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Sociality emerges from solitary behaviours and reproductive plasticity in the orchid bee *Euglossa dilemma*

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The evolution of eusociality and sterile worker castes represents a major transition in the history of life. Despite this, little is known about the mechanisms involved in the initial transition from solitary to social behaviour. It has been hypothesized that plasticity from ancestral solitary life cycles was coopted to create queen and worker castes in insect societies. Here, we tested this hypothesis by examining gene expression involved in the transition from solitary to social behaviour in the orchid bee *Euglossa dilemma*. To this end, we conducted observations that allowed us to classify bees into four distinct categories of solitary and social behaviour. Then, by sequencing brain and ovary transcriptomes from these behavioural phases, we identified gene expression changes overlapping with socially associated genes across multiple eusocial lineages. We find that genes involved in solitary *E. dilemma* ovarian plasticity overlap extensively with genes showing differential expression between fertile and sterile workers—or between queens and workers in other eusocial bees. We also find evidence that sociality in *E. dilemma* reflects gene expression patterns involved in solitary foraging and non-foraging nest care behaviours. Our results provide strong support for the hypothesis that eusociality emerges from plasticity found across solitary life cycles.

1. Introduction

Eusocial insects display striking adaptations of cooperative behaviour that have allowed them to proliferate across terrestrial ecosystems. Theoretical and empirical studies have provided insight into the ultimate causes that govern the evolution of eusocial behaviour. However, the mechanisms that enabled the initial transition from solitary to social behaviour remain poorly understood [1–3]. This is, in part, because molecular studies of insect sociality have been conducted primarily on advanced eusocial species (such as honeybees and ants), leaving the basis of simpler social behaviour relatively understudied [4]. Consequently, investigation of the mechanisms enabling social behaviour in species that form small, flexible social groups may be especially informative, providing unique opportunities to test hypotheses about the evolution of social behaviour [5].

Several models have been proposed to explain the initial transition to eusocial behaviour from a solitary ancestral lineage. Division of labour in insect societies, a hallmark of eusocial behaviour, is often accompanied by dramatic differences in reproductive physiology between queens and workers, and therefore, it has been suggested that reproductive physiology and social behaviour were inherently linked during the evolution of eusociality. For example, the ovarian-ground plan hypothesis [6] explicitly predicts that behaviour and reproductive physiology should be correlated and that the gene networks underlying these correlated cycles of behaviour and physiology in solitary species are coopted to produce the distinct phenotypes observed in queen and worker castes. An alternative model, the maternal heterochrony hypothesis, posits that queen and worker castes evolved via changes in the timing

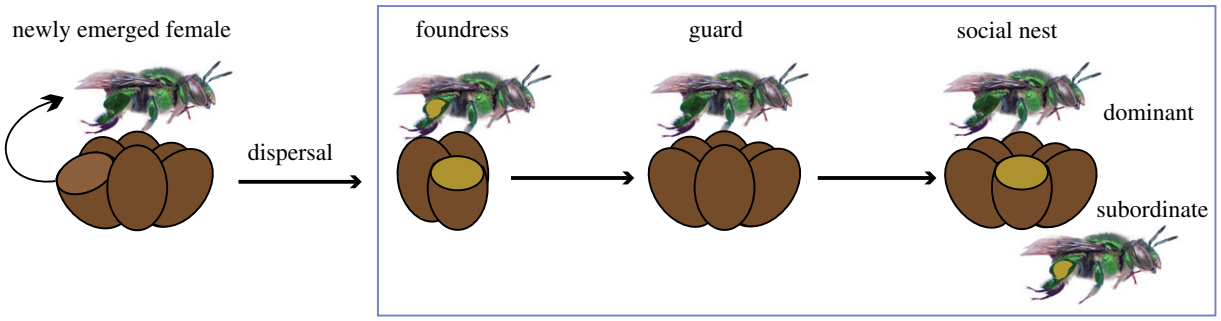

Figure 1. The life cycle of *E. dilemma*. Foraging individuals are shown with yellow pollen in their corbicula (tibial pollen basket). Arrows represent sequential stages of behaviour. The blue box shows which behaviours were sampled in this study. *Euglossa* image adapted from [14]. (Online version in colour.)

of gene expression involved in maternal care, such that individuals performing maternal care, even to non-offspring, should show similar patterns of gene expression, due to shared behavioural tasks instead of differences in reproductive cycles [7]. These two models share the basic prediction that variation across the solitary life cycle serves as the raw material from which to build social behaviours. In this study, drawing on these ideas, we investigate the behaviour and reproductive physiology of an orchid bee (*Euglossa dilemma*), a close relative of the highly eusocial honeybees, that exhibits both solitary and social life phases.

Orchid bees are a diverse group of neotropical pollinators in the corbiculate bee clade. While other corbiculate bees, including honeybees, bumblebees, and stingless bees exhibit obligate eusociality, orchid bees have long been considered solitary [8,9]. However, a growing number of studies indicate that orchid bee species exhibit a range of social behavioural traits, including solitary, communal, and primitively eusocial behaviour [10]. This variation, found within the otherwise highly social corbiculate bees, makes orchid bees particularly interesting for examining transitions from solitary to social behaviour. Our study species, *E. dilemma*, is a primitively eusocial mass-provisioning species with a comparable life history and social system to several other *Euglossa* species that have been studied [11–13].

A single female *E. dilemma* bee initiates a nest by foraging for resin and pollen that she uses to construct and provision approximately 4–11 brood cells, where each brood cell is completed sequentially one at a time (figure 1). After completing the first batch of brood cells, the foundress bee stops regular foraging, ceases reproduction, and enters a behavioural phase in which she remains in the nest, with the entrance sealed with resin. This behaviour, which we refer to as 'guard' behaviour, presumably protects the brood from predators and parasites and represents a protracted stretch of non-reproductive maternal care that lasts for up to two months until offspring emerge. At least one female offspring typically remains in the nest and joins the mother becoming subordinate to her, and the original foundress becomes reproductively dominant.

Both dominant and subordinate females are fertile, mated, and lay eggs; however, dominant females engage in oophagy, replacing the eggs of subordinates with their own, achieving complete or nearly complete reproductive dominance. As in other *Euglossa* species, if the mother has died, two sisters can form a dominant/subordinate relationship [15]. In social nests, *E. dilemma* subordinates forage, while the dominant female remains in the nest.

We address three questions in this study to elucidate possible mechanisms underlying evolutionary transitions from solitary behaviour into social behaviour. First, what changes in gene expression, reproductive physiology, and behaviour underlie transitions (foundress to guard to dominant) across the *E. dilemma* life cycle? Second, are social behaviours (dominant and subordinate) controlled by the same patterns of gene expression involved in the foraging/non-foraging transition seen in the solitary life phases? Finally, we ask whether genes involved in the discrete behaviours found in *E. dilemma* overlap with those involved in similar behaviours in other lineages of social bees, testing the use of a shared genetic toolkit to enable social behaviour.

2. Methods

(a) Sample collection

Euglossa dilemma females were trap-nested around Ft Lauderdale, FL, in small wooden boxes placed on the eaves of buildings. *Euglossa dilemma* is non-native in Florida, having been accidently introduced around 2003 [16]. Following colonization of the nest-boxes, transparent red Plexiglass lids were placed on the boxes to facilitate observation and video recording. Nests were monitored with a combination of video and survey observations. All bees used in the study were marked with small plastic, numbered discs glued to the top of the thorax for individual identification. All nests were naturally colonized by the bees, with observations and video recordings done in the field. Nest observations occurred for a minimum of two weeks to confirm consistent behaviour and nest membership. We classified individuals into four behavioural groups, according to the life cycle shown in figure 1. Foundresses (F) were defined as individuals building new nests in empty boxes without the presence of other bees for the observation period. Guard bees (G) were defined by a general lack of foraging behaviour and a resin-sealed nest entrance during normal foraging hours (sunrise to sunset). In addition, guard bees had only completed and sealed brood cells and were never observed undergoing active provisioning. Dominant (D) and subordinate (S) individuals were primarily assigned based on differences in foraging behaviour and oophagy in nests with multiple individuals. Subordinate individuals were not observed performing oophagy and dominant individuals were rarely observed foraging and never for pollen. Nests typically contain one dominant and one subordinate, though occasionally larger nests with two or three subordinates (but always one dominant) are seen. Social nests in our study consisted of two-bee nests, with the inclusion of one three-bee nest. However, we only included the dominant bee and one subordinate bee from this nest for gene expression analysis described

below. We refer to foundress and guard phases as 'solitary' phases for the purpose of comparison; however, we note that these behavioural phases more accurately represent subsocial behaviour in the sense that the mother is providing extensive parental care (even though she is the only individual bee in the nest at the time).

In order to confirm our general observations and better understand dominance interactions across a broader sample of social nests, we followed the oviposition process in 56 brood cells across 20 social nests using continuous video recordings.

In addition, we conducted detailed observations of within-nest behaviour using continuous video recording in which we followed five social nests and five nests in the guard phase for a period of 3 days per nest (30 days of observation total, 15 for each nest type, from 5.00 to 17.00 each day). For each nest, we documented the patterns of behaviour as well as the number of foraging trips per individual bee and the type of resource collected in each foraging trip. For each nest, we subsampled all the events occurring within 4 h intervals, from 6.00 until 10.00, during one of our observation days chosen at random (20 h total from social nests and 20 h total from guard nests). We found that this subsample of events within this shorter time period is representative of our longer (3-day) observations.

Once sufficient behavioural data were acquired from each nest to confidently assign behaviour, we collected all bees from each nest to perform RNA sequencing. Bees were sampled during the same period of afternoon foraging behaviour (12.00–16.00) on days with qualitatively similar weather. The bees typically forage from sunrise to sunset. To sample the bees, whole nest-boxes were briefly placed on dry ice to incapacitate the bees, which then had their wings removed for a separate phenotypic analysis. Following this, they were immediately frozen in liquid nitrogen. This entire process was completed within minutes of nest-box removal from the field, with minimal disruption to the bees. Flash frozen samples were kept for up to one week in a liquid nitrogen dry shipper before moving to a −80°C freezer for storage until further processing.

(b) Body size and ovary size

We measured body size and ovary size for bees used in our gene expression analysis, in addition to supplementary individuals collected on the same sampling trips (total $n = 63$; $F = 14$, $G = 15$, $S = 18$, $D = 16$). Sample information is found in electronic supplementary material, table S2 in file S2). We used the intertegular distance as a proxy for body size. For ovary size, we created an ovary size index, similar to [17], calculated by taking the length of the longest basal oocyte and dividing it by body size. To test for differences in body size and ovary index among behaviours, we performed ANOVAs in R using Tukey's honestly significant difference (HSD) to assign statistical groupings [18]. We used Levene's test to confirm homogeneity of variances.

(c) Dissections and RNA extractions

We dissected brains (total $n = 32$, $F = 8$, $G = 8$, $S = 7$, $D = 9$) on dry ice and by removing the cuticle around the frons and post-occiput. Next, heads were placed in RNAlater ICE for at least 16 h at −20°C. After RNAlater ICE thaw, brains were dissected on dry ice and immediately transferred to Trizol solution for RNA extraction. We included the entire brain, though we removed the retinas from the optic lobe as well as the ocelli during dissection. Ovaries (total $n = 28$, $F = 7$, $G = 7$, $S = 7$, $D = 7$) were dissected by first removing sections of abdominal cuticle from frozen samples on dry ice. Like the brain samples, abdomens were then thawed in RNAlater ICE for at least 16 h at −20°C before being dissected on dry ice. Ovaries were photographed and placed immediately in Trizol solution. We followed a standard Trizol extraction procedure for RNA isolation, adding glycogen to the brain samples but not the ovary samples to increase yield. RNA was cleaned using an Invitrogen Turbo DNA-free kit and RNA was quantified using a Qubit. Next, we checked RNA quality using a Bioanalyzer (Agilent) before proceeding with library construction on samples which showed high-quality RNA.

(d) Library construction and sequencing

We built sequencing libraries from brain and ovary RNA using the NEBNext Ultra RNA Library Prep Kit along with the Poly-A Magnetic isolation module and dual index (i5 and i7) NEBNext adapters. Libraries were pooled and sequenced at the Vincent J. Coates Genomic Sequencing Laboratory at UC Berkeley using an Illumina HiSeq 4000, with 100 bp paired-end reads. We sequenced a pilot batch of four brain libraries first (two dominant and two subordinate bees) before adjusting sequencing depth and proceeding with the rest of the brain and ovary libraries, which were sequenced as one multiplexed pool. Consequently, the four initial brain samples have higher coverage than the rest but were otherwise treated the same way and collected on the same trips. Overall, libraries from the second batch had an average of 10.7 million sequenced reads and the four pilot libraries had an average of 52.4 million sequenced reads. We account for batch effects and library size differences in differential expression analysis as described in the section below. Following sequencing, we assessed the quality of reads by running FastQC (v. 0.11.7, [19]) which showed uniformly high-quality reads with little drop off towards the end of the reads. The raw sequence data can be found at National Center for Biotechnology Information (NCBI) under the bioproject accession PRJNA523381.

(e) Differential expression

We used Kallisto [20] for producing transcript counts based on genes from the published *E. dilemma* genome [21]. Following transcript quantification, we filtered genes in the ovary and brain dataset separately, so that each of the two datasets consisted of genes that had at least one count per million (CPM) in at least eight of the libraries. For the brain data, this included 10 604 genes and 9960 genes for the ovary data filtered down from the total gene set of 16 127 genes. We used edgeR-robust [22] with default settings and the glmLRT function with false discovery rate (FDR) <0.05 to identify differentially expressed genes (DEGs). We used trimmed mean of M-values (TMM) normalization to account for differences in total amount of reads among libraries. Following the identification of putative technical batches in the brain data during sample clustering, we used SVAseq [23] to identify surrogate variables (SVs) representing these batches for inclusion in the edgeR model. Using the 'be' method in SVAseq, we identified two SVs for the brain that were included in the edgeR model. The first SV corresponds to our pilot samples and the second SV is significantly correlated with differences due to library preparation and collection trip. No putative batches were identified during sample clustering with ovary data and thus, no additional covariates were included with the ovary data in the edgeR model. Further information on methodology can be found in electronic supplementary material, file S1. Further, edgeR output with and without the SV approach is included in electronic supplementary material, file S4. Hierarchical clustering was conducted using Euclidean distance and Ward.D2 clustering using gplots v. 3.0.1 [24].

(f) Cross-study comparisons

To test whether transitions from solitary to social behaviour are correlated with gene expression differences in known toolkit genes, we focused on a list from Okada *et al.* [25], which assembled a set of such genes from across social insects. In

addition, we included genes known to be associated with hormone sensitivity, insect behavioural plasticity, or caste determination (electronic supplementary material, table S5, [26–31]). We also compared the DEG lists identified in our study to other published data obtained for *Megalopta genalis* [32] and *Apis mellifera* [33–35]. These comparisons represent independent origins of eusociality and correspond to different degrees of eusocial complexity. *Apis mellifera* has a complex social system comprising thousands of individuals but, phylogenetically, it is more closely related to *E. dilemma* and may share traits associated with the origin and evolution of eusocial behaviour [9]. By contrast, *M. genalis* has a similar life history to *E. dilemma* even though it represents an independent origin of eusociality [36]. For comparisons to *M. genalis*, we identified orthologous genes between the published *E. dilemma* peptide set and the predicted TransDecoder peptides from Jones *et al.* [32] using a reciprocal best hit (RBH) blastp search (*e*-value $<1\times10^{-5}$). For *A. mellifera* comparisons, we converted our *E. dilemma* gene lists into honeybee identifiers (OGSv 3.2; [37]) using a conversion list generated in Brand *et al.* [21]. We used the functional annotation tool on DAVID 6.8 to perform gene ontology (GO) term analysis with the Benjamini–Hochberg-corrected *p*-values using honeybee OGSv 3.2 identifiers.

3. Results

(a) Reproductive physiology, behaviour, and differential gene expression across the life cycle

To identify possible morphological and physiological differences among behavioural groups, we first measured body size and ovary size. Body size measurements showed no significant differences across behavioural groups ($F_{3,59} = 1.84$, $p = 0.15$; electronic supplementary material, figure S1). The ovary size, however, was significantly different among behavioural groups, with dominant individuals exhibiting the largest ovary size relative to the other behavioural groups. In addition, bees in the guard behavioural group exhibited significantly reduced ovaries relative to all the other behavioural groups ($F_{3,59} = 40.71$, $p < 0.01$, figure 2).

We observed oviposition in 56 brood cells across 20 social nests to better understand reproductive interactions in social nests. In 100% of these observations, we found that, following cell provisioning, the subordinate bee laid an egg and immediately closed the brood cell. Next, the dominant bee reopened the brood cell, ingested the subordinate's egg and replaced it with her own egg. We did not observe the subordinate bee engaging in oophagy or replacement of the dominant bee's eggs. In two cases, the dominant bee replaced her own egg after first replacing the subordinate's egg. The average time from the subordinate's completed oviposition until completed replacement by the dominant was approximately 4 h (average = 03:51:02, s.d. = 04:51:40, range = 00:34:12–34:38:00; electronic supplementary material, table S6). We observed that the subordinate bee was present in the nest during egg replacement by the dominant bee in 91% of cases (51/56). However, we never observed aggression among nest-mates in this process.

We also observed the foraging and within-nest behaviour of guards, dominants, and subordinates to better characterize behaviours outside of reproductive interactions. We observed that guard bees spent most of the day in the nest, with a resin seal over the entrance. They did, however, leave one to two times per day on short trips (average duration: 0:13:32,

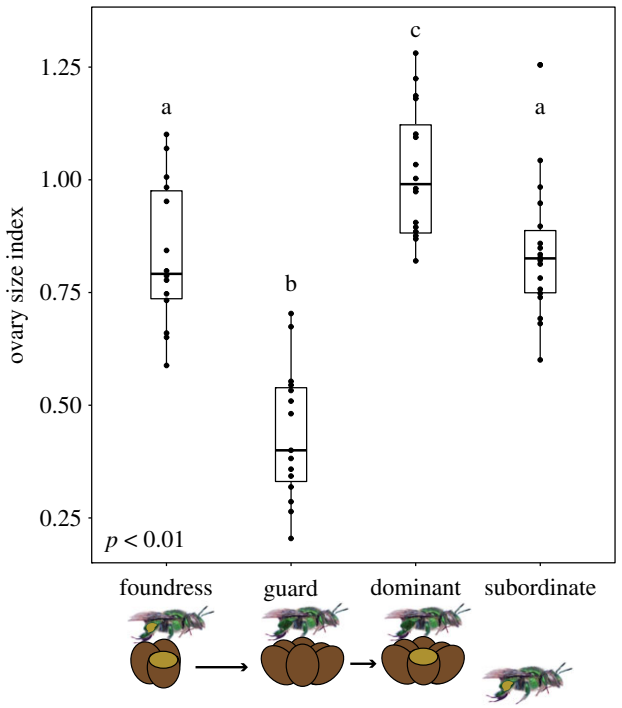

Figure 2. Differences in the ovary size index across behavioural groups. The ovary size index was calculated as the length of the longest basal oocyte divided by intertegular distance. $N = 63$ ($F = 14$, $G = 15$, $D = 16$, $S = 18$). Arrows indicate sequential behaviours. The middle bar of the boxplots represents the mean value. Letters (a–c) denote statistically significant groupings assigned by Tukey's HSD test. (Online version in colour.)

s.d. = 0:05:58, range = 0:05:32–0:34:01, $n = 22$), typically returning without resources (pollen/resin), though one individual was observed returning twice with resin which was immediately applied to the brood cells. These foraging trips likely correspond to nectar feeding. The nest entrance was usually resealed with resin shortly after return to the nest. Inside the nest, guard bees spent most of the time primarily sitting on or facing the door or walking over the brood cells, chewing the resin on the brood cells, and antennating them repeatedly. Individuals showed frequent activity, going back and forth between standing directly in front of the door and chewing on and antennating the brood cells; we observed an average of 40 trips per individual between the brood cells and the door during our 4 h observation periods (s.d. = 24, range = 25–83, $n = 5$). This behaviour appeared to continue throughout the day. Though guards frequently chewed brood cell resin as well as resin on the door and walls of the nest, they did not build new brood cells. We also observed three cases where intruding conspecific females entered or attempted to enter the nest and were repelled by guard bees, which were highly aggressive towards these intruders. In two cases, intruders successfully entered the nest and the guard bees grappled with, bit, and chased the intruders around the nest until they exited. The third intruder began chewing through the resin-sealed door but was repelled when the guard bee pressed her head against the door before the intruder could enter.

We found similar general patterns of behaviour in dominant bees, which also spent most of the day in the nest, save for one to two short non-resource trips a day (average duration: 0:25:34, s.d. = 0:19:32, range = 0:9:59–1:14:47, $n = 15$), though we also observed dominants bringing resin back to

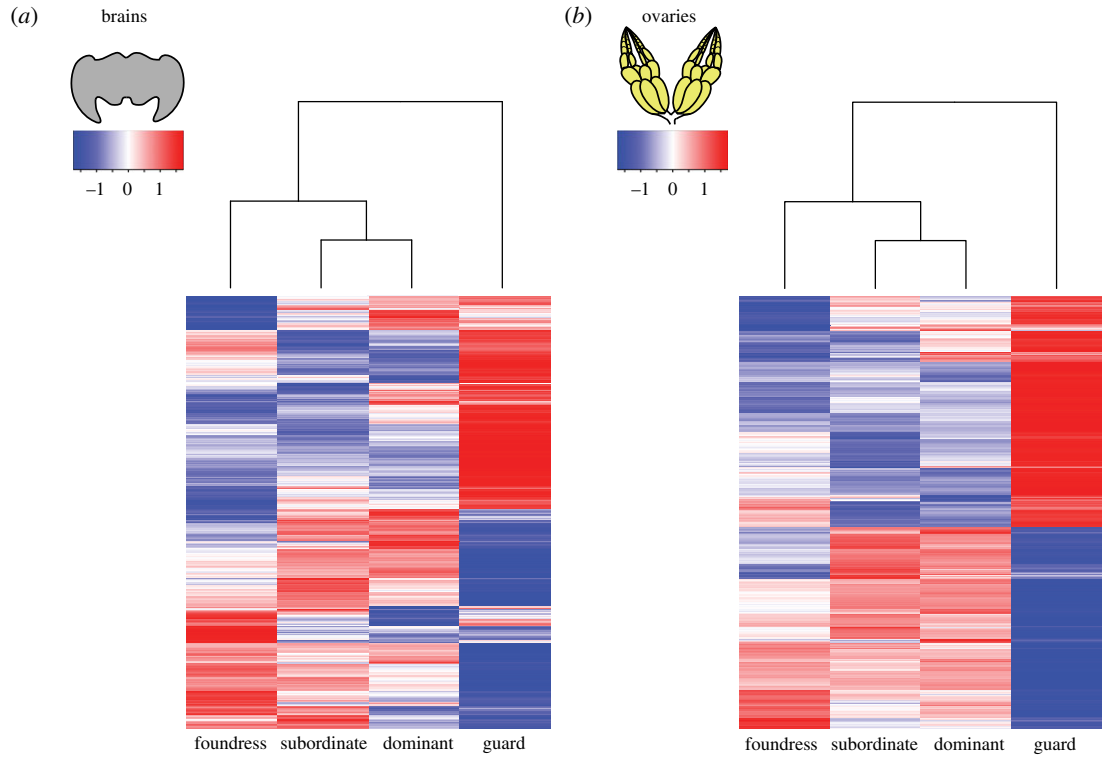

Figure 3. Gene expression patterns across groups for DEGs. Key shows $\log_2$ scaled expression level relative to the mean value for each gene. (*a*) Brain gene expression patterns for 5446 DEGs across all groups. (*b*) Ovary gene expression patterns for 3504 DEGs across all groups. Hierarchical clustering is based on Euclidean distance using the Ward.D2 method. (Online version in colour.)

the nest twice. Within the nest, dominant bees spent most of their time moving back and forth between the door and the brood cells, showing behaviour like the guard bees, chewing and antennating the brood cells. Within the 4 h observation windows, dominants moved back and forth between the brood cells and the door an average of 54 times (s.d. = 33, range: 33–112, $n = 5$). Dominants and subordinates appeared to interact little in the nest, though occasional antennation between individuals was seen when subordinates were leaving or returning to the nest, or when subordinates were depositing pollen in a brood cell. We note that dominants often had to move aside to allow subordinates to enter or leave the nest, as their position at the door blocked entry and exit. Subordinates took an average of eight trips from the nest per day, usually returning with pollen or resin (average duration = 0:42:37, s.d. = 0:20:56, range = 0:07:17–2:17:54, $n = 117$). We never observed aggression among nest-mates. We observed one intruding conspecific female attempt to enter a social nest; the dominant bee placed her head and body in the door preventing access by the intruder. Within the nest, subordinates primarily provisioned and constructed new brood cells. Like guard bees, dominant bees were frequently observed chewing or working with resin but did not participate in constructing or provisioning new brood cells.

Our transcriptome analysis revealed that individuals in the guarding phase represent a highly distinct behavioural group, with thousands of genes differentially expressed across the brain and ovaries relative to the other behavioural groups. Further, hierarchical clustering of mean gene expression patterns grouped guard bees separately from the other three behaviours based on expression levels observed in both brain and ovaries (figure 3). Indeed, most of the DEGs are driven by differences associated with guard behaviour (pairwise DEG comparisons, electronic supplementary material, table S1); 78% of all brain DEGs and 96% of all ovary DEGs are significant only in pairwise comparisons to guards. Functional annotation analysis also revealed several significant terms associated with the switch from foundress to guard, including terms associated with DNA replication, metabolic pathways, and homeobox genes (electronic supplementary material, tables S7 and S8).

Differences in gene expression among the three reproductive groups (dominant, foundress, and subordinate) were less extensive, though all comparisons revealed differentially expressed genes (electronic supplementary material, table S1). In social nests, dominants versus subordinates revealed 204 differentially expressed genes in the brain and 10 in the ovaries.

(b) Social gene expression reflects patterns seen across solitary behaviours

To test whether DEGs observed between social individuals reflect similar patterns of expression as found between the foraging/non-foraging behaviours of foundresses and guards, we examined clustering of all samples based on DEGs found between dominants and subordinates. If gene expression involved in dominant and subordinate differences reflects gene expression patterns underlying transitions in the solitary behavioural phases, we expect samples to fall into two clusters: a foraging cluster (foundresses and subordinates) and a non-foraging cluster (dominants and guards). Largely in line with this prediction, hierarchical clustering based on the 204 brain DEGs between dominants and subordinates successfully sorts 30 of the 32 brain samples into either foraging or non-foraging clusters (figure 4*a*). Further, genes upregulated in the brains of dominants relative to

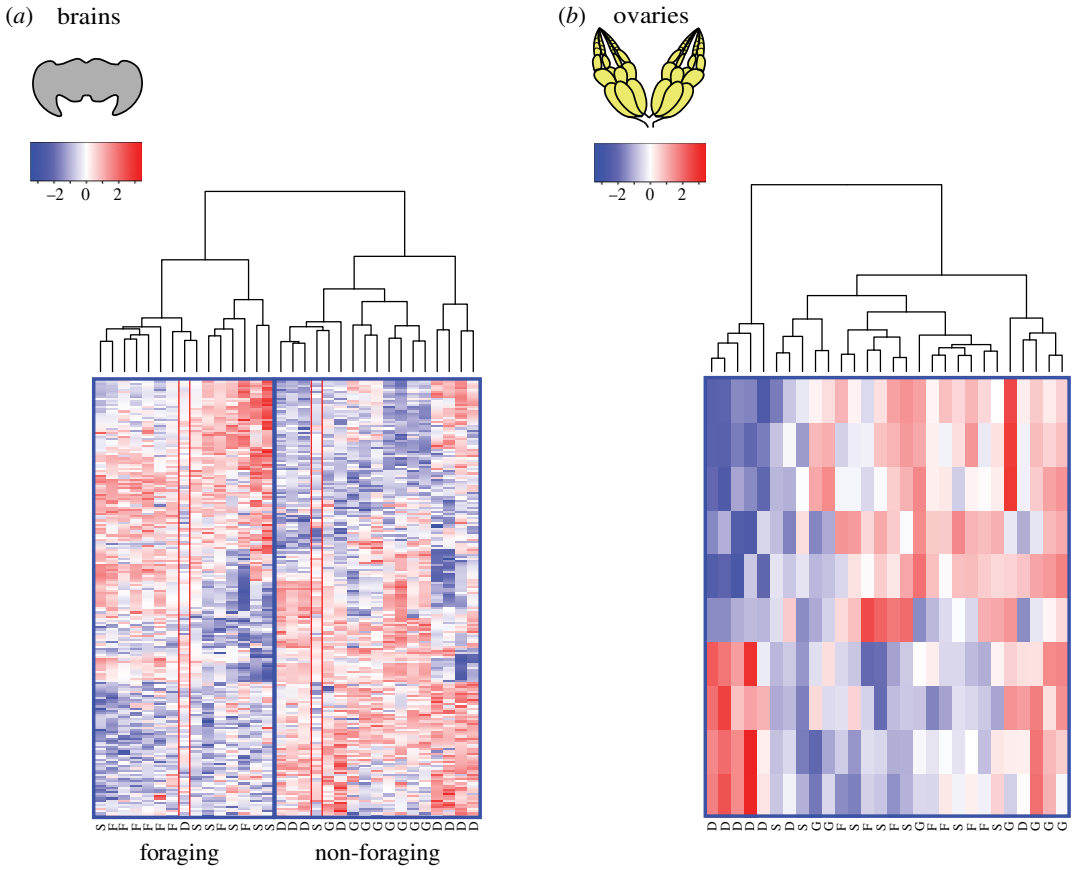

Figure 4. Sample clustering based on social DEGs. Key shows $\log_2$ scaled expression level relative to the mean value for each gene. *D*, *F*, *S*, and *G* labels denote dominant, foundress, subordinate, or guard bees, respectively. (*a*) Heatmap of 204 differentially expressed genes between dominants and subordinates across all brain samples. Thirty of 32 samples sort into predicted foraging or non-foraging clusters. Blue boxes denote behavioural clusters and red highlighting denotes individuals that fall outside of the predicted cluster. (*b*) Heatmap of 10 differentially expressed genes between dominants and foundresses across all ovary samples. Hierarchical clustering was done using Euclidean distance with the Ward.D2 method. (Online version in colour.)

subordinates show a highly significant overlap with genes upregulated in the brains of guards relative to foundresses (hypergeometric test, $p < 0.0001$). Similarly, subordinates show highly significant enrichment for genes upregulated in foundresses relative to guards ($p < 0.0001$). Overall, more than two-thirds of the differentially expressed genes between dominants and subordinates (140/204) show overlap in significance and direction of expression with the foundress/guard comparison. There is also very little overlap outside of the predicted directions, with only 4/204 genes showing a mismatch between behaviours. In total, dominants and subordinates show 60 unique DEGs that are not shared with the foundress and guard comparison, regardless of direction of expression. In contrast with the brains, 10 DEGs in the ovaries do not consistently sort samples by behaviours or into foraging/non-foraging clusters (figure 4*b*).

(c) Toolkit genes and cross-study comparisons

We examined patterns of expression in a selection of 37 genes representing major hormone and signalling pathways known to be associated with caste across a variety of social species (significant DEGs of interest shown in figure 5 along with a full list of toolkit genes in electronic supplementary material, table S5). Overall, we see that the transitions among solitary phases involve genes across many of these pathways known to be associated with insect eusociality. In social *E. dilemma* nests, we see a smaller subset of these genes differentially expressed between dominant and subordinate females and only in the brain (figure 5). However, DEGs between dominants and subordinates are significantly enriched for these toolkit genes ($p < 0.001$, hypergeometric test), while other comparisons do not show significant enrichment (electronic supplementary material, table S5).

We compared DEGs in this study against published studies of social behaviour in bees. Full results are found in the electronic supplemental material, file S3, though we provide an overview here. In *M. genalis*, which exhibits a similar life history to *E. dilemma* but corresponds to an independent origin of eusociality, we find highly significant overlap between DEGs of *E. dilemma* foundress (*F*) and guard (*G*) ovaries and *M. genalis* queen (*Q*) and worker (*W*) abdomens. Comparing *E. dilemma* $F > G$ and *M. genalis* $Q > W$ DEGs, we see striking overlap of 52% of *E. dilemma* DEGs (402/767, $p < 0.0001$). In the other direction, comparing genes of $G > F$ *E. dilemma* and $W > Q$ *M. genalis*, we observe 44% of *E. dilemma* genes overlapping between our DEG lists (364/821, $p < 0.0001$). While brain comparisons revealed fewer overlapping genes, we found a significant overlap between dominant (*D*) versus subordinate (*S*) *E. dilemma* brain DEGs and *Q* versus *W M. genalis* brain DEGs, when considered without respect to direction of expression ($p = 0.038$). Overlapping genes include two neurotransmitter transporters (excitatory amino acid transporter 2 and GABA tranporter 1-A).

We also found significant overlap between genes associated with behaviour and physiology in *E. dilemma* and the honeybee, *A. mellifera*. When comparing the abdomens of honeybee workers with inactivated ovaries (*W*) and egg-laying

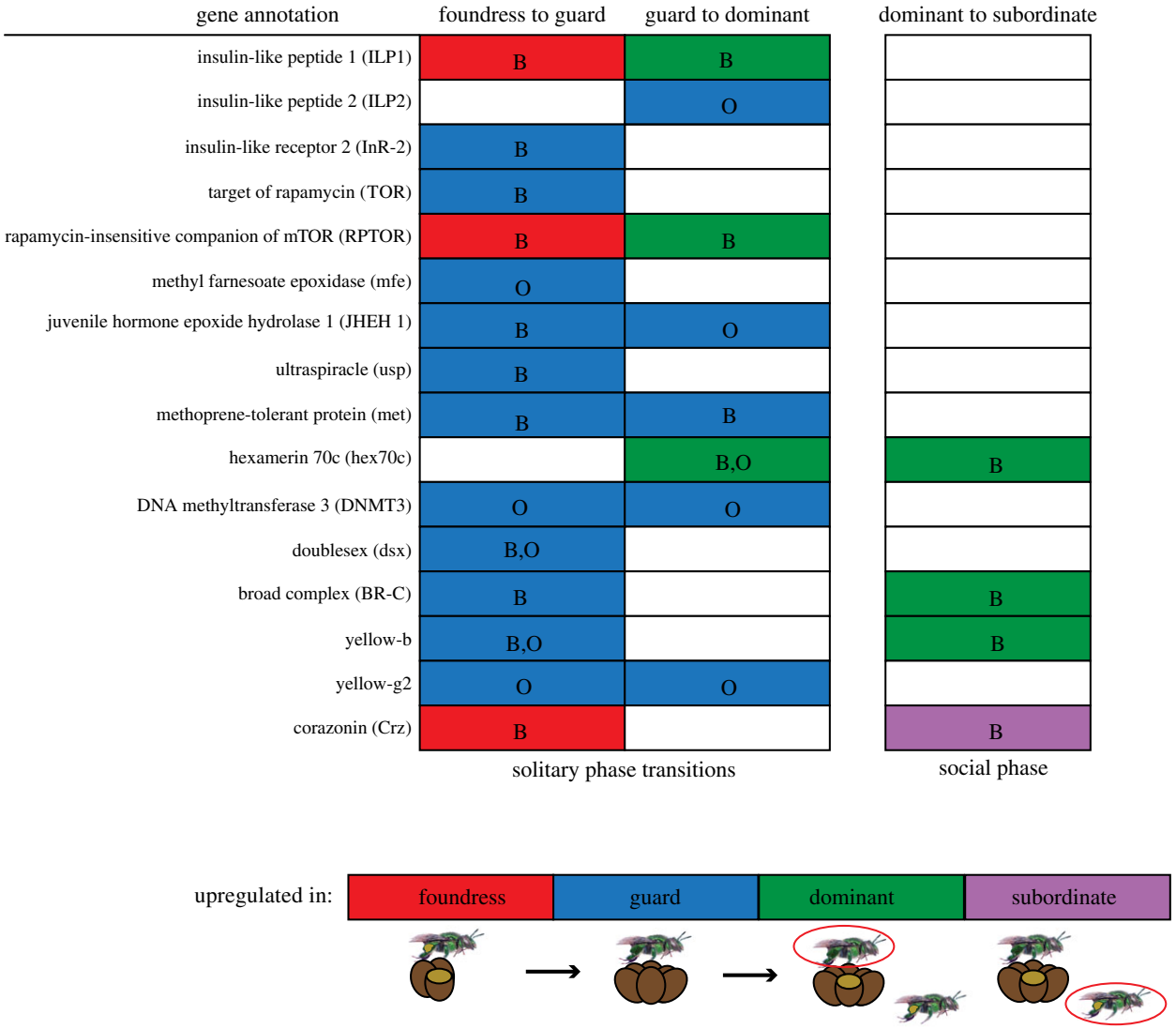

Figure 5. Summary table listing 'toolkit' genes that are differentially expressed across solitary and social phases in the brains and ovaries of *E. dilemma*. Each column represents one pairwise comparison. Highlighted boxes show significant differential expression at that gene in the given comparison (FDR <0.05). 'B' and 'O' labels indicate whether the brain, ovaries, or both tissues exhibit differential expression at the listed gene. In cases where differential expression is in the brain and ovaries, both show the same direction of expression. White blank boxes denote no significant differences in expression. (Online version in colour.)

workers (LW) [35], we see strong overlap with DEGs in *E. dilemma* guard and foundress ovaries, with both $F > G$ compared to $LW > W$ and $G > F$ compared to $W > LW$ comparisons highly significant ($p < 0.0001$), showing hundreds of genes overlapping between the two species (electronic supplemental material, file S3). We do not find significant overlap between our DEG lists and differences between honeybee queen and worker brains [33]. We do note, however, that the overlapping genes identified do include the two neurotransmitter transporters which are also differentially expressed between *E. dilemma* dominants and subordinates and *M. genalis* queens and workers. These are the only shared DEGs we identified among the brains of queens and workers and dominants and subordinates across all three species. We do see significant enrichment in our gene lists for differences between honeybee nurse (N) and forager (Fr) brains [34]. *Euglossa dilemma* $F > G$ brain comparisons show significant overlap with $Fr > N$ honeybees ($p < 0.0001$), though $G > F$ are not significant compared to $N > Fr$ ($p = 0.6$). Dominant and subordinate differences are also significant, though less extensive. $S > D$ brains show significant overlap with $Fr > N$ differences ($p = 0.016$), though the $D > S$ comparison does not show a significant $N > Fr$ overlap ($p = 0.4$). Dominant and subordinate differences also significantly overlap with nurses and forager differences when considered without respect to the direction of expression ($p = 0.0041$).

4. Discussion

In this study, we detail the behavioural, physiological, and transcriptomic basis of solitary and social life phases of the orchid bee *E. dilemma*. Surprisingly, we find that substantial reproductive variation exists across the solitary life phases, with guarding behaviour representing a unique physiological and transcriptomic state across the brain and ovaries. Further, the foundress to guard transition involves differential expression of many genes that have been identified as differentially expressed between social behaviours in other species. DEGs associated with ovarian plasticity across the solitary *E. dilemma* life phases show especially large overlap with DEGs involved in the sterile worker physiology of both *M. genalis* and *A. mellifera*. We note that the large changes

in ovary gene expression we have identified are likely due to high conservation of gene expression patterns underlying the process of oocyte development and ovary activation. However, this suggests that the dramatic reproductive plasticity found in eusocial species may be derived from coopted plasticity found across solitary reproductive cycles [38]. Our results provide strong support for this hypothesis. We also identify genes associated with social behaviour in *E. dilemma* and find support for the hypothesis that *E. dilemma* sociality represents differential expression of a subset of genes, primarily in the brain, underlying solitary patterns of foraging and non-foraging nest care.

(a) Maternal care and the evolution of plasticity

High nest parasitism is thought to exert strong selective pressures and therefore favour maternal care across insects [39]. We hypothesize that the guard phase seen in *E. dilemma* may have evolved as a response to parasitism. There are a host of parasites that target orchid bee nests in their native ranges, including parasitic bees and flies [40–42]. Further, orchid bee species have been documented stealing resin from and usurping conspecifics nests [15,43]. Experiments in other bees and insects with similar guarding behaviour show elevated offspring mortality if the mother is removed during guarding [44,45]. Our observations show that the mother has substantial interaction with the closed brood cells and shows aggressive behaviour towards intruding conspecific females, which suggest that the presence of the mother in the nest could play a role in offspring survival. Recent work on another mass-provisioning bee (*Ceratina calcarata*), which remains with its developing brood, showed that removal of the mother before offspring emergence resulted in substantial changes in offspring gene expression, methylation patterns, and behaviour [46]. Thus, it is possible that selective pressures favouring the mother to remain with the brood could generate behavioural and physiological plasticity that could later be coopted for social behaviour. While much of the differential expression we find between guards and other behaviours is likely attributed to the cessation of reproduction, we also found substantial overlap of *E. dilemma* brain DEGs with DEGs detected between honeybee nurse and forager brains, suggesting a signal of behavioural differentiation that is separate from reproductive status, as honeybee nurses and foragers both have inactivated ovaries.

(b) Behavioural transitions, hormones, and social behaviour in *Euglossa dilemma*

Social behaviour in *E. dilemma* may, in part, be accomplished through the decoupling of reproductive pathways, hormones, and behaviour. The transition from foundress to guard to dominant, for example, seems to show changes in the expression of several juvenile hormone (JH) and ecdysteroid-sensitive genes (jheh, mfe, usp, met), which are differentially expressed between foundresses and guards but ultimately not between dominants and subordinates, which are both reproductive (figure 5; electronic supplementary material, table S5). Further experimental manipulation of these pathways is needed to better understand how hormones are influencing behaviour and physiology in *E. dilemma*. We note that the expression of several of these and other identified 'toolkit' genes are significantly correlated with ovary size and that the nature of these correlations sometimes change between the brain and the ovary datasets, suggesting tissue specificity in response to some of these pathways (electronic supplementary material, table S2 and figure S2). Finally, we do find that dominant *E. dilemma* bees show significantly larger ovary size than subordinate bees (figure 2), though there are few ovary DEGs between them. Further investigation is needed to determine whether these slight differences have important consequences for social behaviour.

Ultimately, our analysis suggests that the evolution of social behaviour in *E. dilemma* was facilitated by the cooption of the brain, but not ovary gene expression patterns from its solitary life cycle for involvement in social behaviour. This scenario is consistent with the maternal heterochrony hypothesis, as brain gene expression differences between social individuals loosely reflect foraging and non-foraging nest care behaviours, as opposed to reproductive differences among individuals (figure 4*a*), though reproduction is clearly correlated with the largest shifts in brain gene expression (figure 3*a*). It is unclear if this pattern of social brain specialization before ovary specialization represents an orchid bee-specific, derived approach to simple sociality or whether these patterns may be more indicative of how early social evolution could have progressed in other species. It also remains unclear whether the pervasive oophagy in social nests represents basic behavioural dominance in the absence of physiological control, or instead if this represents an adaptation to cooperative behaviour, with subordinate oviposition functioning as trophic eggs. Regardless, if the selective environment were to favour a sterile, 'worker-like' subordinate in orchid bee social groups, our results suggest that the reproductive plasticity for such specialization currently exists within the solitary life cycle of *E. dilemma*.

5. Conclusion

Despite the historical classification of orchid bees as solitary, it is now clear that they exhibit more social complexity and variation than previously thought. As the earliest branching clade in the corbiculate bees, orchid bees represent a unique group from which to examine the evolution of sociality. Here, we show that the orchid bee *E. dilemma* shows substantial reproductive and behavioural plasticity undergirded by divergent patterns of gene expression across its life cycle. Further, our results are consistent with the hypothesis that plasticity generated across the solitary life phases may serve as a source of plasticity that can be coopted for sociality. We find evidence in support of this from multiple behaviours within *E. dilemma* and across species when compared with *E. dilemma*. However, outside of a few examples [47], it remains to be seen how widely other taxa show similar transcriptomic changes associated with transitions from reproduction to nest care during solitary life phases. Many bees and other arthropods show egg/nest guarding behaviour like *E. dilemma*. As such, further investigation into the physiological and transcriptomic changes across behaviours in these species may provide further insight into how plasticity is generated and coopted for social behaviour.

Data accessibility. Raw reads used in the project can be found with associated metadata at NCBI under the bioproject accession PRJNA523381. Data for ovary size, body size, behavioural

observations, differential expression, and gene overlap tests are all found in the electronic supplementary material.

Authors' contributions. N.W.S. and S.R.R. designed the experiment. N.W.S. carried out the fieldwork, laboratory work, and data analysis. N.W.S. and S.R.R. wrote the manuscript.

Competing interests. We declare we have no competing interests.

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