering based on behavioral DEGs in the brain was not primarily based on reproductive differences, these differences did clearly influence brain transcriptomes, as seen by the subset of overlapping DEGs between the nonreproductive or reproductive contrast and the pairwise DEGs among all behaviors. Furthermore, the brain gene module most strongly correlated with ovary size variation contained a relatively large number of genes, some of which have been previously found to be associated with social behavior in other species.

FOUNDRESSES AND SUBORDINATES HAVE THE SAME PHYSIOLOGICAL, CHEMICAL, AND TRANSCRIPTOMIC POTENTIAL

Our data strongly suggest that subordinate bees are totipotent and can express the full spectrum of changes seen during the foundress to guard transition. Indeed, natural guards and isolated subordinates are largely indistinguishable by all phenotypes we examined. This, along with the lack of body size differences among behavioral groups, suggests that subordinate behavior is probably not the result of strong developmental differences limiting the plasticity of some individuals. Consequently, it seems unlikely that the foundress versus subordinate trajectories are strictly determined by large nutritional differences, as these would likely be reflected in body size differences (Lawson et al. 2017). However, it is possible that subtle nutritional and/or developmental differences still underly these behaviors and

require additional investigation to uncover. This contrasts with several other well-studied bees showing simple social behavior, such as some halictids and small carpenter bees, in which maternal manipulation of nutrition strongly influences the body size and social trajectory of offspring (Kapheim 2016; Lawson et al. 2017).

This raises the question then, what determines whether a female will disperse or stay as a subordinate? It has been recognized in several orchid bee species that early eclosing females are much more likely to remain in their natal nest and become subordinates compared to later eclosing females (Augusto and Garófalo 2009; Andrade-Silva and Nascimento 2012). Although this generally appears to be true in *E. dilemma*, the pattern is not always consistent (N Saleh, personal observation) and does not explain why the number of subordinates varies among nests. In addition, this observation is somewhat confounded by the fact that, because nests rarely grow beyond one to two subordinates, later eclosing females may have little opportunity to remain in the nest. Thus, correlates with eclosion order cannot easily be teased apart without further experiments.

In the social allodapine bee *Exoneura bicolor*, eclosion order and not developmental or nutritional factors is the proximate determinant of dominance status, so posteclosion hierarchy determination is known to occur in bees (Schwarz and Woods 1994). Furthermore, in *Euglossa townsendi* social nests, individuals can transition back and forth between dominant-like behaviors and subordinate-like behaviors, irrespective of age, such that the social hierarchy shifts over time among a group of individuals (Augusto and Garófalo 2004). In *E. dilemma*, one possibility is that newly emerged females could undergo some decision-making process, integrating information about the local availability of nesting resources (e.g., pollen, resin), the current state of the nest, the age, and condition of current occupants, and the likelihood of inheriting the nest, before remaining as a subordinate or dispersing as a foundress. Experimental manipulation of these factors is a necessary next step in clarifying the role of developmental versus posteclosion factors in determining offspring trajectory.

WHY DO *E. DILEMMA* SOCIAL GROUPS LACK NONREPRODUCTIVE WORKERS?

Considering the question posed by Roberts and Dodson (1967), "why, then, has there been no evolution of distinct worker and reproductive castes among these bees?" we can, from a proximate perspective, rule out the hypothesis that individuals lack the reproductive plasticity to express worker-like physiology. The data presented here show that subordinates will exhibit nonreproductive phenotypes that involve genes associated with worker physiology in eusocial species. This leaves us with the question then, if subordinate reproductive physiology can be dynamically regulated, why does not this happen in social groups, leading to nonreproductive workers? Other bees, such as *M. genalis*, can form small social groups comparable in size to those in *E. dilemma* that still contain nonreproductive workers (Kapheim et al. 2012). Thus, social group size itself does not have to impose a limit on the evolution of nonreproductive workers.

One possibility is that subordinate eggs are functioning as a type of trophic egg and that disrupting oophagy may have fitness costs on social individuals. Many stingless bee species, despite their derived form of eusociality, have workers with activated ovaries that lay trophic eggs for the queen (Wille 1983). Furthermore, trophic egg-laying workers appear to be ancestral in the stingless bees, though, in contrast with orchid bees, they have repeatedly evolved nonreproductive workers (Gruter 2018). Consequently, comparative analysis of orchid bee and stingless bee oophagy behaviors may be especially useful in understanding how trophic eggs evolve and function and whether these traits influence the evolution of nonreproductive workers. Ultimately, additional data are needed to investigate the evolutionary forces (or lack thereof) that have shaped reproductive interactions in orchid bee social groups.

Conclusions

These data show that *E. dilemma* females are highly flexible, with each individual capable of large behavioral and reproductive changes regardless of their initial foundress or subordinate trajectory. Furthermore, these changes involve genes associated with worker physiology in eusocial species, suggesting that *E. dilemma* subordinates are capable of worker-like nonreproductive physiology. As such, *E. dilemma* represents a unique case in the corbiculate bees where functional reproductive division of labor has evolved via behavioral but not physiological specialization.

AUTHOR CONTRIBUTIONS

N.W.S. and S.R.R. designed the project. N.W.S and J.H. collected samples. N.W.S. performed sample processing and data analysis. All authors participated in writing and revising the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA ARCHIVING

Code and data files to reproduce the analysis in the manuscript have been deposited in Dryad (https://doi.org/10.25338/B8TK96) and Zenodo (https://doi.org/10.5281/zenodo.6502386). Sequencing data can be found at NCBI Bioproject accession PRJNA750777.

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Supporting Information

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Figure S1. Four possible outcomes (A-D) for the behavior and ovary size of subordinates that have been isolated. Yellow in brood cells or on the hindleg (corbicula) represents ongoing pollen provisioning. The red "X" on the dominant in the first nest illustration indicates that individuals were removed with one subordinate left remaining. The predicted ovary size of the isolated subordinate performing each behavior is shown beneath nest illustrations in each box. Green boxes indicate totipotent trajectories and red boxes indicate non-totipotent trajectories. **A)** A trajectory that indicates totipotency, with the isolated subordinate showing guarding behavior after completing a first brood. This individual shows reduced ovary size as seen during natural guarding behavior. **B)** A trajectory that does not indicate physiological totipotency, showing that the isolated subordinate shows guarding behavior but does not show a reduction in ovary size. **C)** A trajectory that does not indicate totipotency, where the isolated subordinate forages for and provisions brood cells until offspring emerge, bypassing guarding behavior and ovary size changes. In this case, the isolated subordinate becomes the new dominant. **D)** If the isolated subordinate disperses or dies following isolation, totipotency cannot be determined. It is possible that disappearing females could found their own nest or join another nest as a subordinate.

Figure S2. NMDS plot of CHC variation across behaviors with rarer polymorphism individuals included (four individuals shown in the lower left corner). Unique color/symbol combinations represent the different behavioral groups. Stress value for NMDS configuration = 0.02.

Figure S3. Mutlidimensional scaling plot showing relationships among brain samples with behavior coded. Ellipses are drawn to show the outlier that was dropped for technical issues and the samples that were retained in analysis due to expected biologically relevant variation. Data for plot consists of all transcripts with at least 1 CPM across at least 5 samples, per the analysis detailed in the main text.

Figure S4. First brood size of natural guards and isolated subordinates. Statistical significance determined using a Kruskal-Wallis test ($p = 0.012$). The box plots show the median value in each group.

Figure S5. Intertegular distance among reproductives, natural guards, and isolated subordinates. The p-value is calculated using a Kruskal-Wallis test ($p = 0.26$). The box plots show the median value in each group.

Figure S6. Ovary size index differences between guarding females (natural guards and isolated subordinates) collected for this study compared with natural guards from Saleh and Ramírez, 2019. Ovary size index consists of the length of the longest basal oocyte divided by intertegular distance. For the Saleh and Ramírez, 2019 data, all natural guards with ovary size data and intertegular distance present in published supplementary material were used, though not all individuals were used for the RNAseq analysis in that study. The boxplot shows the median value in each group. P-Value was <0.01.

Figure S7. Variable importance plot from random forest analysis. A higher "Mean Decrease in Accuracy" value indicates a more important variable for classifying samples into the specified categories. For the compounds, which make up the specific variables in the analysis, the number after the "C" indicates the carbon length. Compounds with an underscore ("_1") indicate an alkene instead of an alkane. The "a" and "b" indicate that the double bond for the specific alkene is in a different position from the other alkene of the same carbon length.

Figure S8. Spearman correlation plot among all eigengene values from the brain and ovary WGCNA along with the ovary size index measures. Each square in the grid contains a circle where the size of the circle corresponds to the degree of correlation between the two columns which intersect at that square. Blue color represents a positive correlation while red is negative. "O" columns refer to the ovary module eigengene from the numbered module and "B" refer to the brain module eigengene from the numbered module.

Figure S9. Correlation between eigengene values and ovary size index measurements from module 10, containing 116 genes detected with WGCNA of ovary transcriptome data. Spearman correlation coefficient is shown. The P-value ($p<0.001$) on the plot was FDR adjusted for 26 ovary size comparisons.

Figure S10. Correlation between eigengene values and ovary size index measurements from module 3, containing 1,256 genes detected with WGCNA of brain transcriptome data. Spearman correlation coefficient is shown. The P-value ($p = 0.028$) on the plot was FDR adjusted for 26 ovary size comparisons.

Appendix 1. Evaluating robustness of differential expression results.

Figure A1. Mutlidimensional scaling plot showing relationships among brain samples with collection year color-coded. Data for plot consists of all transcripts with at least 1 count per million across at least 5 samples, per the analysis detailed in the main text.

Figure A2. Hierarchical clustering of samples based on expression of the 99 pairwise DEGs identified among behaviors in the brain when the three year-1 samples were removed. Color key shows the $\log_2$ scaled expression relative to the mean value for each gene. Sample clustering was based on using Euclidean distance with the Ward.D2 clustering method.

Figure A3. Mutlidimensional scaling plot showing relationships among samples with collection year color-coded. Data for plot consists of all transcripts with at least 1 count per million across at least 5 samples, per the analysis detailed in the main text.

Figure A4. Hierarchical clustering of samples based on expression of the 372 pairwise DEGs identified among behaviors in the ovaries when the three year-1 samples were removed. Color key shows the $\log_2$ scaled expression relative to the mean value for each gene. Sample clustering was based on using Euclidean distance with the Ward.D2 clustering method.

Figure A5. Mutlidimensional scaling plot showing relationships among brain samples with behavioral phase color coded. Data for the plot consists of all transcripts with at least 1 count per million across at least 5 samples, per the analysis detailed in the main text.

Figure A6. Mutlidimensional scaling plot showing relationships among ovary samples with behavioral phase color coded. Data for the plot consists of all transcripts with at least 1 count per million across at least 5 samples, per the analysis detailed in the main text.

Figure A7 Hierarchical clustering of independently collected samples from Saleh and Ramirez 2019, based on the expression of the 412 pairwise DEGs identified among behaviors in the ovaries in this study. Color key shows the $\log_2$ scaled expression relative to the mean value for each gene. Sample clustering was based on using Euclidean distance with the Ward.D2 clustering method. Behaviors are color-coded and a letter below each column also corresponds to this information, where d = dominant, s = subordinate, f = foundress, and g = natural guard.

Figure A8. Hierarchical clustering of independently collected samples from Saleh and Ramirez 2019, based on the expression of the 132 pairwise DEGs identified among behaviors in the brains in this study. Color key shows the $\log_2$ scaled expression relative to the mean value for each gene. Sample clustering was based on using Euclidean distance with the Ward.D2 clustering method. Behaviors are color-coded and a letter below each column also corresponds to this information, where d = dominant, s = subordinate, f = foundress, and g = natural guard.

Table S1. Data summary for the 14 nests manipulated in the subordinate isolation experiment.

Table S2. Random forest confusion (classification) matrix for CHC data.

Table S3. Number of DEGs in each pairwise behavioral comparison in the brain and ovaries. Genes upregulated in the first behavioral category in the comparison relative to the second behavioral category are shown outside of the parentheses, with downregulated genes shown inside the parentheses.