RNA-seq analysis of workers' brain reveals that queen and brood affect bumble bee worker reproduction via similar genetic pathways

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Abstract

Worker reproduction in social insects is often regulated by the queen's presence but can be regulated by other colony members, such as the brood and nestmates. Adults and brood may induce the same outcomes in subordinates but may use different mechanisms. Here, we compared gene expression patterns in bumble bee workers (Bombus impatiens) in response to the queen, the brood, both or none. RNA-seq analysis of workers' brain identified 27 differentially expressed genes regulated by the queen and the brood. Expression levels of 8 candidate genes were re-tested using qRT-PCR in worker brain and fat body. Our results show that the brood's effect on gene expression is substantially weaker than the queen, and a greater impact on gene expression was caused by the combined presence of the queen and the brood. All the genes that were explained by the brood presence were also regulated by the queen presence. A significant amount of the variation in gene expression was explained by the queen, that regulated the expression of key regulators of reproduction and brood care across insects, such as neuroparsin and vitellogenin. A comparison of the data with similar datasets in the honeybee and the raider ant revealed that neuroparsin is the only differentially expressed gene shared by all species. These data highlight the need to consider components other than the queen when examining mechanisms regulating worker sterility and provide information on key genes regulating reproduction that are likely to play an important role in the evolution of sociality.

Introduction

One of the most intriguing features defining eusocial insects is the reproductive division of labor among female castes, with reproduction being monopolized by the queen/s whereas workers act as sterile helpers (Michener, 2007). Worker reproduction is often inhibited by the queen's (or the dominant female) presence, however, it can also be regulated by colony members other than the queen, such as the brood and nestmates, and by various chemical and behavioral means (Leonhardt, Menzel, Nehring, & Schmitt, 2016; Ronai, Vergoz, & Oldroyd, 2016; Schultner, Oettler, & Helantera, 2017). For example, in the honey bee *Apis mellifera* , worker reproduction is inhibited by the queen via highly specific queen pheromones (Slessor, Kaminski, King, & Winston, 1990), by pheromones produced by the brood (Le Conte, Arnold, Trouiller, Masson, & Chappe, 1990), and also via policing behavior by workers who attack nestmates with activated ovaries (Visscher, 1996). In the primitively eusocial bees, *Bombus terrestris* and *Bombus impatiens* , worker reproduction is behaviorally and chemically regulated by the queen during the early phase of colony development (Duchateau & Velthuis, 1988; Orlova, Treanore, & Amsalem, 2020), by nestmate workers (Amsalem & Hefetz, 2011; Bloch & Hefetz, 1999; Roseler, Roseler, & Van-Honk, 1981; Van-Honk, Roeseler, Velthuis, & Hogeveen, 1981), and also by the presence of young larvae (Starkey, Brown, & Amsalem, 2019). However, whether reproductive inhibition by different members of the colony is also mediated via different genetic mechanisms in the subordinates is yet to be explored.

Adults (queen and workers) may inhibit subordinate reproduction by exerting aggression (Rittschof & Grozinger, 2021), limiting their access to nutrition by selective trophallaxis (Ratnieks, 1988) or by producing pheromones that advertise their fecundity and relatedness to workers (Amsalem, 2020; Leonhardt et

al., 2016). They may also decrease the reproductive output of competitors post reproduction by oophagy of eggs (Ratnieks, 1988; Ratnieks & Visscher, 1989). The brood, being immobilized, is unable to coerce adults, and instead may exhibit begging behavior that results in adults spending more time in brood care than in reproduction. Alternatively, the brood, like adults, can signal its quality and relatedness to workers, leading females to increase their inclusive fitness by investing in care (Boer & Duchateau, 2006; Le Conte, Mohammedi, & Robinson, 2001).

Findings in several species show that adults and brood inhibit reproduction in subordinates differentially. For example, in *Apis mellifera,* one of the only species where brood pheromones were studied, brood pheromones increase brood care and foraging behavior that reduce worker fecundity, while some of the chemical signals produced by the queen mandibular glands (QMP) operate directly on worker reproduction via dopaminergic pathways (Beggs et al., 2007). In *Bombus impatiens* , the queen's presence inhibits both worker ovary activation and egg laying, while the presence of young larvae reduce egg laying, but not ovary size in workers (Starkey, Brown, et al., 2019). Finally, larvae (but not eggs) delay the time to worker egg laying in subnests separated from the queen in the ant *Novomessor cockerelli* (Ebie, Holldobler, & Liebig, 2015), while the queen inhibits worker reproduction using fertility signals on her cuticle and Dufour's gland (Smith, Hölldobler, & Liebig, 2008, 2012). These studies may suggest that adults and brood, while both are capable of manipulating worker reproduction, operate via different mechanisms to achieve that goal.

Previous studies on the genetic mechanisms regulated in subordinates have mostly focused on individual genes or did not directly compare the impacts of the queen and the brood. These have found both similarities and differences in gene expression patterns induced by the queen and the brood. For example, *krüppel homolog 1* (*kr-h1*), a gene regulated by juvenile hormone (Shpigler et al., 2014) was downregulated in the brain of subordinate workers following exposure to *Bombus terrestris* queen and dominant workers (Shpigler et al., 2010),*Bombus impatiens* queen (Orlova, Starkey, & Amsalem, 2020) and*Apis mellifera* QMP (Grozinger, Sharabash, Whitfield, & Robinson, 2003). Another gene encoding to the major yolk protein invested in worker ovaries, *vitellogenin* , was upregulated in the fat body of honeybee workers in response to QMP (Fischer & Grozinger, 2008), but was downregulated in *Bombus impatiens* workers in the presence of the queen or the brood (Orlova, Starkey, et al., 2020). Furthermore, within *Bombus impatiens* , the impact of the queen on*vitellogenin* expression levels in workers was fivefold higher compared to the impact by the brood (Orlova, Starkey, et al., 2020). In a study comparing transcriptomic differences in response to brood pheromone and QMP in honeybee workers (Alaux et al., 2009), only a few genes overlapped between the two data sets, suggesting the genetic mechanisms targeted by signals produced by them are different.

Bumble bees are an excellent system to examine the genetic mechanisms regulating fecundity since worker reproduction is dynamic, reversible, regulated by multiple colony members and by different means of communication (Amsalem, Grozinger, Padilla, & Hefetz, 2015). Bumble bees are primitively eusocial species that form annual colonies during which the workers maintain their ability to reproduce and lay eggs. Colonies are founded in the spring by a single queen. During the first part of the life cycle, workers are reproductively inhibited by the queen using a combination of behavioral and chemical means, whereas later, during the competition phase, workers form a dominance hierarchy and dominant workers activate their ovaries and compete with other females over male production (Duchateau & Velthuis, 1988). The presence of young brood has been shown to regulate worker egg-laying behavior, with similar effects induced by female and male larvae, either related or unrelated to workers (Starkey et al, 2019). Furthermore, physical contact between the queen and workers (Alaux, Jaisson, & Hefetz, 2004; Padilla, Amsalem, Altman, Hefetz, & Grozinger, 2016), among workers (Amsalem & Hefetz, 2010), and between workers and brood (Starkey, Derstine, & Amsalem, 2019) is crucial for reproductive inhibition to take place. Whether the queen, brood and workers induce similar effects in workers remain unknown. However, several recent findings suggest this is not the case. In a previous study comparing the impacts induced by the queen and the brood, we found that both queen and young larvae are able to inhibit worker egg-laying while pupae have an opposite effect (Amsalem et al., 2017; Padilla et al., 2016; Starkey, Brown, et al., 2019). In addition, only queens were able to inhibit workers' ovary activation, suggesting young larvae and queens trigger different physiological pathways (Padilla et al., 2016; Starkey, Brown, et al., 2019). We further looked at the expression of four genes and found

both synergetic and additive effects of the queen and the brood on worker brain gene expression (Orlova, Starkey, et al., 2020).

Here, we expanded on these studies by conducting a whole transcriptome analysis of workers' brain to examine the genetic mechanisms regulating reproduction by the brood and the queen. We grouped 2 newly emerged workers with an active queen, young brood, both, or none and sampled them after 3 days. We conducted RNA-seq analysis of workers' brain, dissected the worker ovary and further tested candidate genes using qRT-PCR in both the brain and the fat body of workers in a second set of samples. We hypothesized that the queen and brood each affect different genetic mechanisms in accordance with their physiological impact on workers and predict that the combined presence of the queen and the brood will have a larger effect on gene expression compared to any of them alone.

Material and Methods

Bumble bee rearing

Bombus impatiens colonies were obtained from Koppert Biological Systems (Howell, MI, USA) and were maintained in the laboratory in the dark, temperature of 28-30°C, 60% relative humidity and supplied*ad libitum* with 60% sugar solution and fresh pollen collected by honeybees, purchased from Koppert. These colonies were used for collecting egg-laying queens, larvae and newly emerged workers that were used for the treatments listed below.

Two newly emerged workers (< 24 h) were placed in small plastic cages (11 cm diameter x 7 cm height) with unlimited sugar solution and fresh pollen and were assigned to one of the following treatments: (1) an active queen (CQ); (2) young brood (CB); (3) both active queen and young brood (CBQ); and (4) no queen or brood (C). Workers were flash frozen in dry ice by the end of the third day and kept at -80°C until further analysis. At this age, workers are too young to activate their ovaries or lay eggs, ensuring that gene expression patterns are not mediated by the worker reproductive state. Worker ovarian activation was examined in all samples (14-16 pairs per treatment). From these, we used 6 pairs per treatment for whole transcriptome analysis of workers' brain (a total of 24 libraries) and additional 8-10 pairs per treatment for retesting selected candidate genes in the brain and fat body using RT-qPCR.

Cages with brood (CB and CBQ) were supplied with young larvae (first and second instars). Two to three batches of larvae were collected 4-7 days after eggs were laid. Larvae hatch approximately 4-5 days after eggs are laid and the first and second instars last approximately 1-2 days each (Cnaani, Schmid-Hempel, & Schmidt, 2002). Since eggs are laid in batches (6-10 eggs per batch), it is impossible to count the number of offspring without ruining the batch. The exact number of larvae per cage was counted in 18 of the pairs containing brood (out of 30) by the end of the experiment and was confirmed to be on average 8.9±1.1 per cage in the CB treatment (n=10 pairs), and 9.6±2.3 per cage in the CBQ treatment (n=8 pairs). In a previous study, we showed that young larvae are able to reduce worker egg-laying and that the sex of the larvae or their relatedness to workers have no impact on the resulting outcomes (Starkey, Brown, et al., 2019). While workers were too young to lay any eggs, eggs were laid by the queens. In the CQ treatment, eggs laid during the experiment were removed daily (to prevent the presence of brood), while in the CBQ treatment, eggs laid during the experiment were remained in the cage.

Brain, fat body and ovary dissection

Worker head was placed on dry ice under a stereomicroscope. The cuticle and head tissues around the brain were removed using fine-tipped forceps until the brain was exposed. The brain remained frozen during the entire procedure. Brains were placed in 350 µl of lysis buffer and were homogenized using a pellet pestle motor.

The abdomen was kept frozen until dissection and were rapidly opened under stereomicroscope by making a triangle cut in the ventral part using a dissecting scissor. The abdomen content (i.e., gut, ovaries, stinger) was placed in a drop of water for further measurement of ovary size, whereas the abdomen cuticle containing the fat body attached to it was placed in a 500 µl of lysis buffer containing sterile beads. The fat body was

homogenized using a fast prep machine. The brain and fat body samples were kept at -80°C until RNA extraction.

The two ovaries were separated from the drop of water containing the abdomen content. We measured the length of the three largest oocytes (at least one from each ovary). The score was averaged per bee and is presented in mm. This was done in order to ensure ovaries were not differentially activated across the treatments.

RNA extraction

The homogenized brains or fat bodies from each pair were pooled together before extraction. Total RNA was extracted using RNeasy Mini kit (Qiagen) according to manufacturers' instructions with an additional step of DNAse treatment to eliminate DNA contamination. RNA quality and quantity were assessed using NanoDrop One^{C} (Thermo Fisher Scientific).

Whole transcriptome sequencing, cleanup and analysis

Sample preparation and sequencing were performed by the Genome Core Facility at Penn State according to standard RNA sequencing protocol. Twenty-four libraries of brain samples (each contains a pool of two bees) were constructed using Illumina TruSeq Stranded mRNA kit. Each library was uniquely barcoded and pooled with the other libraries. The pools were sequenced on three NextSeq 550 High Output 75 nt single read sequencing runs to control for a bias between runs.

The quality of the raw data was assessed using FastQC (Andrews, 2010) and visualized using MultiQC (Ewels, Magnusson, Lundin, & Käller, 2016). The single reads were filtered for quality (Phred score below 25 were removed) and length (removal of reads smaller than 36 bp). TruSeq3-SE adapters were removed using Trimmomatic-v0.39 (Bolger, Lohse, & Usadel, 2014).

Cleaned reads from each library were mapped to the *Bombus impatiens* genome BIMP_2.2 version, release 102 (Sadd et al., 2015) using STAR-v2.7 aligner (Dobin et al., 2013) implemented in RSEM-v1.3.3 (Li & Dewey, 2011). The expected gene counts resulted from RSEM were exported using *tximport* (Soneson, Love, & Robinson, 2015) to be used in DESEq2-v1.28 (Love, Huber, & Anders, 2014). Analyses were conducted using R version 3.5.2. The count matrix was filtered by keeping rows with count sum greater than one across all samples and the data were rlog transformed (Love et al., 2014) for exploratory analysis and visualization.

We used the SVA-v3.36.0 package (Leek, Johnson, Parker, Jaffe, & Storey, 2012) to estimate batch effect. One surrogate variable was specified to be estimated. This variable was not related to any of the factors controlled in the experiment (*i.e.* , treatment, colony, ovary activation). To control for this unknown variable, we included it in the model together with the colony identity and the treatment using the DESeq function. Differentially expressed genes (DEGs) were identified using contrast between pairs of all treatments and considered significant below a false discovery rate threshold of 5% (padj < 0.05). We performed principal component analysis (PCA) using the DEGs using the function prcomp from stats R package (Becker, Chambers, & Wilks, 1988). The plots were built using ggplot2 package (Villanueva & Chen, 2019). The percentages of the variance in the DEGs explained by the queen or brood presence, colony of identity and the residuals were calculated using variancePartition-v1.22.0 (Hoffman & Schadt, 2016). In this model we subtracted the effect of the surrogate variable and used the residuals to calculate the variance explained by the colony of identity, the queen (in both the QC and QBC treatments) and the brood (in both the BC and QBC treatments). The heatmap was performed using pheatmap-v1.0.12 package (Kolde & Kolde, 2015) and the columns were ordered by treatment. Gene ontology terms annotation of the DEGs was performed using InterProScan (https://www.ebi.ac.uk/interpro/search/sequence/). Comparison of the DEGs in the current study with DEGs in similar studies (Grozinger et al., 2003; Libbrecht, Oxley, & Kronauer, 2018; Ma, Rangel, & Grozinger, 2019) was done by searching for homologous genes against the databases used in these studies using blastp or tblastn.

RT-qPCR analysis

The expression of eight genes that were identified as differentially expressed in the workers' brain transcriptome were retested in a new set of samples in workers' brain and fat body using RT-qPCR.

Design of forward and reverse primers for each gene was performed using Primer-BLAST (Ye et al., 2012) and the specificity was checked against*Bombus impatiens* genome. Primers were designed in-between exons to eliminate DNA amplification in case of contamination. A list of all primers can be found in Table S1.

The conversion of total RNA to cDNA was performed using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems^{TM}) following manufacturer's instructions. Levels of expression were quantified using RT-qPCR on a QuantStudio 5 system (Thermo Fischer Scientific). For each sample, 2 µl of cDNA were placed together with 0.2 µl of each forward and reverse primers (10 µmol), 4.6 µl of water and 5 µl of SYBR GreenER^{TM} qPCR SuperMix (Invitrogen^{TM}). Two housekeeping genes were used as control: *arginine kinase* and *phospholipase A2* (Amsalem et al., 2017; Padilla et al., 2016). Negative controls were included in all plates: a reaction using cDNA that was converted without the reverse transcriptase enzyme and water in place of RNA sample in the mix. PCR product quality and specificity were verified using melt curve analysis. Samples were run in triplicates and were averaged for use in the statistical analysis. Expression levels of candidate genes were normalized to the geometric mean of the two housekeeping genes using the $2^{-[?][?]Ct}$ method.

Statistics

Differences in oocyte size and RT-qPCR gene expression levels were examined using JMP? 15 (SAS Institute Inc., Cary, NC). The effects of treatment on oocyte size and gene expression were examined using standard least square. A linear mixed model was fit with the treatment as fixed term and worker colony identity as random effect using the REML method. Shapiro-Wilk test was used to examine fit for normal distribution. Non-normal data were log transformed (oocyte size, gene expression of *centrosomin* in the brain; *SLCO2A1* and*selenoprotein* in the fat body). Post hoc pairwise comparisons among the four treatments were performed using Tukey test HSD.

Results

According with their young age, all workers in our study had inactivated ovaries (oocyte size smaller than 0.6 mm) and no significant differences were found in the oocyte size of workers from different treatments ($F_3 = 1.36$, $p > 0.005$; Figure S1). This analysis was important to ensure that differences in gene expression do not stem from the reproductive status of workers.

All pairwise comparisons between the four treatments resulted in a total of 27 differentially expressed genes (DEGs) (Figure 1, Table S2). One, 6 and 16 genes were differentially regulated in the brain of workers that were kept with the brood (CB), the queen (CQ) or with both (CBQ) as compared to control (C) that were queenless and broodless, respectively. A heatmap, representing color-coded expression levels (rlog transformed) of these DEGs in all pairwise comparisons (Figure 1), demonstrates overall similarity between the two queenright (CQ, CBQ) and the two queenless groups (C, CB). Gene expression pattern in the queen group (CQ) was often intermediate as compared to the queenless groups (C, CB) and the group containing both queen and brood (QBC).

Principle component analysis with the 27 DEGs demonstrates that the samples are grouped by treatment (Figure 2) and most of the variance between the treatments is explained by the presence of the queen or by the presence of the queen and the brood, but not by the brood alone. A closer look into the split of variance for each of the DEGs (Figure 3a), shows that some of the variance is not explained by factors controlled in the study (residuals) and the explained variance is primarily attributed to the queen presence, followed by a smaller contribution of the colony identity and the brood. Among the genes whose their variance was explained mostly by the queen presence were *neuroparsin-A*(upregulated in workers, 65% of the variance was attributed to the queen), solute carrier organic anion transporter family member 2A1 (SLCO2A1, upregulated in workers, 52%), *vitellogenin*(downregulated in workers, 49%), *microtubule-actin cross-linking factor 1 (Macf1,* upregulated in workers, 46%), Mucin-5AC (upregulated in workers, 44%)

and an uncharacterized gene (LOC100748013, downregulated in workers) that 52% of the variance in its expression was attributed to the queen. Another gene of interest is ecdysteroid-regulated 16 kDa protein (ESR16, 31%, upregulated in workers). An effect attributed to the brood presence was found in three of these genes: SLCO2A1 (16%), LOC100748013 (18%) and mucin-5AC (12%) in the same directionality as the queen. The potential function of these genes is reviewed in the discussion. The differences in expression in a few more genes were mostly attributed to the colony identity that explained up to 44% of the variance in selected genes (Figure 3b). While some genes were regulated by the queen only, all the genes regulated by the brood were also regulated by the queen and often, to a larger degree. This analysis also revealed the genes that were highly significant between the treatments, but their significance was not attributed to any of the factors we controlled for. These genes were of low interest to us. Gene Ontology analysis of the 27 DEGs showed enriched representation of genes associated with transmembrane transport, lipid transport and protein binding (Table S3).

The DEGs in this study were compared to three similar data sets that identified brain gene expression differences in workers exposed to queen and brood presence or pheromones. These included a microarray study from 2003 comparing brain gene expression in *Apis mellifera* workers exposed to QMP, queen presence or none (Grozinger et al., 2003), and two RNA-seq studies in *Apis mellifera* (Ma et al., 2019) and the clonal raider ant *Ooceraea biroi* (Libbrecht et al., 2018). Ma et al. 2019 compared workers' brain exposed to two different brood pheromones (ester brood pheromone and (E)-beta-ocimene), and Libbretch et al. 2018 compared workers' brain in the reproductive and non-reproductive stages of the colony life cycle which are equivalent to the presence and absence of larvae. The comparison with these studies revealed that 17 genes (out of the 27) were also differentially expressed in at least one of the other studies (Table 1). Eight genes identified in the current study were also differentially regulated in *Apis mellifera* workers exposed to both brood and queen pheromones. However, the largest overlap was between our data and *Apis mellifera* workers exposed to QMP which resulted in overlap of 15 DEGs. *neuroparsin-A* was the only gene differentially expressed in all data sets.

To further explore these genes, we selected 8 of them and retested them using RT-qPCR in a new set of samples and within two tissues – the brain and the fat body of workers (Figure 4).

Only two of these genes followed the brain expression pattern observed in the transcriptome analysis: *neuroparsin-A* was significantly upregulated and *vitellogenin* was significantly downregulated in workers' brain in the presence of the queen, either with or without brood as compared to controls (*neuroparsin-A* : $F_{3,27.44} = 9.11$, $p = 0.001$; *vitellogenin* : $F_{3,27.88} = 8.88$, $p < 0.001$). *Vitellogenin* and *mucin-5AC* also differed significantly in the fat body. *Vitellogenin* was downregulated and *mucin-5AC* was upregulated in the presence of the queen but not in the presence of the brood (*vitellogenin* : $F_{3,27.41} = 25.06$,$p < 0.001$; *mucin-5AC:* $F_{3,26.74} = 4.02$, $p = 0.017$) (Figure 4; data obtained in the RNAseq analysis for the same genes are provided in Figure S2).

Discussion

In the current study, we examined whether worker reproduction is regulated by the brood and the queen through similar or distinct genetic pathways. To do that, we placed newly emerged workers together with the brood, queen, both or none for three days. The young age of workers guaranteed that the ovaries remained undeveloped by the end of the experiment as confirmed in Figure S1. Thus, changes found in gene expression were not the consequence of ovary activation. Similar studies in social insect species that compared queenright and queenless workers that also differ in their reproductive status often find large number of differentially expressed genes (Harrison, Hammond, & Mallon, 2014; Holman, Helanterä, Trontti, & Mikheyev, 2019). The limited number of genes identified in the current study (*i.e.* , only 16 genes differed between queenright and queenless workers and 27 DEGs in total) suggests that the impact of the queen is smaller than assumed and is likely to target a small group of genes that lead to substantial physiological and molecular differences in workers down the road. That being said, RNA-seq studies of insect brain often yield a low number of DEGs. In *Apis mellifera* workers, only 58 genes were differentially expressed in response to the ester brood pheromone (Ma et al., 2019) and in a study that examined brain transcriptome of reproductive and non-reproductive

workers of the paper wasp*Polistes canadensis* and the dinosaur ant *Dinoponera quadriceps* (Patalano et al., 2015), the authors have identified 67 and 147 DEGs, respectively. The limited differences in these studies were found despite additional differences between the treatment groups (e.g., the females differed in age, specialized in different tasks, or had developed their ovaries). Thus, the small number of genes identified in the current study is well within the normal range.

Our data showed that most of the impact on workers' brain gene expression is attributed to the queen, while the impact of the brood is weaker, and all the genes that were regulated by the brood were also regulated by the queen, suggesting the queen and the brood regulate worker reproduction via similar genetic pathways. This is in line with the physiological impacts queen and brood have on worker reproduction in*Bombus impatiens* , where the queen inhibit ovary activation and suppress worker egg laying, but the presence of young larvae can only reduce egg laying in workers (Orlova, Starkey, et al., 2020; Starkey, Brown, et al., 2019). Additionally, the combined impact of the brood and the queen was larger than each one of them separately, in line with previous study showing that the impact of the queen and the brood on worker ovary activation and egg laying is larger than either the queen or the brood alone (Orlova, Starkey, et al., 2020). This may indicate that workers refrain from reproduction only after they gathered information from multiple sources and the impact of the queen is unlikely to be manipulative (Amsalem, 2020).

Our study further identified key genes that are likely to play an important role in the regulation of worker reproduction. As evidence by the similarity in brain gene expression pattern in different species, these genes are not specific to *Bombus impatiens* or even to bumble bees, and while their functional role is yet to be explored, they are likely to play an important role in the evolution of social behavior.

Among these, a few genes stand out. *Neuroparsin-A* is part of a large group of small proteins discovered in the pars intercerebralis-corpora cardiaca complex and involved in the hormonal regulation of insect reproduction. These are commonly termed as 'parsins' and include also insulin-related peptides, ovary maturating parsins and pacifastins (Badisco et al., 2007). In solitary insects,*neuroparsin* have been shown to have an anti-gonadotropin effect (*Schistocerca gregaria*) (Badisco et al. 2007) and to inhibit vitellogenesis and juvenile hormone levels (*Locusta migratoria*) (Girardie, Boureme, Couillaud, Tamarelle, & Girardie, 1987). In social species, it was further shown to regulate reproduction and brood care. In the queenless ant species, *Ooceraea biroi,* females alternate between brood care/sterility and reproduction according to the presence of larvae in the colony. The presence of larvae was found to increase*neuroparsin* and decrease *vitellogenin* expression in workers (Libbrecht et al. 2018). Similarly, when workers compete to replace the queen in the ant *Harpegnathos saltator* , the losers exhibit high levels of *neuroparsin* and low levels of*vitellogenin* in their brain compared to the workers that will become the new queens (Opachaloemphan et al., 2021). In honeybees,*neuroparsin* is known as *queen brain-selective protein 1 (Qbp-1)* and is also influenced by brood presence. Specifically,*Qbp-1* is down-regulated in workers exposed to the larval pheromone E-beta-ocimene compared to workers exposed to the ester brood pheromone (Ma et al. 2019). In line with these studies, our data show that *neuroparsin* was strongly impacted by the presence of the queen. And its strongest effect was in the presence of both the queen and the brood, suggesting an additive effect. Along these lines,*vitellogenin* , the main yolk protein invested in the ovary of workers (Klowden 2013), showed the opposite pattern and, as noted in solitary insects, maybe also regulated by *neuroparsin* , leading to worker sterility and reduction in worker aggression (Amsalem, Malka, Grozinger, & Hefetz, 2014; Amsalem et al., 2017; Padilla et al., 2016). These two genes are strong candidates to serve a key to understanding the mechanistic regulation of worker reproduction by the queen and the brood across social species.

Other genes of interest are*mucin-5AC,* that was upregulated in workers in both the brain and the fat body (Figure 4), and *solute carrier organic anion transporters* (*SLCO2A1*). Both of these genes were affected by the combined presence of the queen and the brood (Figure 1, Table S2). *mucin-5AC* is a gel-forming glycosylated protein known to protect the mucosa body from infection, dehydration and physical or chemical injury in vertebrates (Quintana-Hayashi et al., 2015). However, in insects, its function is not well known. Recently, eight mucin genes were characterized in *Locusta migratoria* .*mucin-5AC* was detected in different tissues, including the fat body, but not in the ovaries and reducing its expression in*Locusta migratoria* via

RNA interference resulted in no visible phenotype during molting (Zhao et al., 2020). The upregulation of this gene in both the brain and the fat body of workers in response to the queen presence calls for further investigating of its role in social species.

SLCO2A1 acts as prostaglandins transport. Prostaglandins (PGs) are lipid signal molecules known to regulate reproduction and immune response in insects (Stanley, 2005; Stanley & Kim, 2019) PGs and steroid hormones are important for the insect follicle maturation and may be critical for female ovipositing, though this is not the case in at least one species (Stanley & Kim, 2019). These transporters were upregulated in workers in the presence of the queen and the brood with a stronger impact by the queen. Whether this gene should be upregulated or down regulated in order to achieve reproductive inhibition is not clear. Another gene of interest, *ESR-16* (*ecdysteroid-regulated 16kDa protein*), was mostly affected by the queen (upregulated in workers, 31% and 9% of the variance are attributed to the queen and the brood, respectively), due its potential regulation of reproduction-related hormones. *ESR-16* belongs to NPC2 family (Uniprot) which is involved in cholesterol transport. This gene, too, was upregulated in workers that were kept with queen and brood compared to the control. It was also upregulated in honeybee larvae that were transplanted into queen cell as compared to control (He et al., 2017). These larvae also showed increased of JH and upregulation of*vitellogenin* and were suggested to regulate hormonal pathways related to queen development. We would expect workers that their reproduction is inhibited to show reduction in JH, however the role of JH in the honeybee and bumble bees are also different, with JH acting as gonadotropin in bumble bees and most insects but evolved to regulate task specialization in advanced eusocial insects (Amsalem et al., 2014)

Interestingly, analysis of the same genes in the same tissue using RNA-seq and RT-qPCR provided only a modest overlap, with two genes out of eight DEGs in the RNA-seq analysis showing significant differences also in RT-qPCR (Figures 4, S2). These differences may stem from the fact we used two different set of samples that may vary slightly (i.e., the experiment was replicated to allow the extraction of both the brain and the fat body) and also from controlling for the batch effect in the RNA-seq data. However, incomplete match has been obtained also in a previous study where the exact same RNA samples were used (Amsalem, Galbraith, Cnaani, Teal, & Grozinger, 2015). It has been debated if validating RNA-seq using RT-qPCR is truly needed, especially given the ease and increased quality of sequencing nowadays. It is likely that highly differential genes (e.g., *neuroparsin A* and*vitelogenin*) will show the same pattern of expression in both methods, however small differences in expression are more accurately captured using RNA-seq.

Overall, our study shows that the brood and the queen may impact worker reproduction using similar genetic pathways, and that the impact of the brood alone is weak but the combined impact of the queen and the brood is larger. We further identified and discussed the role of selected genes in regulating worker reproduction, in particularly*neuroparsin* that is upregulated in the presence of the queen and the brood and is associated with sterility. These genes are likely to have an important regulatory role in social insects and female reproductive division of labor.

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Data Accessibility

The sequences determined in this study have been deposited in the NCBI's Gene Expression Omnibus and are accessible through GEO Series Accession XXX (will be deposited upon acceptance of manuscript). Transcript counts, raw data of ovarian activation, and qRT–PCR gene expression are available in Dryad at DOI XXX (will be deposited upon acceptance).

Author Contributions

EA designed the study. PS and JS conducted the experiments. PS and DG analyzed the genomic data. PS and EA wrote the manuscript. All co-authors commented on the final draft.

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Table 1: Comparison of the 27 differentially expressed genes (DEGs) identified in the current study with three similar data sets: Grozinger et al. 2003 compared DEGs in *Apis mellifera* worker's brain of three treatments: in the presence of the queen, in its absence, and when exposed to queen mandibular pheromone (QMP) ; Ma et al. 2009 compared DEGs in *A. mellifera* worker's brain exposed to two different brood pheromones (EBO and BP); and Libbretch et al. 2018 compared DEGs in the brain of workers of the clonal raider ant*Ooceraea biroi* in the reproductive stage (absence of young larvae) and the non-reproductive stages (presence of young larvae). An overlap between the DEGs identified in these studies and the current study

was indicated in the table with yes/no. Whenever data were available, we also provide the directionality of the expression.

Accession number	**Ma et al. 2019 – *A. mellifera***	**Grozinger et al. 2003 – *A. mellifera***	**Libbrecht et al. 2018 – *Ooceraeae biroi***	**Annotation**
Up in Cvs. CB	**Up in Cvs. CB**	**Up in Cvs. CB**	**Up in Cvs. CB**	**Up in Cvs. CB**
LOC112213952/ LOC100740924	NO	No Homologue	NO	*coiled-coil domain-containing protein 113/ uncharacterized LOC100740924*
Up in C vs. CQ	**Up in C vs. CQ**	**Up in C vs. CQ**	**Up in C vs. CQ**	**Up in C vs. CQ**
LOC100747176	NO	NO	YES	*vitellogenin*
LOC100748013	Up in EBO vs Control	QR < QL, Down in QMP treatment	NO	uncharacterized LOC100748013, transcript variant X1
LOC100749564	Up in EBO vs BP	QMP > QL, Up in QMP treatment	NO	*inositol oxygenase (MIOX)*
Down in C vs. CQ	**Down in C vs. CQ**	**Down in C vs. CQ**	**Down in C vs. CQ**	**Down in C vs. CQ**
LOC100740321	NO	NO	NO	*mitochondrial 2-oxoglutarate/malate carrier protein-like (SLC25A11)*
LOC100745608	NO	NO	YES	*cyclin-dependent kinase inhibitor 1C-like (CDKN1C)*
LOC100747366	Up in EBO vs BP	QR > QL	YES	*neuroparsin-A-like isoform X1*
Up in C vs. CBQ	**Up in C vs. CBQ**	**Up in C vs. CBQ**	**Up in C vs. CBQ**	**Up in C vs. CBQ**
LOC100747176	NO	NO	YES	*vitellogenin*
LOC100750219	NO	NO	NO	*histone H3-like*
LOC100742865	No Homologue	No Homologue	NO	*selenoprotein M (SELENOM)*
Down in C vs. CBQ	**Down in C vs. CBQ**	**Down in C vs. CBQ**	**Down in C vs. CBQ**	**Down in C vs. CBQ**
LOC100742261	Up in EBO vs BP	QR < QL	NO	*solute carrier organic anion transporter family member 2A1 (SLCO2A1)*

Accession number	**Ma et al. 2019 – *A. mellifera***	**Grozinger et al. 2003 – *A. mellifera***	**Libbrecht et al. 2018 – *Ooceraeae biroi***	**Annotation**
LOC100740321	NO	NO	NO	*mitochondrial 2-oxoglutarate/malate carrier protein-like (SLC25A11)*
LOC100746138	NO	NO	NO	*uncharacterized LOC100746138*
LOC100747366	Up in EBO vs BP	QR > QL	YES	*neuroparsin-A-like isoform X1*
LOC100745101	NO	NO	NO	*mucin-5AC*
LOC100743455	NO	NO	NO	*retinol-binding protein pinta*
LOC100742540	NO	QMP > QL, Down in QMP treatment	NO	uncharacterized protein LOC100742540 isoform X5
LOC100740422	NO	QMP > QL, Up in QMP treatment	NO	*monocarboxylate transporter 10 isoform X1*
LOC100740130	NO	Down in QMP treatment	NO	*microtubule-actin cross-linking factor 1 isoform X6 (macf1)*
LOC100740900	NO	NO	NO	*protein white isoform X1*
LOC100746862	Down in EBO vs Control	NO	NO	*ecdysteroid-regulated 16 kDa protein (ESR16)*
LOC100749924	NO	Up in QMP treatment	NO	*centrosomin isoform X1*
LOC105681220	NO	QR < QL, QMP < QL	NO	*alpha-(1,3)-fucosyltransferase C-like (FucTC)*
Down in CB vs. CQ	**Down in CB vs. CQ**	**Down in CB vs. CQ**	**Down in CB vs. CQ**	**Down in CB vs. CQ**
LOC112213952/ LOC100740924	NO	NO	NO	*coiled-coil domain-containing protein 113/ uncharacterized LOC100740924*
LOC100740321	NO	NO	NO	*mitochondrial 2-oxoglutarate/malate carrier protein-like (SLC25A11)*

Accession number	**Ma et al. 2019 – *A. mellifera***	**Grozinger et al. 2003 – *A. mellifera***	**Libbrecht et al. 2018 – *Ooceraeae biroi***	**Annotation**
LOC100745608	NO	NO	YES	*cyclin-dependent kinase inhibitor 1C-like (CDKN1C)*
Up in CB vs. CBQ	**Up in CB vs. CBQ**	**Up in CB vs. CBQ**	**Up in CB vs. CBQ**	**Up in CB vs. CBQ**
LOC100747176	NO	NO	YES	*vitellogenin*
LOC100750219	NO	NO	NO	*histone H3-like*
LOC100742002	NO	No Homologue	No Homologue	*general odorant-binding protein 56d-like (Obp56d)*
LOC105680747	NO	NO	NO	*cGMP-dependent protein kinase 1 isoform X2 (PRKG1)*
Down in CB vs. CBQ	**Down in CB vs. CBQ**	**Down in CB vs. CBQ**	**Down in CB vs. CBQ**	**Down in CB vs. CBQ**
LOC100742261	Up in EBO vs BP	QR < QL	NO	*solute carrier organic anion transporter family member 2A1 (SLCO2A1)*
LOC100746138	NO	NO	NO	uncharacterized LOC100746138
LOC100747366	Up in EBO vs BP	QR > QL	YES	*neuroparsin-A-like isoform X1*
LOC100740426	NO	QR > QL, Up in QMP treatment	NO	*prolyl 3-hydroxylase 1 isoform X1 (P3H1)*
LOC100742638	NO	No Homologue	NO	*plancitoxin-1 isoform X1*
LOC100740620	Up in EBO vs BP	Up in QMP treatment	NO	*solute carrier family 22 member 21-like isoform X2 (SLC22A21)*
LOC100745056	NO	No Homologue	YES	uncharacterized protein LOC100745056
LOC112213952/ LOC100740924	NO	NO	NO	*coiled-coil domain-containing protein 113/ uncharacterized LOC100740924*

Accession number	Ma et al. 2019 – *A. mellifera*	Grozinger et al. 2003 – *A. mellifera*	Libbrecht et al. 2018 – *Ooceraeae biroi*	Annotation
LOC100740321	NO	NO	NO	*mitochondrial 2-oxoglutarate/malate carrier protein-like (SLC25A11)*
LOC100743567	NO	Up in QMP treatment	NO	uncharacterized protein LOC100743567 isoform X2
Up in CQ vs. CBQ	**Up in CQ vs. CBQ**	**Up in CQ vs. CBQ**	**Up in CQ vs. CBQ**	**Up in CQ vs. CBQ**
LOC100750219	NO	NO	NO	*histone H3-like*

Figure 1

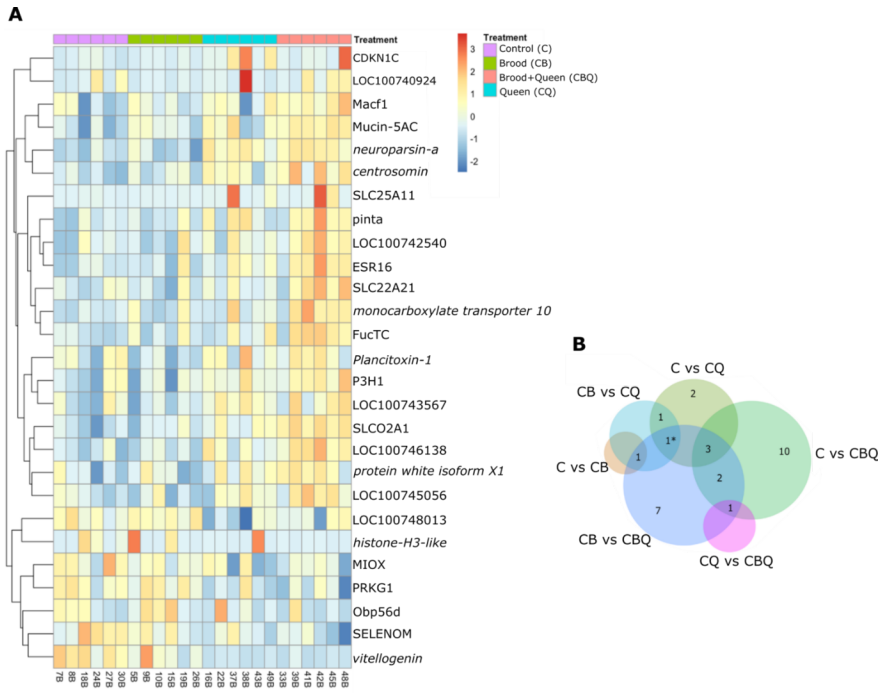

Figure 1: Whole transcriptome analysis of*Bombus impatiens* workers' brain in the presence of the queen and the brood. A) Heatmap representing color-coded expression levels of differentially expressed genes (DEGs) in worker's brain in all pairwise comparisons among the four treatments. Each column represents the individual samples ordered by the treatment and each row represent the expression level of selected gene. **B)** Venn diagram showing the number of DEGs in workers' brain in all pairwise comparisons among the four treatments. Data are based on 24 libraries of workers' brain (6 replicates per treatment). Pair of newly emerged workers were assigned to four treatments and kept for three days with the queen (CQ), young brood (CB), the queen and young brood (CBQ), or alone (C).

Figure 2

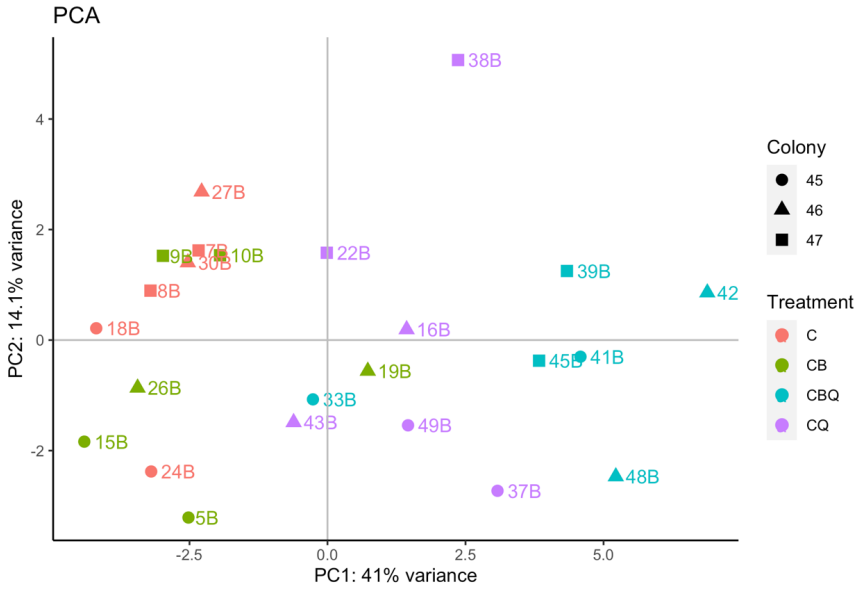

Figure S2: Principal component analysis of the samples used for RNA-seq brain analysis featuring all samples divided according to the treatment (marked with different colors) and the colony of origin (marked with different symbols). The plot is based on the 27 differentially expressed genes. Data are based on 24 libraries of workers' brain (6 replicates per treatment). Pair of newly-emerged workers were assigned to four different treatments: with the queen (CQ), young brood (CB), the queen and young brood (CBQ), or alone (C) and were sampled after 3 days. Workers were equally sampled from 3 different colonies.

Figure 3

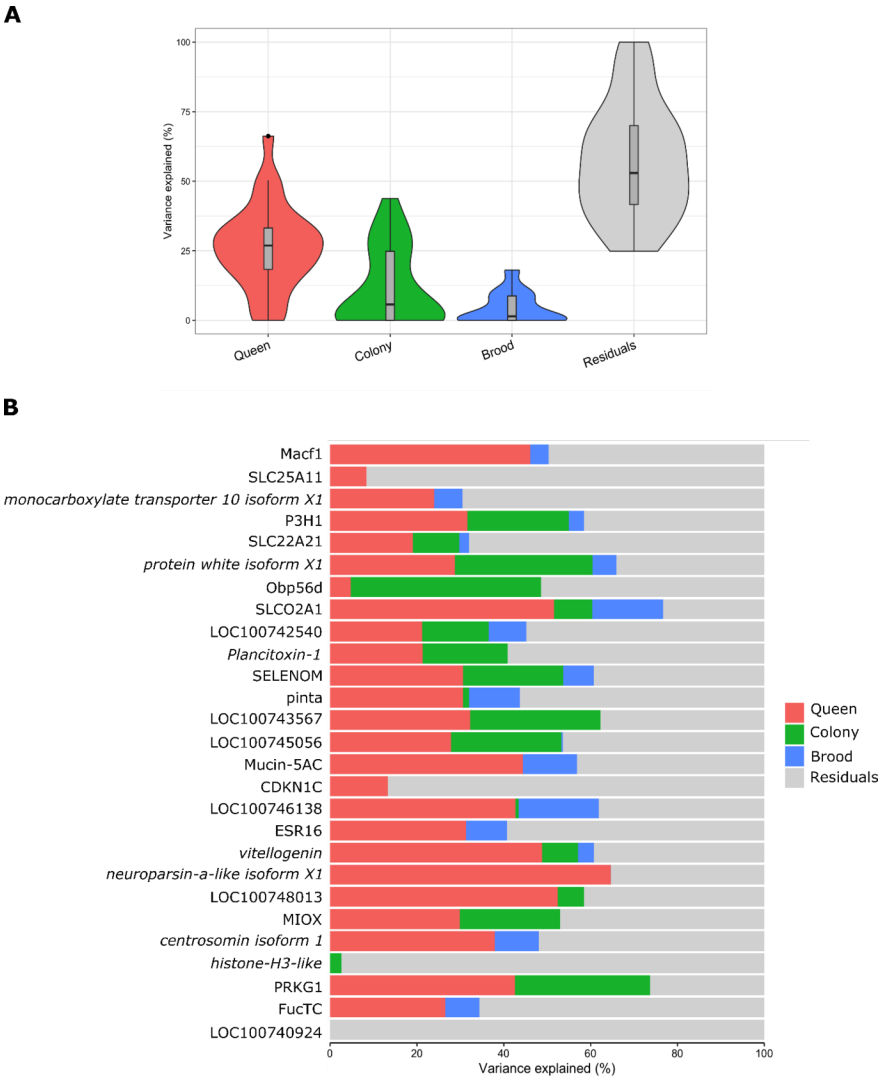

Figure 3: The percentage of variance in the differentially expressed genes explained by selected variables. A) The percentage of variance explained by the presence of the brood and the queen, the identity of the worker parental colony and the residuals; B) the percentage of variance explained by the queen, brood, colony, and residuals for each of the 27 differentially expressed genes identified in the study. Data are based on 24 libraries of worker's brain (6 replicates per treatment). Pair of newly emerged workers were assigned to four treatments and kept for three days with the queen (CQ), young brood (CB), the queen and young brood (CBQ), or alone (C). Workers were equally sampled from 3 different colonies.

Figure 4

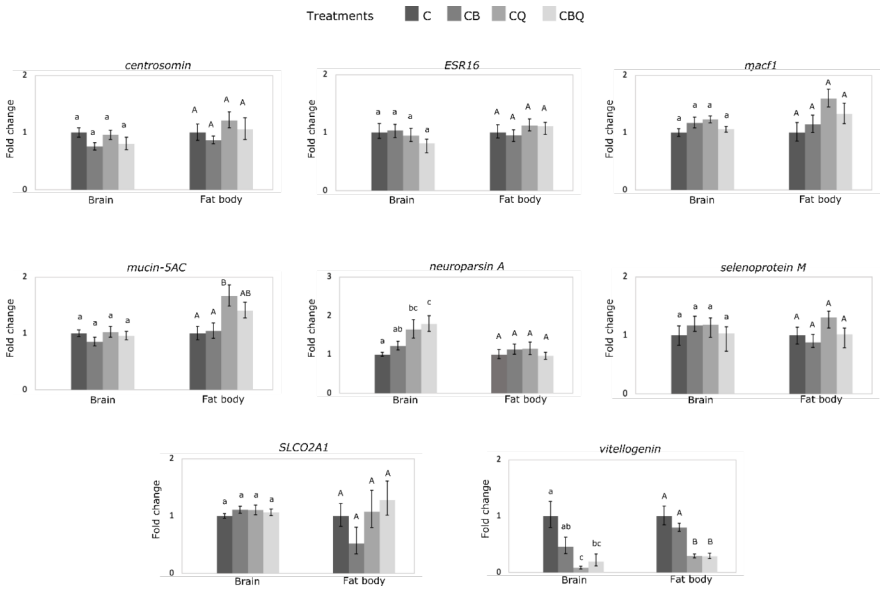

Figure 4: RT-qPCR analysis of selected genes in*Bombus impatiens* workers' brain and fat body in the presence of the queen and the brood. Expression levels of selected genes from RNA-seq analysis were examined in workers' brain and fat body tissues. Pair of newly emerged workers were assigned to four treatments and kept for three days with the queen (CQ), young brood (CB), the queen and young brood (CBQ), or alone (C). Different letters above columns indicate statistical differences at $\alpha = 0.05$.