Current Research in Insect Science 3 (2023) 100056

Contents lists available at ScienceDirect

Current Research in Insect Science

journal homepage: www.elsevier.com/locate/cris

Differential gene expression underlying the biosynthesis of Dufour's gland signals in *Bombus impatiens*

Nathan Derstine*, David Galbraith, Gabriel Villar, Etya Amsalem

Department of Entomology, Center for Chemical Ecology, Center for Pollinator Research, Huck Institutes of the Life Sciences, Pennsylvania State University, University Park, PA 16802, U.S.A

ARTICLE INFO

Keywords:
Signal biosynthesis
Dufour's gland
Social insects
RNA-seq

ABSTRACT

Pheromones regulating social behavior are one of the most explored phenomena in social insects. However, compound identity, biosynthesis and their genetic basis are known in only a handful of species. Here we examined the gene expression associated with pheromone biosynthesis of two main chemical classes: esters and terpenes, using the social bee *Bombus impatiens*. We conducted chemical and RNA-seq analyses of the Dufour's gland, an exocrine gland producing a plethora of pheromones regulating social behavior in hymenopteran species. The Dufour's gland contains mostly long-chained hydrocarbons, terpenes and esters that signal reproductive and social status in several bee species. In bumble bees, the Dufour's gland contains queen- and worker-specific esters, in addition to terpenes and terpene-esters only found in gynes and queens. These compounds are assumed to be synthesized de novo in the gland, however, their genetic basis is unknown. A whole transcriptome gene expression analysis of the gland in queens, gynes, queenless and queenright workers showed distinct transcriptomic profiles, with thousands of differentially expressed genes between the groups. Workers and queens express genes associated with key enzymes in the biosynthesis of wax esters, while queens and gynes preferentially express key genes in terpene biosynthesis. Overall, our data demonstrate gland-specific regulation of chemical signals associated with social behavior and identifies candidate genes and pathways regulating caste-specific chemical signals in social insects.

1. Introduction

Reproductive division of labor between a primary egg-layer and non-reproductive females is a defining feature of eusocial insects (Wilson, 1971), and is often maintained using chemical signals produced in exocrine glands (Amsalem, 2020; A. Hefetz, 2019). Such signals have been identified in numerous insect species (Hoover et al., 2003; Steiger and Stokl, 2018; Steitz and Ayasse, 2020), and while they can be quite diverse (Amsalem, 2020; A. Hefetz, 2019), they are commonly derivatives of fatty acid biosynthesis (Yew and Chung, 2015). Fatty acid derivatives such as hydrocarbons, wax esters, fatty alcohols, and acids are frequently represented as signal molecules, and thus may share a genetic and biosynthetic origin. In contrast, terpenes, another frequently represented class of signal molecules, derive from a separate pathway (Bellés et al., 2005). Despite the importance of chemical signals for regulating reproduction and social behavior, their molecular and biosynthetic underpinnings are often poorly understood. Better understanding of the genetic basis of signal biosynthesis enables direct manipulations of key genes to examine mechanisms of signal production (Yan and Liebig, 2021), and is a crucial step towards understanding the evolution of signals and sociality.

Signals that are derivatives of fatty acid biosynthesis are common across social insect species. For instance, hydrocarbons serve primarily to prevent insect desiccation (Menzel et al., 2019), but evolved as signals reflecting species, caste, age, task, reproductive and social status in many species (Blomquist and Bagnères, 2010; Derstine et al., 2018; Dietemann et al., 2003; Endler et al., 2004; Oi et al., 2015; Smith et al., 2009, 2013). Likewise, various wax esters comprise the brood pheromone of honey bees (Yves Le Conte et al., 2001), and play a role in the reproductive signaling of worker bumble bees (Amsalem et al., 2009), and macrocyclic lactones (cyclic esters) serve as queen pheromones and indicators of reproductive state in halictid bees (Kingwell et al., 2021; Steitz and Ayasse, 2020; Steitz et al., 2019). Furthermore, the main component of the honey bee queen mandibular pheromone is a fatty acid derived hydroxy-acid which regulates worker reproduction (Erika Plettner et al., 1998; Slessor et al., 1990). The common biosynthetic origin of these molecules and their role in regulating reproduction suggests an underlying genetic link.

* Corresponding author.
E-mail address: ntd34@psu.edu (N. Derstine).

https://doi.org/10.1016/j.cris.2023.100056
Received 28 June 2022; Received in revised form 29 March 2023; Accepted 4 April 2023

The Dufour's gland (DG) is a female-specific exocrine gland which produces a range of chemical signals in Hymenopterans (Mitra, 2013). Many of these regulate disparate aspects of social life, such as inhibiting worker reproduction (Steitz and Ayasse, 2020) and trail following (Blatrix et al., 2002), and thus provide an excellent opportunity to study the genetic basis of social signals and their biosynthesis. Depending on the species, the DG contains various compounds such as hydrocarbons, esters, lactones, terpenes and terpene esters, and fatty acids (Keeling et al., 2004). In bumble bees and honey bees, worker- and queen-specific esters in the DG act as sterility and fertility signals, respectively, advertising female fecundity (Amsalem et al., 2013; Amsalem et al., 2009; Dor et al., 2005). In honey bees, the esters are produced de-novo in the gland (Katzav-Gozansky et al., 2000) while the hydrocarbons are synthesized in oenocytes and transported into the gland via lipophorins (Soroker and Hefetz, 2000), indicating that the gland is not only a reservoir for the signals but also the place where the active components of the signal are being produced.

Bumble bees provide an excellent model species to examine the biosynthesis of reproductive signaling. The variation found between castes and reproductive states in the DG secretion, and the ability to replicate this variation by manipulating the social environment and mating status facilitates comparative experiments. Furthermore, the composition of the DG has been extensively studied in several bumble bee species, and contains saturated and unsaturated hydrocarbons, wax and acetate esters, fatty acids, terpenes, and terpene esters in blends unique to species, age, reproductive state, and caste (Amsalem et al., 2009; Cahlíková et al., 2004; Derstine et al., 2021; Tengö et al., 1991). In *B. terrestris*, wax esters in the DG disappear in workers as their ovaries develop and were suggested to signal sterility (Amsalem et al., 2009), reduce aggression (Amsalem and Hefetz, 2010) and/or advertising the risky work of forager bees (Amsalem et al., 2013). In *B. impatiens*, worker-specific wax esters correlate with age and ovarian activation, while queens and gynes (young, non-reproductive queens) produce terpenes and terpene esters and long-chain wax esters not found in workers (Derstine et al., 2021), suggesting divergent biosynthetic pathways that makes *B. impatiens* an excellent model to study the genetic basis of signal production.

Despite the importance of DG esters and terpenes as signal molecules, little is known of the genes underlying their biosynthesis The synthesis of wax esters, formed from the condensation of fatty acids and alcohols, involves the elongation of fatty acids, conversion of fatty acyl-CoAs to fatty alcohol, and final esterification step. Two enzymes are likely involved in the esterification: a fatty acyl reductase (FAR), and a wax synthase (WS) which is a specialized acyltransferase (Teerawanichpan and Qiu, 2010; Wenning et al., 2017). FAR enzymes are responsible for the production of primary fatty alcohols, which can serve as signals themselves and also as precursors to a variety of esters (Matoušková et al., 2008; Valterová et al., 2019). Insects have multiple FAR enzymes with varying levels of substrate specificity, and FAR enzymes in hymenopterans have undergone an expansion implicated in shaping species-specific pheromone communication (Tupec et al., 2019). While current evidence suggests that a WS catalyzes the esterification of a fatty acyl-CoA and a primary fatty alcohol to produce a wax ester, genes with wax synthase activity have only been identified in non-insect organisms, such as mammals, plants, yeasts, and bacteria, (Yang et al., 2012). In many organisms, a multi-functional gene in the acyltransferase family (such as diacylglycerol acyltransferase or DGAT) catalyzes the production of wax esters, but also mono, di, or triglycerides (Kalscheuer and Steinbüchel, 2003; F. Li et al., 2008; Teerawanichpan and Qiu, 2010; Wenning et al., 2017; Yen et al., 2005). In *B. impatiens*, worker-specific wax esters are formed from acid and alcohol components less than 16 carbons long, as opposed to the longer queen-specific wax esters, indicating a chain-shortening beta-oxidation process specific to workers prior to esterification. Overall, the caste specificity of the wax esters in *B. impatiens* Dufour's gland likely results from differences in beta-oxidation activity and the expression of FAR enzymes, which supply primary alcohols of varying lengths to be esterified by a relatively non-specific wax synthase/DGAT.

The diterpenes produced by the queen caste are synthesized via isoprenoid pathway and share a biosynthetic origin with the terpenes serving as sex pheromones in the labial glands of *Bombus* males (Brabcova et al., 2015; Prchalova et al., 2016; Valterová et al., 2019). This pathway is conserved among eukaryotes and produces the building blocks of all terpenes and terpenoids, giving rise to an enormous diversity of semiochemicals including monoterpenes, diterpenes, sesquiterpenes, and also juvenile hormones (Bellés et al., 2005; Miziorko, 2011). Many of these semiochemicals function in sexual communication (Andreadis et al., 2015; Beran et al., 2016; Darragh et al., 2021; Engel et al., 2016). While the isoprenoid pathway is conserved, the downstream enzymes responsible for conversion of terpene precursors into any of the myriad final terpene products (such as β-springene in *B. impatiens* gynes) are likely taxon-specific and few specific genes have been identified. Even so, diterpene-producing tissue in termites exhibits high expression of hydroxymethylglutaryl-CoA synthase (HMGS) and hydroxymethylglutaryl-CoA reductase (HMGR), rate-limiting enzymes in the isoprenoid pathway (Hojo et al., 2012). It is likely that these genes are highly expressed in gynes, which produce the largest amount of diterpenes.

To examine the biosynthesis and genetic basis of wax esters and terpenes in the Dufour's gland of *B. impatiens* females, we analyzed the chemical composition of the gland and conducted RNAseq analyses of the gland in queen, gynes and workers (both queenright and queenless). We examine variation in gene expression associated with caste, mating status, and fertility. We hypothesized that: 1) genes in the fatty acid biosynthetic pathway related to chain shortening and beta-oxidation would be upregulated in workers groups which produce shorter wax esters than queens, and would be highest in queenright workers, which produce more of these compounds compared to queenless workers, 2) FAR and acyltransferase genes involved in ester formation would be downregulated in queens that produce little to no wax esters, and 3) genes in the isoprenoid biosynthetic pathway would be upregulated in gynes which produce the most terpenes compared to queens and workers.

2. Methods

2.1. Insect rearing and sampling

Bees were collected from *B. impatiens* colonies that were obtained from Koppert Biological Systems (Howell, Michigan, USA). Colonies were maintained in nest-boxes at a constant darkness, temperature of 28–30 °C and 60% relative humidity and supplied ad libitum with 60% sugar solution and honeybee-collected pollen (purchased from Koppert). These colonies were used as a source for mated, egg-laying queens (hereafter "queens"), newly-emerged, unmated queens (hereafter "gynes"), and newly-emerged workers. Upon emergence, workers were assigned to one of two treatments: queenright workers (QRW) and queenless and brood-less workers (QLW). QRW were individually labeled and remained in their natal colony in the presence of the queen, brood, and nestmates until collection at the desired age, while QLW were housed in a plastic cage (11 cm diameter × 7 cm height) in groups of 3–6 workers until collection. Both groups of workers were sampled at the age of 7 days. Since the presence of brood can impact worker reproduction (Starkey et al., 2019), QLW cages were monitored daily for egg laying, and newly-laid eggs were removed. Gynes were collected upon emergence from late-season colonies and housed individually in small cages until sampling on days 3–4. Queens were sampled from colonies with ongoing egg-laying and worker production, indicating that they were successfully mated, and were > 8 weeks old from the emergence of the first worker. The time points of the treatment groups were selected to capture differences in phenotype of both ovarian activation and the DG secretion based on previous work (Derstine et al., 2021).

These experimental procedures were repeated twice; once to generate samples for chemical analysis of the DG, and a second time to generating samples for RNA extraction and subsequent RNAseq analysis. For chemical analyses, QRW (n = 15), QLW (n = 15), and gynes (n = 20) were sampled from a total of 8 colonies (minimum 4 bees per colony). Queens (n = 20) each originated from a different colony. RNA was extracted from pools of 5–10 workers (58 QRW and 48 QLW), 3–7 gynes (n = 33) and single queens (n = 6) and collected from 15 colonies resulting in 24 samples (6 QLW, 6 QRW, 6 gynes and 6 queens). Detailed information on the number of glands used per sample and their colony of origin is found in Table S1.

2.2. Ovarian activation

Oocyte size was measured in all samples. Ovaries were removed and placed in a drop of distilled water, and the largest three terminal oocytes across both ovaries (at least one from each ovary) were measured with an eyepiece micrometer (Amsalem and Hefetz, 2010). The average terminal oocyte was used as a measure of ovary activation for each bee.

2.3. Chemical analysis

Bees were dissected under a stereomicroscope in distilled water and the gland was removed. Glands were extracted for 24 h in 150 μL hexane containing internal standard of eicosane (C_{20}) at 20 ng/μL, before being analyzed by gas-chromatography flame ionization detection (GC-FID).

DG components of *B. impatiens* females were identified in a previous study (Derstine et al., 2021). To quantify the components in this study, DG extracts were analyzed on a Trace 1310 GC (Thermo Fisher, Waltham, MA USA) equipped with a flame-ionization detector (FID) and a TG-5MS column (0.25 mm id x 30 m x 0.25 μm film thickness; Thermo Fisher). The oven temperature was programmed at 60°C for 1 min, increased to 120°C at 15°C/min, then increased to 300°C at 4°C/min and held at 300 °C for 5 min. The injector port and FID were held constant at 250°C and 320°C, respectively.

2.4. RNA extraction

DGs were dissected by removing the whole sting complex of a dry-ice anesthetized bee and placing it in a drop of RNAlater (Invitrogen, Carlsbad, CA) to keep the tissue cold and prevent degradation. Individual DGs were removed from the sting complex using clean forceps and pooled according to treatment group in sterilized 2 mL FastPrep tubes containing six 2 mm zirconia beads and 250 μL Trizol (Invitrogen, Carlsbad, CA). Individual DGs were pooled to obtain sufficient amounts of RNA for downstream analyses. Worker samples contained RNA from 5 to 10 glands, gynes from 5 to 7 glands, and queens from a single gland (Table S1). Samples were frozen at −80° C and stored until processing. Total RNA was isolated using a PicoPure kit according to the manufacturer instructions. RNA quantity was measured using a NanoDrop One^{TM}.

2.5. RNA-sequencing and differential gene expression analysis

Twenty-four cDNA libraries were prepared using Illumina TruSeq protocols (n = 6 per treatment in QLW, QRW, gyne, and queen groups). RNA-sequencing was performed using single end 75 nt read lengths at the Penn State Genomics Core Facility on an Illumina NextSeq 550 system. FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016) were used to identify potential sequencing issues such as low quality reads. According to the recommended usage of Trimmomatic (Bolger et al., 2014), reads with a Phred score below 25 and length below 36 bp were removed. The reads were then processed with Trimmomatic v0.039 to remove TruSeq3-SE adapter sequences and to trim low-quality bases from the ends of the reads. The processed reads were aligned to the *Bombus impatiens* genome assembly GCF_000188095.2 (Sadd et al., 2015) using STAR v2.7 (Dobin et al., 2013) and read counts were calculated using the standard RSEM pipeline (B. Li and Dewey, 2011), producing count data for 10,633 genes, with an average of 18,309,015 reads per sample.

Differential gene expression analyses were conducted using the packages edgeR (Robinson et al., 2010) and limma (Law et al., 2016; Ritchie et al., 2015) in R v. 4.1.3. First, lowly expressed genes (1064 which had a sum of transcript counts < 20 across all samples) were removed from analyses. The *voom* method (Law et al., 2014) was used to estimate the mean-variance relationship of log transcript counts per million (logCPM) and create a precision weight for every normalized observation. These weights are used when fitting linear models to the gene expression data to account for variable standard deviation inherent in comparing count data across a large scale. Differentially expressed genes (DEGs) were identified between specific contrasts of treatment groups (queen vs. gyne, queen vs. QRW, queen vs. QLW, gyne vs. QRW, gyne vs. QLW, queens vs. workers), and multiple testing was controlled using Benjamini and Hochberg's false discovery rate at 0.05 to adjust P-value cutoffs (Benjamini and Hochberg, 1995). We analyzed the contrasts of QLW vs. QRW to examine the effect of reproductive status and social condition on worker gene expression, queen vs. gyne to examine the same effects in queens, and queen vs QLW to examine the effect of caste on gene expression in reproductively active bees. T-distributed stochastic neighbor embedding (t-SNE) was used to visualize sample clustering based on the full gene count matrix with the Rtsne and ggplot2 packages (Krijthe and Van der Maaten, 2015; Wickham, 2011). A heatmap of selected DEGs was created using the *heatmap.2* function from the gplots package (Warnes et al., 2016). An upset plot of DEG sets was created using the UpSetR package (Conway et al., 2017). Volcano plots of selected contrasts were made using the EnhancedVolcano package (Blighe et al., 2021). Gene set enrichment analysis (GSEA) was performed using TopGo's classic algorithm and p-values from Fisher's exact method (Alexa and Rahnenfuhrer, 2022). GO annotations associated with *B. impatiens* genes were retrieved using the web server g:Profiler (Raudvere et al., 2019). KEGG pathway enrichment analysis was performed using the *gseKEGG* function from the clusterProfiler package (Wu et al., 2021).

2.6. Statistical analyses

Statistical analyses of DG compounds were performed using R (version 4.0.5) in RStudio (version 1.2.5033). To examine chemical differences in the DG secretion between females of different caste (queens, workers) and social conditions (QRW, QLW), permutational analysis of variance (PERMANOVA) was performed using the *adonis* function from the vegan package (Oksanen et al., 2013) on relative peak areas, with social condition and caste as grouping variables. Pairwise comparisons between groups was performed with *pairwise.perm.manova* function from the RVAideMemoire package (Hervé and Hervé, 2020) using the Wilks test and FDR correction for multiple testing. Only peaks > 0.5% of the total peak area in at least one group were included. Non-metric multidimensional scaling plots (NMDS) using esters, alkanes, alkenes, or all compounds were used to visualize the results.

Mean terminal oocyte size was compared between treatment groups using a one-way ANOVA followed by Tukey's HSD post-hoc test. When comparing the percentage of chemical classes between treatment groups, the data were not always normally distributed (Shapiro-Wilks test > 0.05) or had unequal variances (Levene's test > 0.05), and thus relative and absolute amounts of chemical classes were compared between treatment groups using the non-parametric Kruskal-Wallis test. Dunn's post-hoc test from the dunn.test package (Dinno and Dinno, 2017) and Benjamini and Hochberg multiple comparison correction were performed to provide contrasts between treatment groups.

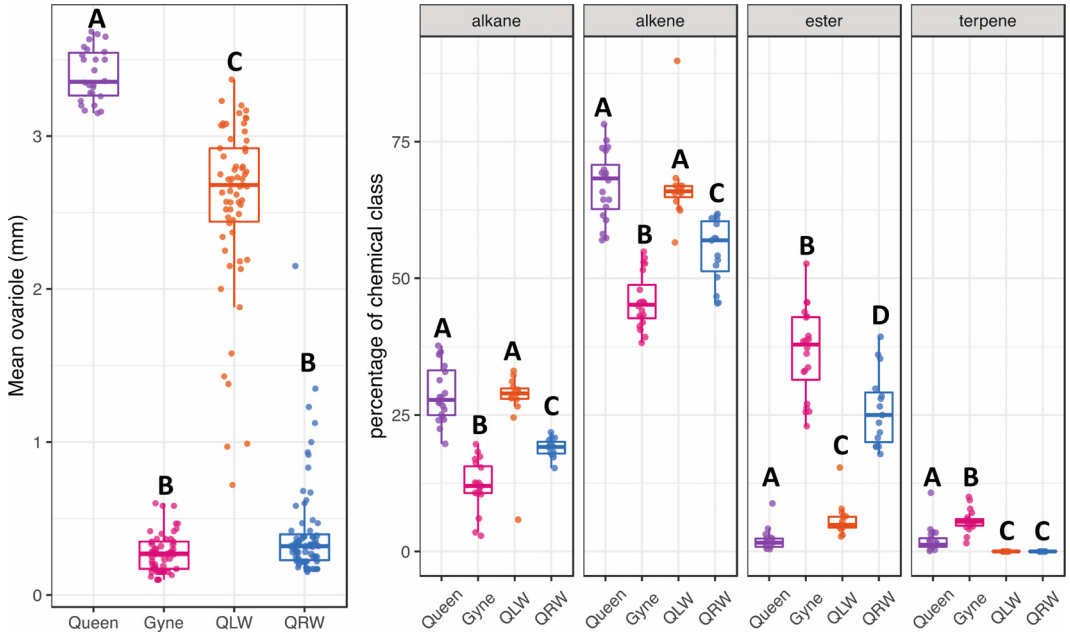

Fig. 1. – A - Average terminal oocyte length of bees used in chemical and RNAseq in four groups of *B. impatiens* females: 7-days-old queenright workers (QRW, $n = 73$), 7-days-old queenless workers ($n = 63$), 3–4 days old gynes ($n = 53$) and active queens ($n = 26$). B – Percentages of specific chemical classes of the *B. impatiens* DG secretion in four groups of *B. impatiens* females: 7-days-old queenright workers (QRW, $n = 15$), 7-days-old queenless workers ($n = 15$), 3–4 days old gynes ($n = 20$) and active queens ($n = 20$). Different letters denote significant differences at $p < 0.05$ (mean ovariole - One-way ANOVA with Tukey HSD post-hoc test; percentage of chemical class - Kruskal-Wallis with Dunn's post-hoc test).

3. Results

3.1. Social condition and reproductive phenotype

Ovarian activation was significantly different between treatment groups (One-way ANOVA, $F_{3,210} = 797.8$, $p < 0.001$). Queens and queenless workers (QLW) displayed higher ovary activation compared to gynes and queenright workers (QRW) (Fig 1a). On average, queens had significantly larger terminal oocytes than gynes (3.4 ± 0.03 and 0.29 ± 0.03, mean mm ± SE, respectively, ANOVA followed by Tukey HSD post-hoc test, $p < 0.001$), and QLW had significantly larger terminal oocytes than QRW (2.56 ± 0.07 and 0.41 ± 0.04, mean mm ± SE, respectively, ANOVA followed by Tukey HSD post-hoc test, $p < 0.001$). In groups with activated ovaries, queen terminal oocyte size was also larger than QLW (ANOVA followed by Tukey HSD post-hoc test, $p < 0.001$). Gynes and QRW ovaries were not significantly different.

3.2. Chemical analysis

Chemical analysis of *B. impatiens* DG secretion revealed a highly caste specific chemical composition that varies with life stage, reproductive, mating and social condition. The DG secretion varied significantly in the proportion of alkanes (Kruskal Wallis test, $\chi^2 = 50.47$, df = 3, $p < 0.001$), alkenes (Kruskal Wallis test, $\chi^2 = 51.79$, df = 3, $p < 0.001$), esters (Kruskal Wallis test, $\chi^2 = 59.41$, df = 3, $p < 0.001$), and terpenes (Kruskal Wallis test, $\chi^2 = 60.84$, df = 3, $p < 0.001$). Groups with activated ovaries had higher proportions of alkanes (Kruskal-Wallis with Dunn's post hoc test, $p < 0.05$) and alkenes ($p < 0.05$), while groups with inactivated ovaries, QRW and gynes, had a significantly higher proportion of esters than those with activated ovaries ($p < 0.05$). Terpenes were present in queens and gynes, but not workers, and were higher in gynes compared to any other female group ($p < 0.05$). Mean proportions of each chemical class and overall test statistics are provided in Table S2, while post-hoc test statistics are provided in tables S3–6. NMDS analyses show that differences in the relative composition of DG compounds distinguish between groups based on caste (queens vs. workers), life stage, mating and reproductive status (queens vs. gynes) and social and reproductive condition (QRW vs. QLW) (Figure S1). PERMANOVA analyses of the relative composition of individual components of the DG secretion showed significant differences between all groups, regardless of chemical class (FDR corrected p-values all < 0.01, Table S7).

3.3. RNAseq results

Of the 9569 genes used in differential expression analyses, more than a third were differentially expressed between queens and gynes (1381 upregulated and 1271 downregulated). A similar number were differentially expressed between queen and worker castes (1405 upregulated and 1344 downregulated). In contrast, there were no significant DEGs between the worker treatments, which also clustered together in a t-SNE plot (Fig 2). The RNA samples were generated using a different number of individuals that were pooled together to allow a sufficient amount of RNA for sequencing. While worker and gyne samples were pooled (5–10 and 3–7 glands per sample for worker and gyne samples, respectively), each queen sample was generated using a single gland. To make sure this approach didn't introduce a bias in our data, we compared the coefficient of variation between the samples and show that while there is a negative correlation between the number of glands in the pool and the coefficient of variation (Pearson correlation −0.5, $t = -2.7$, df = 22, $p = 0.01$, Fig S2), this relationship is driven by the queen samples. When the queen samples are removed the correlation is no longer significant (Pearson correlation −0.38, $t = -1.6$, df = 16, $p = 0.12$). To further explore this, we mapped the number of glands per sample onto a cluster dendrogram and show that even within treatment group, the number of glands per sample does not predict sample relationships (Fig S3). A heatmap of the 100 most DEGs (ranked by log-odds ratio) for each contrast shows striking differences between queens, gynes, and workers, but clustering of QRW and QLW treatment groups (Fig 3). Volcano plots show the large magnitude of expression differences between all treatment groups except between QRW and QLW (Fig S4). Interestingly, the number and magnitude of DEGs between queens and gynes were greater than between any queen-workers comparison, and varied up to a log fold

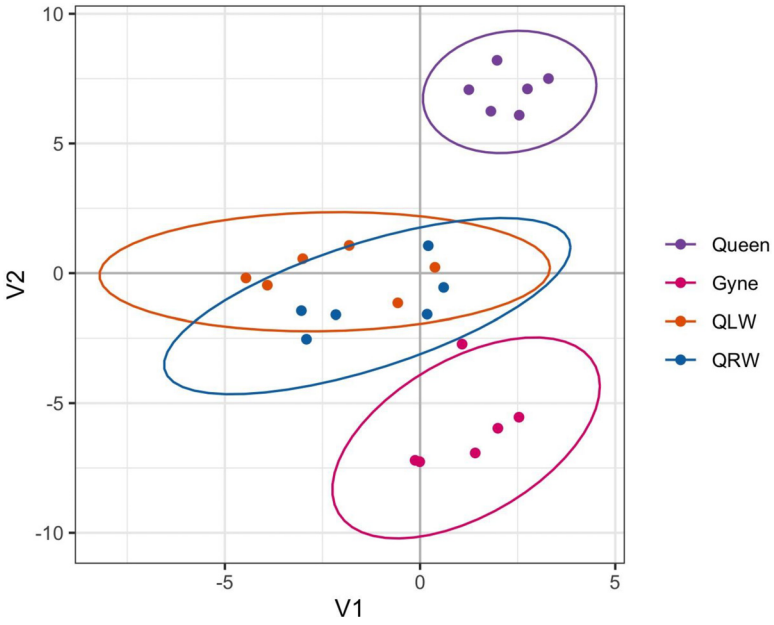

Fig. 2. – t-SNE plot showing clustering of Dufour's gland RNAseq samples based on treatment group using all gene expression data.

change of 10 (Fig S4). An Upset plot (Fig 4) shows the overlap of DEGs between different contrasts, and identifies the number of genes which vary in response to caste (239 DEGs in queens and gynes vs. workers), reproduction (82 DEGs between queens and QLW vs. gynes and QRW), and mating status (526 DEGs in queens vs. gynes and workers).

To identify genes responsible for the differences in DG secretion between the treatment groups, we examined genes related to the fatty acid biosynthetic pathway which produce wax esters (produced by workers and gynes, proposed biosynthesis is provided in Fig 5), and the mevalonate/isoprenoid biosynthetic pathway which produces terpenes (produced mostly by gynes, but also by queens, Fig 6). Since QRW and QLW were indistinguishable in terms of gene expression, they were grouped together as one variable in the plots identifying genes involved in biosynthesis.

3.4. Candidate genes related to wax ester biosynthesis in females

Candidate genes related to fatty acid biosynthesis included enzymes involved in 5 discrete steps of wax ester biosynthesis. The first step, fatty acid synthesis, is responsible for the earliest steps in the construction of fatty acid chains from acetyl-CoA units (Fig 7A). Here, two DEGs involved in this process were recovered, while expression of fatty acid synthase genes did not vary between treatments. We found 7 DE genes involved in β-oxidation, which shortens fatty acid chains by two carbons. These tended to be more expressed in workers and queens than gynes (Fig 7B). Fatty acyl reductases (FAR) produce fatty alcohols from fatty acyl-CoAs and depending on the specific gene, varied between all treatment groups (Fig 7C). About half of the FAR genes (7 of 15) were exclusively upregulated in workers compared to queens and gynes. The remaining FAR genes varied in their expression patterns between groups with six being downregulated in workers compared to queens, gynes or both, and the two others being upregulated in both workers and queen compared to gynes (Fig 7C). Desaturases (DESAT) introduce double-bonds to fatty acyl-CoA and all three DE genes were highest in gynes, differing mostly between gynes vs. queens and workers, but not between queens and workers (Fig 7D). Finally, acyltransferases (mono and di-acylglyceroltransferases MGAT/DGAT), synthesize mono, di, and triglycerides, in addition to a possible role as wax synthases and were most highly expressed in gynes (3 of 4 genes) (Fig 7E). Elongases lengthen the chain of fatty acyl-CoA beyond 16 or 18 carbons and tended to be more highly expressed in gynes than workers or queens (Fig S5). As the long chain wax esters differentiating the queen caste are comprised of acid and alcohol portions up to 18 carbons, these are more likely to be involved in cuticular hydrocarbon than wax ester biosynthesis.

3.5. Genes regulating terpenes in females

Differentially expressed genes related to isoprenoid/mevalonate biosynthesis included genes involved early in the pathway and several genes downstream in the pathway. The early genes (acetoacetyl-CoA thiolase - AACT, HMGS, HMGR, phosphomevalonate kinase - PMK) construct the precursors to all terpenes and terpenoids and function in the production of isopentyl diphosphate from acetyl-CoA, while downstream genes like farnesyl diphosphate synthase (FPPS) condenses isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) to make geranyl and subsequently farnesol diphosphate, the precursor of all future farnesyl compounds. Farnesol dehydrogenase genes oxidize farnesol to farnesal, for instance in the production of juvenile hormone that serves as a gonadotropin in female insects (Mayoral et al., 2009; Rivera-Perez et al., 2014). Genes related to the isoprenoid/mevalonate pathway were among the most highly expressed genes we detected (Fig S6). Genes in the early isoprenoid/mevalonate pathway, including HMGR, the rate-limiting enzyme for the whole pathway, tended to be most highly expressed in gynes, which also produced the largest amounts of terpenes and terpene esters. The farnesol dehydrogenase genes were all most highly expressed in queens, while gynes and workers did not differ (Fig 8). Gene IDs and annotations corresponding to genes displayed in Figs. 7 and 8 are given in Table S8, and statistical values of each group contrast given in Supplementary File S1.

3.6. Enriched KEGG pathways and gene ontology terms

Several KEGG pathways relevant to chemical signal biosynthesis were significantly enriched in lists of differentially expressed genes between treatment groups. Between gynes and workers the pathways "biosynthesis of unsaturated fatty acids" (KEGG pathway bim01040), "fatty acid elongation" (bim00062), and "biosynthesis of unsaturated fatty acids" (bim01212) were enriched (Fig S7). The "fatty acid elongation" pathway was also enriched in the queen vs gyne gene list (Fig S8).

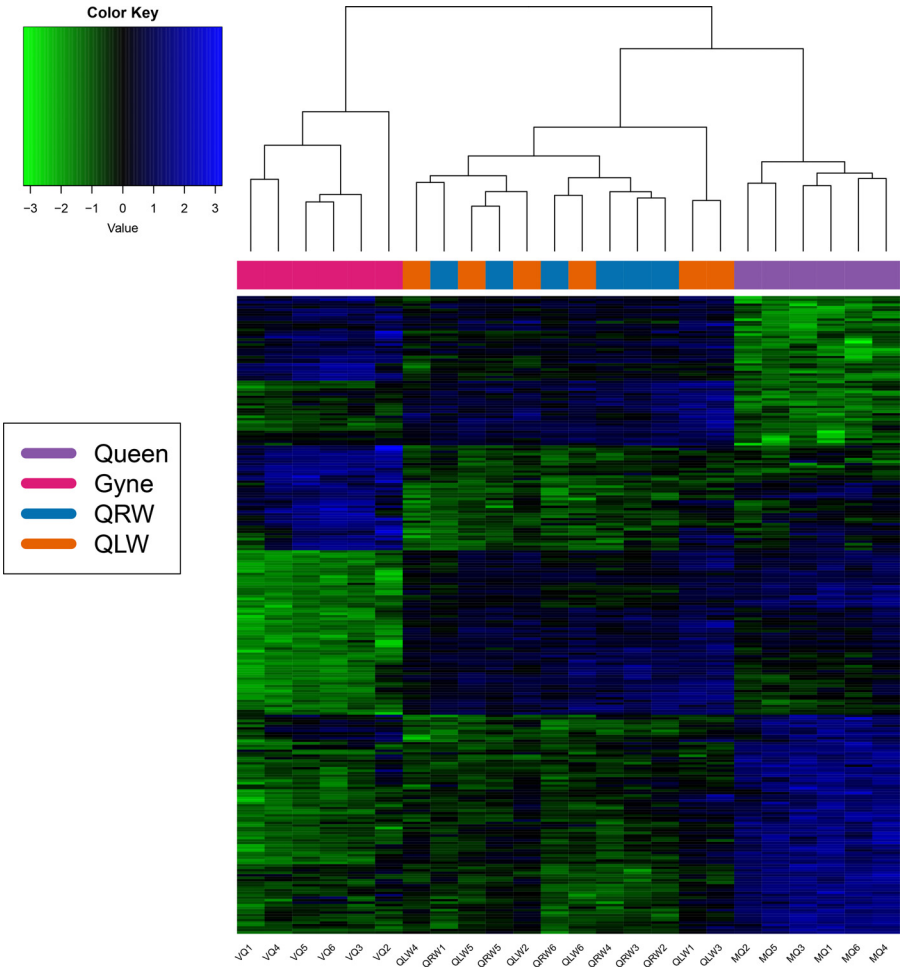

Fig. 3. – Heatmap showing the scaled (mean = 0, sd = 1), log2 expression values for a subset of the differentially expressed genes for each sample of the RNAseq experiment (6 samples per treatment and a total of 24 libraries). Rows are genes and columns are samples. The subset of genes was created by selecting the top 100 differentially expressed genes based on the highest absolute value of fold change for each contrast. This list produced 297 unique genes used in the heatmap. Colors at the tips of the dendrogram represent the treatment group of the samples.

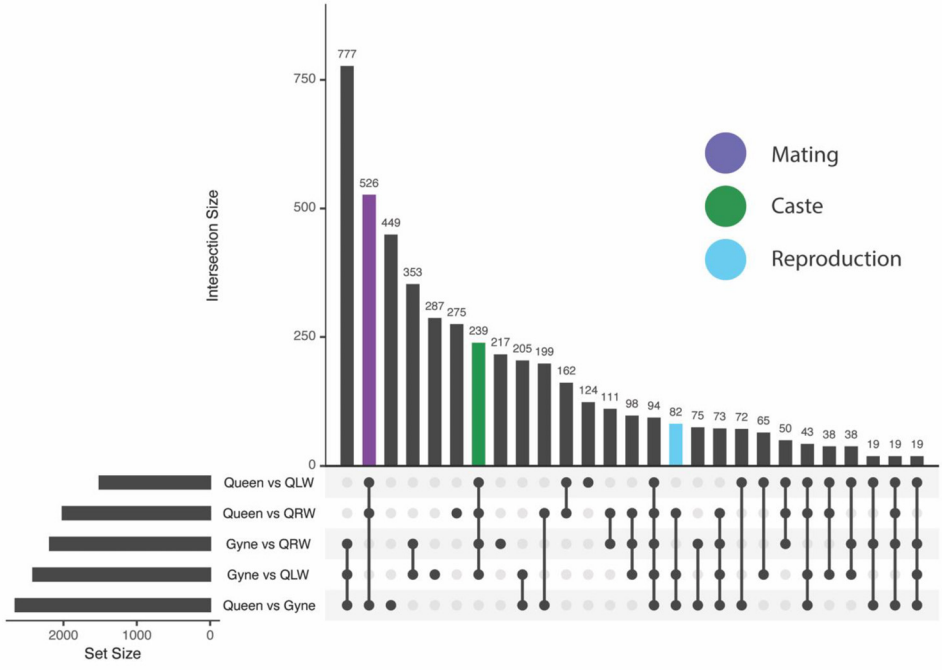

Fig. 4. – Upset plot showing the overlap of differentially expressed genes in the Dufour's gland between all pairwise comparisons. Colored bars represent gene lists likely to be enriched for genes related to mating status (purple), caste (green), and reproductive state (blue), selected by identifying overlap with comparisons sharing a given trait. Pairwise comparisons were between treatment the treatment groups "queen" (mated, egg-laying queens > 2 months old), "gyne" (unmated, non-reproductive 6-day old gynes), "QRW" (7-day old queenright workers), and "QLW" (7-day old queenless workers).

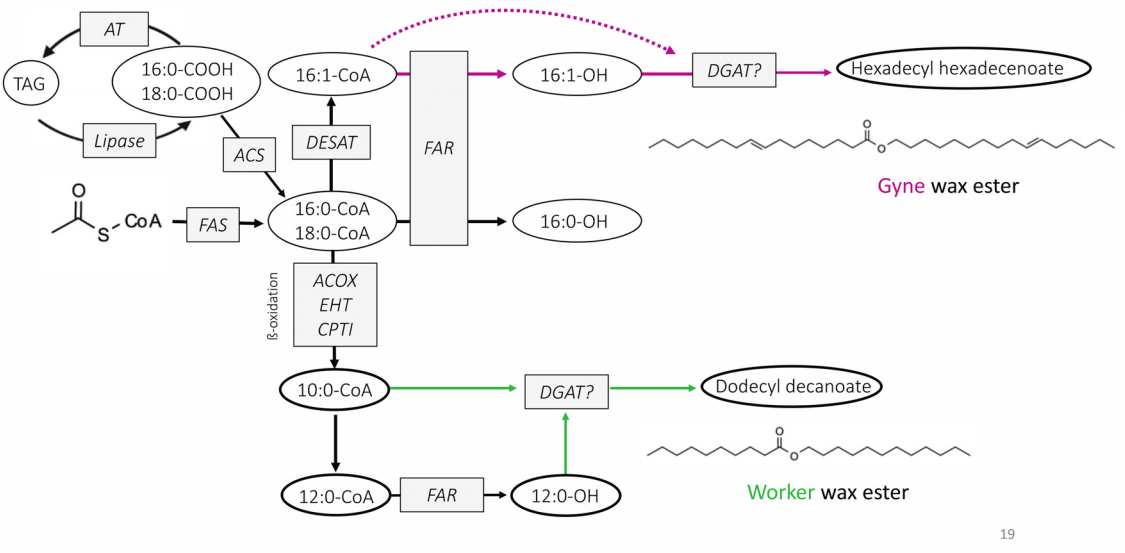

Fig. 5. – Proposed biosynthetic pathway of gyne-like or worker-like wax esters (figure adapted from Buček et al. 2017). Enzymes abbreviations in the pathway are: *FAS* (fatty acid synthase), *ACOX* (acyl-CoA oxidases, *EHT* (enoyl-CoA hydratases), *ACT* (3-ketoacyl-CoA thiolases), *CPTI* (carnitine O-palmitoyltransferase 1, liver isoform), *DESAT* (fatty acyl desaturases), *FAR* (fatty acyl reductases), *AT* (acyltransferases), *ACS* (acyl-CoA synthases).

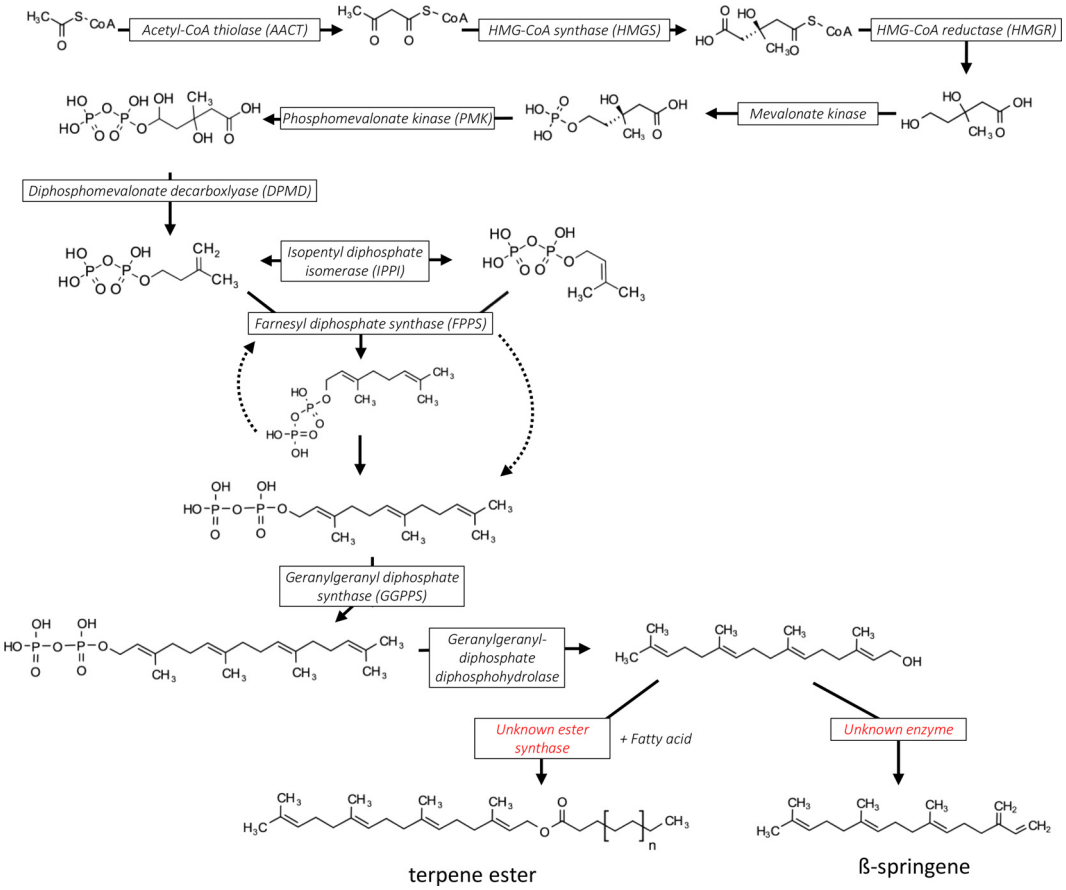

Fig. 6. – Proposed biosynthetic pathway of terpenes and terpene esters which are unique to the queen caste (adapted from Buček et al. 2017). The figure depicts how geranylgeraniol could either be reduced to produce *β*-springene by an unknown enzyme or combined with a fatty acid to produce a terpene ester.

Enrichment of GO classification terms in DEG lists of each pairwise comparison of treatment groups shows significant enrichment in many terms related to fatty acid metabolism, catabolism, lipid transport, and proteasome processes, among others. A full list of significantly enriched GO terms for each DEG list is given in tables S9–15.

4. Discussion

In the current study we examine the chemical composition and patterns of gene expression in the Dufour's gland of *Bombus impatiens* with regard to caste, reproductive, and mating status. We focus on genes

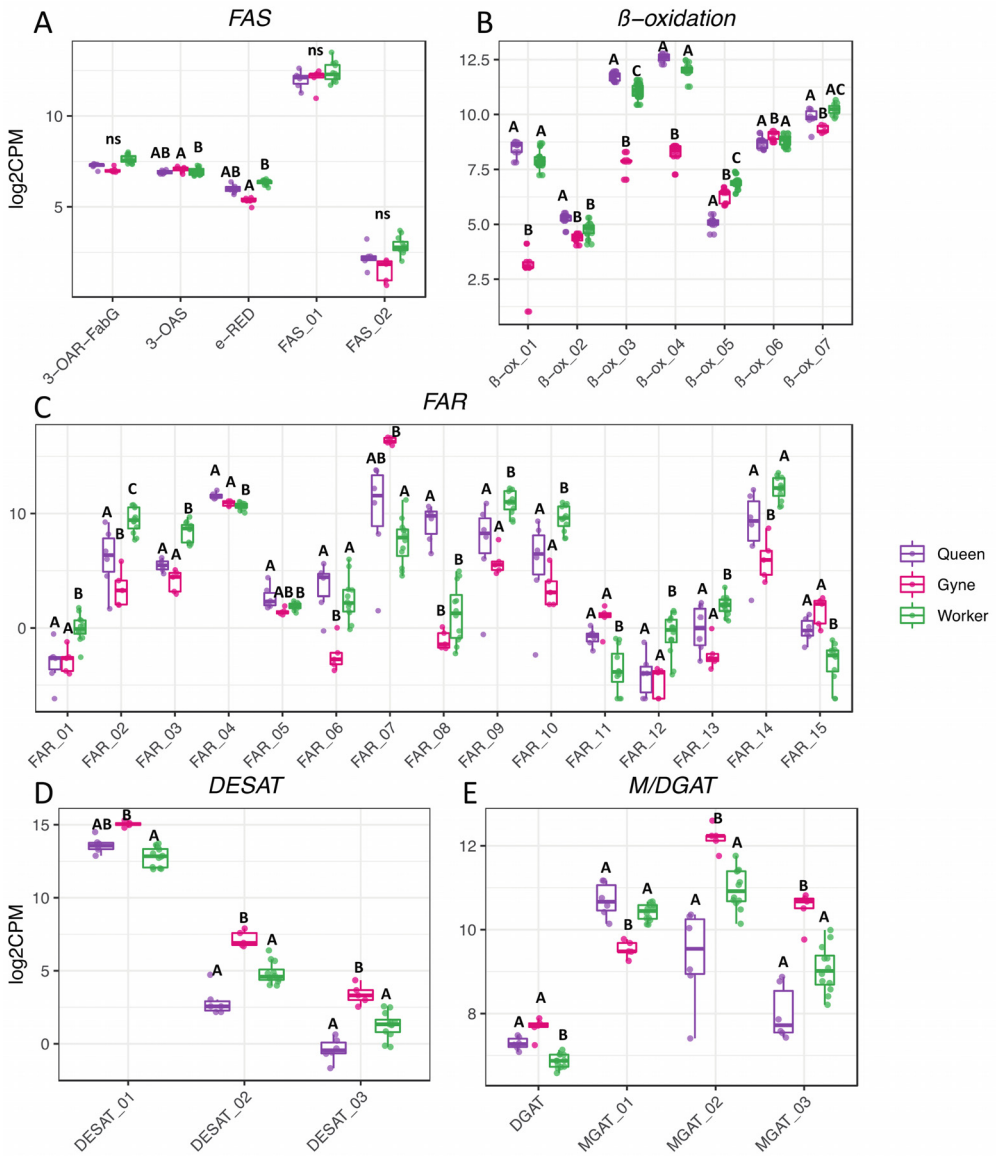

Fig. 7. – DEGs from RNAseq analyses involved in fatty acid biosynthesis. Gene IDs and annotations of labeled genes are given in Table S8. Data are presented as boxplots overlaid with each data point. Letters above columns denote statistical differences of pairwise contrasts using Benjamini and Hochberg adjusted p-values at $\alpha = 0.05$.

predicted to participate in the biosynthesis of esters, terpenes, and terpenoids that likely act as signals mediating social or mating behaviors. As predicted, we found that many of the enzymes involved in fatty acid and isoprenoid/mevalonate biosynthesis were significantly expressed and differentially regulated across castes, highlighting the role of the gland in semiochemical biosynthesis and providing many candidate genes for further investigation. Overall, differential expression analyses showed large differences between the DG of gynes and workers (~25% of detected genes) and queens and workers (~30% of detected genes), while QRW and QLW were indistinguishable. We find partial support for our initial hypotheses regarding patterns of gene expression and the different chemical phenotypes between queens, gynes, and workers. For instance, chain-shortening genes in the fatty acid pathway were highly expressed in workers, as predicted, but also highly expressed in queens, which may be due to the gene serving multiple functions. In the second hypothesis, that FAR and acyltransferase genes would be downregulated in queens and upregulated in gynes and workers, correlating with the amount of wax esters in their DG, we find general support in genes annotated as acyltransferases, but due to the high number of FAR genes showing differential expression between groups and uncertainty regarding their specific substrates, a clear pattern related to chemical phenotype is not discernible. We again find partial support for the third hypothesis, that genes in the isoprenoid biosynthetic pathway would be upregulated in gynes, specifically in the expression levels of HMGS and HMGR which are early genes that likely regulate the whole pathway.

The chemical analyses of the DG composition confirm it is distinct among queens, gynes, QLW, and QRW in line with previous work in *B. impatiens* (Derstine et al., 2021). In gynes and queens, this is primarily explained by the production of longer chain wax esters (e.g., hexadecyl hexadecenoate) and terpene compounds (β-springene and isomers in addition to unidentified terpene esters that are absent in workers). However, also the relative amounts of various chain length alkanes and alkenes is specific to caste and reproductive state. Further differentiating the castes are a series of worker-specific dodecyl esters which are produced in larger amounts by queenright compared to queenless workers and are not produced by queens. These worker-specific esters are found in higher proportion in QRW with inactive ovaries (Derstine et al., 2021).

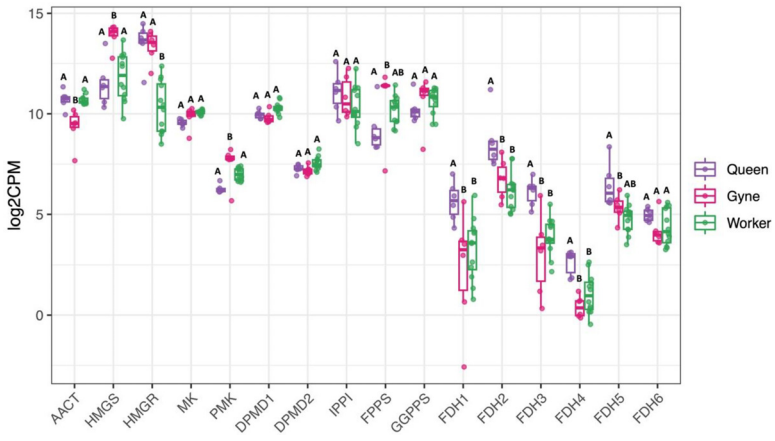

Fig. 8. – DEGs from RNAseq analyses in the isoprenoid/mevalonate biosynthetic pathway. Gene IDs and annotations of labeled genes are given in Table S8. Data are presented as boxplots overlaid with each data point. Letters above columns denote statistical differences of pairwise contrasts using Benjamini and Hochberg adjusted p-values at $\alpha = 0.05$.

Linking chemical and gene expression phenotypes, we found many candidate genes likely involved in the biosynthesis of DG compounds. Among these were FARs and M/DGATs involved in wax ester biosynthesis, as well as high expression of genes in the isoprenoid/mevalonate pathway that leads to terpenes. Fatty-acyl desaturases, a class of enzymes in the fatty acid biosynthesis pathway which introduce double bonds, were all upregulated in gynes compared to queens and workers. This matches the chemistry data from the Dufour's gland showing that gynes produce the largest amounts of both unsaturated hydrocarbons and wax esters with unsaturated alcohol or acid moieties. Similarly, the acid moiety of most of the long chain wax esters found in gynes contains unsaturations. Worker wax esters are further differentiated from queens and gynes by decreased length, which could be explained by an upregulated β-oxidation chain-shortening process. This iterative reduction in fatty acid chain length by two carbons occurs in both mitochondria and peroxisomes, and β-oxidation genes involved in both locations are differentially expressed in the Dufour's gland (Demarquoy and Le Borgne, 2015). However, the peroxisomal genes (β-ox_01–04, Fig 7b) are more involved in biosynthetic as opposed to catabolic processes, and accordingly, show much larger fold change differences in workers and queens compared to gynes.

Interestingly, we find that caste is much more explanatory of chemical phenotypes than reproductive state, a finding highlighted by the lack of DEGs between QRW and QLW which vary drastically in ovarian activation. Gene expression data did not explain the quantitative differences in the DG composition of worker groups, or more generally reflect any differences related to reproductive state, although the DG opens into the oviduct and is associated with the reproductive system of females. It is possible that differences in DG composition between QRW and QLW could result from the differential secretion of certain compounds, while the production remains the same. For instance, QLW which have a lower percentage of esters could be secreting them at a higher rate than QRW in line with a reproductive signaling function. Alternatively, regulation of these compounds could happen at the protein instead of the transcript level, which we would not detect with RNA sequencing.

Wax esters in the Dufour's gland could be biosynthesized via two different routes, either de novo, or from fatty acids transported from outside the gland. In the first option, fatty acids synthesized outside the gland are stored as triacylglycerol (TAG) in fat body tissue. Specific lipases act on TAGs to produce diacylglycerol (DAG), which is bound to the transport protein lipophorin and transported to various tissues (Majerowicz and Gondim, 2013; Schal et al., 1998). Once inside the cell, lipases release fatty acids from DAG where they are converted to their final form by the necessary array of enzymes. In the de novo route, fatty acids are constructed from acetate units inside the cell, where the same array of enzymes would convert them to their final form. Thus, expression of genes early in the fatty acid biosynthetic pathway provide evidence for de novo biosynthesis. This may be indicated by the expression of fatty acid synthase (FAS), which combines acetate units to produce 16 and 18 carbon fatty acyl-CoA molecules. While not differentially expressed between the groups, FAS has relatively high expression compared to many genes found in the study. Furthermore, the expression of numerous elongases which extend fatty acyl-CoA molecules beyond 16 and 18 carbons is consistent with de novo hydrocarbon synthesis. Previous work in honey bees found that the hydrocarbons are likely transported into the DG while the esters are synthesized de novo (Gozansky et al., 1997). This has yet to be established in *Bombus*.

Similarly, early genes in the terpene pathway, including AACT, HMGS, and HMGR, the rate limiting enzyme of the isoprenoid pathway (Friesen and Rodwell, 2004), were highly expressed relative to other genes we detected, providing strong evidence for de novo terpene synthesis. The pathways first steps are shared by almost all organisms and result in the production of isopentyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) from acetyl-CoA (Miziorko, 2011). These two molecules act as the substrates for a variety of enzymes, which can be functionalized and combined in various iterations to produce the plethora of mono-, sesqui-, and di-terpenes and terpenoids (Darragh et al., 2021; Miziorko, 2011; Morgan, 2010), including those commonly found in various bumble bee exocrine glands, such as β-springene, dihydrofarnesol, and terpene esters such as the fatty acid esters of geranylcitronellol or farnesol (Derstine et al., 2021; Abraham Hefetz et al., 1996; Orlova et al., 2021). Overall, the high levels of expression and numerous DEGs in fatty acid and isoprenoid biosynthetic pathways are consistent with de novo pheromone synthesis of esters and terpenes in the Dufour's gland.

Wax esters, an important class of compounds in the Dufour's gland (Abdalla and Cruz-Landim, 2001; Cane, 1981; Mitra, 2013), are biosynthesized by likely conserved enzymes in the acyltransferase family. However, no specific wax synthases have yet been identified in insects. In mice, wax esters are biosynthesized by two acyltransferases, a multifunctional *DGAT1*, which has moderate ability to produce wax esters, primarily catalyzing the production of triacylglycerols, and a "dedicated" wax synthase, which produces wax monoesters but has little ability to catalyze other types of esters (Cheng and Russell, 2004; Yen et al., 2005). Plants and bacteria have either a specific wax synthase enzyme, or multifunctional acyltransferases such as DGATs which both catalyze fatty acid ester formation and triacylglycerols. In the honey bee *A. mellifera*, the monoesters found in beeswax are typically composed of C16-C20 acids and C24–32 or larger alcohols, and are synthesized by an acyltransferase enzyme, as shown by studies using radio-labeled precursors (Blomquist and Ries, 1979). A candidate wax synthase gene in the acyltransferase family was identified in the white wax scale *Ericerus pela* by comparing gene expression levels in instars, but has yet to be functionally characterized (Yang et al., 2012). Here, we identified several

differentially expressed acyltransferase genes which could function as wax synthases (Fig 7E) but will require follow-up functional validation studies to conform their role.

As a chemical class, terpenes are famously diverse, and thus we expect the genes involved in their biosynthesis to be less specific than in wax ester biosynthesis. To narrow down the focus, we selected a specific tissue (e.g., the Dufour's gland is not predicted to participate in the biosynthesis of other common insect terpenes, like the corpora allata is in producing juvenile hormone) and focused on the rate limiting enzymes. Two genes responsible for terpene synthesis, HMGS and HMGR, were DE in queens and/or gynes compared to workers, potentially explaining the presence of terpenes and terpenes esters that are absent in workers. Data from other species of *Bombus* show transcript abundances of isoprenoid biosynthesis genes (AACT, HMGR, FFPS) correlated with observed differences between male pheromone components of *B. lucorum,* which are primarily fatty-acid derived compounds (ethyl tetradecenoate and ethyl dodecanoate), and *B. terrestris,* which are primarily isoprenoid derived (E-2,3-dihydrofarnesol, geranylcitronellol, and 2,3-dihydrofarnesal) (Prchalova et al., 2016). However, while gynes clearly have the largest amount and proportion of terpene compounds, many of the genes in the pathway often have similar expression as workers, which produce none. HMGR is the rate-limiting enzyme in this pathway, and is under extensive regulation at the level of transcription, translation, and post-translation (Friesen and Rodwell, 2004). This coupled with the diverse downstream products of terpene synthesis help to explain the expression seen in worker groups which do not produce terpenes.

Biosynthetic studies of other organisms with terpene pheromones also found expression of early mevalonate pathway genes in pheromone producing tissues, coupled with highly specific but often unknown downstream enzymes such as terpene synthases. This is the case in the psychodid fly *Lutzomyia longipalpis*, which produces a blend of homosesquiterpenes that function as a male sex pheromone (González-Caballero et al., 2014; González-Caballero et al., 2013), and in the terpene based male labial gland components of *Bombus terrestris* (Bucek et al., 2016; Prchalova et al., 2016). Until recently, no terpene synthases had been identified in insects, but it has now been shown in several lineages that insects have co-opted known genes in the isoprenoid pathway. For instance, enzymes with terpene synthase activity in flea beetles (Beran et al., 2016), stink bugs (Lancaster et al., 2018, 2019) and a *Heliconius* butterfly (Darragh et al., 2021) derive from an isoprenyl diphosphate synthase (IDS), and represent an independent evolution of this function from plants and microorganisms.

Understanding the biosynthesis and genetic basis of insect pheromones allows the study of their evolution, which can inform important processes like the evolution of social behavior. In social insects, reproductive division of labor is regulated in part by chemical communication, with different castes signaling fertility or producing pheromones which regulate ovarian activation or egg-laying (A. Hefetz, 2019; Y. Le Conte and Hefetz, 2008). In many species, these females have the same genotype, and thus the different pheromones or pheromone blends they produce are likely the result of differential gene expression. In honey bees, queens regulate worker reproduction with a multi-component pheromone blend from the mandibular gland, the major components of which are 9-oxo-2-decenoic acid (9-ODA) and 9-hydroxy-2-decenoic acid (9-HDA) (Hoover et al., 2003; Slessor et al., 1990), while the major product in workers is 10-hydroxy-2-decenoic acid (10-HDA) (E Plettner et al., 1995; Slessor et al., 1988). Research to understand the caste-specific biosynthesis of these compounds identified the hydroxylation step as the probable divergence point between queen and worker chemical phenotypes, and posited that the key enzymes were CYP450 hydroxylases with different substrate affinities (Erika Plettner et al., 1998). This was confirmed in later work that identified differential expression between CYP4AA1 (worker hydroxylation pattern) and CYP18A1 (queen hydroxylation pattern) as producing the observed patterns of caste-specific hydroxy acid biosynthesis (O. Malka et al., 2009). Furthermore, this expression can be plastic, with queenless workers able to switch pathways and produce more queen-like secretions (Crewe and Velthuis, 1980; Osnat Malka et al., 2006; E Plettner et al., 1993). These insights have helped explain biological phenomena such as the interesting case of the honey bee subspecies *A. m. capensis*, whose workers are capable of becoming social parasites in part by upregulating the queen specific hydroxylation pathway to produce higher amounts 9-ODA and 9-HDA (F. N. Mumoki et al., 2019; Fiona N Mumoki et al., 2021). Additionally, the evolution of queen pheromones is thought to be an important step in the evolution of eusociality, and understanding the genes involved is necessary for comparative studies with solitary ancestors that seek to understand the evolutionary origin of pheromones regulating reproduction (Treanore et al., 2021).

Finally, matching gene expression to chemical phenotypes is complicated by the multifunctional nature of many of the enzymes involved, and the limited functional information available for the genes of interest. For example, the synthesis of dodecyl esters produced by *B. impatiens* workers are likely to involve activity by FAR and DGAT in the final synthetic step. However, DGAT genes likely catalyze not only wax ester formation but also triacylglycerides. Additionally, FAR enzymes play a role in the reduction of fatty-acyl molecules, a process taking place in all castes, regardless of caste-specific pheromone production, and little is known about the substrate specificity of the numerous FAR enzymes. Furthermore, it is unknown if similar signals produced by different castes (e.g., shorter chain esters in workers and long chain/terpen esters in gynes) are synthesized by the same enzymes. Therefore, multifunctional enzymes pose a challenge and do not necessarily result in direct matches between chemical and gene expression phenotypes. Future work to functionally validate select genes could help resolve these issues. The FAR and MGAT genes which show strong differential expression between gynes and workers (Fig 7C and 7E) would be excellent candidates.

Overall, our data provide ample opportunities to further the understanding of signal evolution and their genetic basis in social insects with functional studies targeting specific genes, and experiments designed to assess the specific reactions and substrate specificity catalyzed by the many candidate enzymes described. As genes involved in the production of social signals are more likely to have undergone recent selection, it would also be fruitful to compare rates of evolution and gather evidence about the type of selection experienced by these genes during the transition from their solitary past.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Nathan Derstine: Conceptualization, Investigation, Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing. **David Galbraith:** Software, Investigation, Data curation, Writing – review & editing. **Gabriel Villar:** Conceptualization, Investigation, Writing – review & editing. **Etya Amsalem:** Conceptualization, Investigation, Writing – review & editing, Resources, Supervision, Funding acquisition.

Data availability

Raw sequence reads and metadata have been deposited at NCBI under BioProject PRJNA912882. Chemical data from the analysis of the Dufour's gland secretion, terminal oocyte measurements of bees used in the study, and unfiltered counts of gene expression for all samples are availble in Supplementary Data File S1.

Acknowledgments

This work was funded by NSF-CAREER IOS-1942127 to EA. We would like to thank two anonymous reviewers for comments which improved the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2023.100056.

References

Abdalla, F.C., Cruz-Landim, C., 2001. Dufour gland in the hymenopterans (apidae, formicidae, vespidae); a review. Braz. J. Biol. 6 (1), 95–106.

Alexa A., & Rahnenfuhrer J. (2022). Package 'topGO' enrichment analysis for gene ontology. R package version 2.50.0.

Amsalem, E., 2020. One problem, many solutions: female reproduction is regulated by chemically diverse pheromones across insects. Adv. Insect Phys. 59, 131.

Amsalem, E., Hefetz, A., 2010. The appeasement effect of sterility signaling in dominance contests among Bombus terrestris workers. Behav. Ecol. Sociobiol. Print 64 (10), 1685–1694. doi:10.1007/s00265-010-0982-4.

Amsalem, E., Shpigler, H., Bloch, G., Hefetz, A., 2013. Dufour's gland secretion, sterility and foraging behavior: correlated behavior traits in bumblebee workers. J. Insect Physiol. 59 (12), 1250–1255. doi:10.1016/j.jinsphys.2013.09.007.

Amsalem, E., Twele, R., Francke, W., Hefetz, A., 2009. Reproductive competition in the bumble-bee Bombus terrestris: do workers advertise sterility? Proc. Biol. Sci. 276 (1660), 1295–1304. doi:10.1098/rspb.2008.1688.

Andreadis, S.S., Cloonan, K.R., Myrick, A.J., Chen, H., Baker, T.C., 2015. Isolation of a female-emitted sex pheromone component of the fungus gnat, lycoriella ingenua, attractive to males. J. Chem. Ecol. 41 (12), 1127–1136. doi:10.1007/s10886-015-0650-2.

Andrews, S., 2010. FastQC: a quality control tool for high throughput sequence data. Babraham Bioinformatics. Babraham Institute, Cambridge.

Bellés, X., Martín, D., Piulachs, M.D., 2005. The mevalonate pathway and the synthesis of juvenile hormone in insects. Annu. Rev. Entomol. 50, 181–199.

Benjamini, Y., Hochberg, Y., 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Methodol. 57 (1), 289–300.

Beran, F., Jiménez-Alemán, G.H., Lin, M.-y., Hsu, Y.-C., Mewis, I., Srinivasan, R., … Reinecke, A., 2016a. The aggregation pheromone of phyllotreta striolata (coleoptera: chrysomelidae) revisited. J. Chem. Ecol. 42 (8), 748–755. doi:10.1007/s10886-016-0743-6.

Beran, F., Rahfeld, P., Luck, K., Nagel, R., Vogel, H., Wielsch, N., … Heckel, D.G., 2016b. Novel family of terpene synthases evolved from trans-isoprenyl diphosphate synthases in a flea beetle. Proc. Natl. Acad. Sci. 113 (11), 2922–2927.

Blatrix, R., Schulz, C., Jaisson, P., Francke, W., Hefetz, A., 2002. Trail pheromone of ponerine ant Gnamptogenys striatula: 4-methylgeranyl esters from Dufour's gland. J. Chem. Ecol. 28 (12), 2557–2567.

Blighe K., Rana S., & Lewis M. (2021). EnhancedVolcano: publication-ready volcano plots with enhanced colouring and labeling. 2020. R Package Version 1.8. 0. In.

Blomquist, G.J., Bagnères, A.G., 2010. Insect hydrocarbons: biology, biochemistry, and Chemical Ecology. Cambridge University Press.

Blomquist, G.J., Ries, M.K., 1979. The enzymatic synthesis of wax esters by a microsomal preparation from the honeybee Apis mellifera L. Insect Biochem. 9 (2), 183–188.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30 (15), 2114–2120. doi:10.1093/bioinformatics/btu170.

Brabcova, J., Demianova, Z., Kindl, J., Pichova, I., Valterova, I., Zarevucka, M., 2015. Characterisation of acetyl-CoA thiolase: the first enzyme in the biosynthesis of terpenic sex pheromone components in the labial gland of bombus terrestris. ChemBioChem 16 (7), 1047–1051. doi:10.1002/cbic.201402591.

Bucek, A., Brabcova, J., Vogel, H., Prchalova, D., Kindl, J., Valterova, I., Pichova, I., 2016. Exploring complex pheromone biosynthetic processes in the bumblebee male labial gland by RNA sequencing. Insect Mol. Biol. 25 (3), 295–314. doi:10.1111/imb.12221.

Cahlíková, L., Hovorka, O., Ptáček, V., Valterová, I., 2004. Exocrine gland secretions of virgin queens of five bumblebee species (Hymenoptera: apidae, Bombini). Z. Naturforsch. C 59 (7–8), 582–589.

Cane, J.H., 1981. Dufour's gland secretion in the cell linings of bees (Hymenoptera: apoidea). J. Chem. Ecol. 7 (2), 403–410. doi:10.1007/BF00995762.

Cheng, J.B., Russell, D.W., 2004. Mammalian wax biosynthesis. II. Expression cloning of wax synthase cDNAs encoding a member of the acyltransferase enzyme family. J. Biol. Chem. 279 (36), 37798–37807. doi:10.1074/jbc.M406226200.

Conway, J.R., Lex, A., Gehlenborg, N., 2017. UpSetR: an R package for the visualization of intersecting sets and their properties. Bioinform.

Crewe, R., Velthuis, H., 1980. False queens: a consequence of mandibular gland signals in worker honeybees. Naturwissenschaften 67, 467–469.

Darragh, K., Orteu, A., Black, D., Byers, K.J., Szczerbowski, D., Warren, I.A., … Fernanda Garza, S., 2021. A novel terpene synthase controls differences in anti-aphrodisiac pheromone production between closely related Heliconius butterflies. PLoS Biol. 19 (1), e3001022.

Demarquoy, J., Le Borgne, F., 2015. Crosstalk between mitochondria and peroxisomes. World J. Biol. Chem. 6 (4), 301–309.

Derstine, N.T., Gries, R., Zhai, H., Jimenez, S.I., Gries, G., 2018. Cuticular hydrocarbons determine sex, caste, and nest membership in each of four species of yellowjackets (Hymenoptera: vespidae). Insectes Soc. 65 (4), 581–591. doi:10.1007/s00040-018-0649-0.

Derstine, N.T., Villar, G., Orlova, M., Hefetz, A., Millar, J., Amsalem, E., 2021. Dufour's gland analysis reveals caste and physiology specific signals in *Bombus impatiens*. Sci. Rep. 11 (1), 2821. doi:10.1038/s41598-021-82366-2.

Dietemann, V., Peeters, C., Liebig, J., Thivet, V., Holldobler, B., 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant Myrmecia gulosa. Proc. Natl. Acad. Sci. U. S. A. 100 (18), 10341–10346. doi:10.1073/pnas.1834281100.

Dinno, A., Dinno, M.A., 2017. Package 'dunn. test. CRAN Repos 10.

Dobin, A., Davis, C.A., Schlesinger, F., Drenkow, J., Zaleski, C., Jha, S., … Gingeras, T.R., 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29 (1), 15–21. doi:10.1093/bioinformatics/bts635.

Dor, R., Katzav-Gozansky, T., Hefetz, A., 2005. Dufour's gland pheromone as a reliable fertility signal among honeybee (Apis mellifera) workers. Behav. Ecol. Sociobiol. (Print) 58 (3), 270–276. doi:10.1007/s00265-005-0923-9.

Endler, A., Liebig, J., Schmitt, T., Parker, J.E., Jones, G.R., Schreier, P., Holldobler, B., 2004. Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. Proc. Natl. Acad. Sci. U. S. A. 101 (9), 2945–2950. doi:10.1073/pnas.0308447101.

Engel, K.C., Stokl, J., Schweizer, R., Vogel, H., Ayasse, M., Ruther, J., Steiger, S., 2016. A hormone-related female anti-aphrodisiac signals temporary infertility and causes sexual abstinence to synchronize parental care. Nat. Commun. 7, 11035. doi:10.1038/ncomms11035.

Ewels, P., Magnusson, M., Lundin, S., Kaller, M., 2016. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics 32 (19), 3047–3048. doi:10.1093/bioinformatics/btw354.

Friesen, J.A., Rodwell, V.W., 2004. The 3-hydroxy-3-methylglutaryl coenzyme-A (HMG–CoA) reductases. Genome Biol. 5 (11), 1–7.

González-Caballero, N., RodrÌguez-Vega, A., Dias-Lopes, G., Valenzuela, J.G., Ribeiro, J.M.C., Carvalho, P.C., … Cuervo, P., 2014. Expression of the mevalonate pathway enzymes in the Lutzomyia longipalpis (Diptera: psychodidae) sex pheromone gland demonstrated by an integrated proteomic approach. J. Proteomics 96, 117–132.

González-Caballero, N., Valenzuela, J.G., Ribeiro, J.M.C., Cuervo, P., Brazil, R.P., 2013. Transcriptome exploration of the sex pheromone gland of Lutzomyia longipalpis (Diptera: psychodidae: phlebotominae). Parasites Vectors 6 (1), 56. doi:10.1186/1756-3305-6-56.

Gozansky, T.K., Soroker, V., Hefetz, A., 1997. The biosynthesis of Dufour's gland constituents in queens of the honeybee (Apis mellifera). Invert. Neurosci. 3 (2–3), 239–243.

Hefetz, A., 2019. The critical role of primer pheromones in maintaining insect sociality. Z. Naturforsch. C. 74 (9–10), 221–231. doi:10.1515/znc-2018-0224.

Hefetz, A., Taghizadehr, T., Francke, W., 1996. The exocrinology of the queen bumble bee Bombus terrestris (Hymenoptera: apidae, Bombini). Z. für Naturforsch. C 51 (5–6), 409–422.

Hervé M., & Hervé M.M. (2020). Package 'RVAideMemoire'. See https://CRAN.R-project.org/package=RVAideMemoire.

Hojo, M., Maekawa, K., Saitoh, S., Shigenobu, S., Miura, T., Hayashi, Y., … Maekawa, H., 2012. Exploration and characterization of genes involved in the synthesis of diterpene defence secretion in nasute termite soldiers. Insect Mol. Biol. 21 (6), 545–557. doi:10.1111/j.1365-2583.2012.01162.x.

Hoover, S.E., Keeling, C.I., Winston, M.L., Slessor, K.N., 2003. The effect of queen pheromones on worker honey bee ovary development. Naturwissenschaften 90 (10), 477–480. doi:10.1007/s00114-003-0462-z.

Kalscheuer, R., Steinbüchel, A., 2003. A Novel Bifunctional Wax Ester Synthase/Acyl-CoA:diacylglycerol Acyltransferase Mediates Wax Ester and Triacylglycerol Biosynthesis inAcinetobacter calcoaceticus ADP1*. J. Biol. Chem. 278 (10), 8075–8082. doi:10.1074/jbc.M210533200.

Katzav-Gozansky, T., Soroker, V., Hefetz, A., 2000. Plasticity in caste-related exocrine secretion biosynthesis in the honey bee (*Apis mellifera)*. J. Insect Physiol. 46, 993–998.

Keeling, C.I., Plettner, E., Slessor, K.N., 2004. Hymenopteran semiochemicals. Top. Curr. Chem. 239, 133–177. doi:10.1007/b95452.

Kingwell, C., Böröczky, K., Steitz, I., Ayasse, M., Wcislo, W., 2021. Cuticular and dufour's gland chemistry reflect reproductive and social state in the facultatively eusocial sweat bee megalopta genalis (hymenoptera: halictidae). J. Chem. Ecol. 47 (4), 420–432.

Krijthe J.H., & Van der Maaten L. (2015). Rtsne: t-distributed stochastic neighbor embedding using Barnes-Hut implementation. *R Package Version 0.13,* URL https://github.com/jkrijthe/Rtsne.

Lancaster, J., Khrimian, A., Young, S., Lehner, B., Luck, K., Wallingford, A., … Marek, P.E., 2018. De novo formation of an aggregation pheromone precursor by an isoprenyl diphosphate synthase-related terpene synthase in the harlequin bug. Proc. Natl Acad. Sci. 115 (37), E8634–E8641.

Lancaster, J., Lehner, B., Khrimian, A., Muchlinski, A., Luck, K., Köllner, T.G., … Tholl, D., 2019. An IDS-type sesquiterpene synthase produces the pheromone precursor (Z)-α-Bisabolene in Nezara viridula. J. Chem. Ecol. 45 (2), 187–197.

Law, C.W., Alhamdoosh, M., Su, S., Dong, X., Tian, L., Smyth, G.K., Ritchie, M.E., 2016. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. F1000Research 5. doi:10.12688/f1000research.9005.3.

Law, C.W., Chen, Y., Shi, W., Smyth, G.K., 2014. voom: precision weights unlock linear model analysis tools for RNA-seq read counts. Genome Biol. (R29) 15.

Le Conte, Y., Hefetz, A, 2008. Primer pheromones in social hymenoptera. Annu. Rev. Entomol. 53, 523–542. doi:10.1146/annurev.ento.52.110405.091434.

Le Conte, Y., Mohammedi, A., Robinson, G.E, 2001. Primer effects of a brood pheromone on honeybee behavioral development. Proc. R. Soc. Lond. B Biol. Sci. 268 (1463), 163–168.

Li, B., Dewey, C.N., 2011. RSEM: accurate trascript quantification from RNA-Seq data with or without a reference genome. BMC Bioinf. 12 (1–16).

Li, F., Wu, X., Lam, P., Bird, D., Zheng, H., Samuels, L., … Kunst, L., 2008. Identification of the wax ester synthase/acyl-coenzyme A: diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in Arabidopsis. Plant Physiol. 148 (1), 97–107. doi:10.1104/pp.108.123471.

Majerowicz D., & Gondim K.C. (2013). Insect lipid metabolism: insights into gene expression regulation. Recent Trends Gene Expr 147–189.

Malka, O., Karunker, I., Yeheskel, A., Morin, S., Hefetz, A., 2009. The gene road to royalty–differential expression of hydroxylating genes in the mandibular glands of the honeybee. FEBS J. 276 (19), 5481–5490. doi:10.1111/j.1742-4658.2009.07232.x.

Malka, O., Shnieor, S., Hefetz, A., Katzav-Gozansky, T., 2006. Reversible royalty in worker honeybees (Apis mellifera) under the queen influence. Behav. Ecol. Sociobiol. Print 61 (3), 465–473. doi:10.1007/s00265-006-0274-1.

Matoušková, P., Luxová, A., Matoušková, J., Jiroš, P., Svatoš, A., Valterová, I., Pichová, I., 2008. A Δ9 desaturase from Bombus lucorum males: investigation of the biosynthetic pathway of marking pheromones. ChemBioChem 9 (15), 2534–2541.

Mayoral, J.G., Nouzova, M., Navare, A., Noriega, F.G., 2009. NADP+-dependent farnesol dehydrogenase, a corpora allata enzyme involved in juvenile hormone synthesis. Proc. Natl. Acad. Sci. 106 (50), 21091–21096.

Menzel, F., Morsbach, S., Martens, J.H., Räder, P., Hadjaje, S., Poizat, M., Abou, B., 2019. Communication versus waterproofing: the physics of insect cuticular hydrocarbons. J. Exp. Biol. 222 (23), jeb210807.

Mitra, A., 2013. Function of the Dufour's gland in solitary and social Hymenoptera. J. Hymenopt. Res. 35, 33–58. doi:10.3897/jhr.35.4783.

Miziorko, H.M., 2011. Enzymes of the mevalonate pathway of isoprenoid biosynthesis. Arch. Biochem. Biophys. 505 (2), 131–143.

Morgan, E.D., 2010. Biosynthesis in Insects. Royal society of chemistry.

Mumoki, F.N., Yusuf, A.A., Pirk, C.W.W., Crewe, R.M., 2019. Hydroxylation patterns associated with pheromone synthesis and composition in two honey bee subspecies Apis mellifera scutellata and a. m. capensis laying workers. Insect Biochem. Mol. Biol. 114, 103230. doi:10.1016/j.ibmb.2019.103230.

Mumoki, F.N., Yusuf, A.A., Pirk, C.W.W., Crewe, R.M., 2021. The biology of the cape honey bee, apis mellifera capensis (hymenoptera: apidae): a review of thelytoky and its influence on social parasitism and worker reproduction. Ann. Entomol. Soc. Am. 114 (2), 219–228. doi:10.1093/aesa/saaa056.

Oi, C.A., Van Oystaeyen, A., Caliari Oliveira, R., Millar, J.G., Verstrepen, K.J., van Zweden, J.S., Wenseleers, T., 2015. Dual effect of wasp queen pheromone in regulating insect sociality. Curr. Biol. 25 (12), 1638–1640. doi:10.1016/j.cub.2015.04.040.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'hara, R., … Wagner, H., 2013. Package 'vegan. Commun. Ecol. Package Version 2 (9), 1–295.

Orlova, M., Villar, G., Hefetz, A., Millar, J.G., Amsalem, E., 2021. A gland of many uses: a diversity of compounds in the labial glands of the bumble bee Bombus impatiens suggests multiple signaling functions. Res. Sq. Prerint doi:10.21203/rs.3.rs-689566/v1.

Plettner, E., Slessor, K., Winston, M., Robinson, G., Page, R., 1993. Mandibular gland components and ovarian development as measures of caste differentiation in the honey bee (Apis mellifera L.). J. Insect Physiol. 39 (3), 235–240.

Plettner, E., Slessor, K.N., Winston, M.L., 1998. Biosynthesis of mandibular acids in honey bees (Apis mellifera): de novo synthesis, route of fatty acid hydroxylation and caste selective β-oxidation. Insect Biochem. Mol. Biol. 28 (1), 31–42.

Plettner, E., Sutherland, G., Slessor, K., Winston, M., 1995. Why not be a queen? Regioselectivity in mandibular secretions of honeybee castes. J. Chem. Ecol. 21 (7), 1017–1029.

Prchalova, D., Bucek, A., Brabcova, J., Zacek, P., Kindl, J., Valterova, I., Pichova, I., 2016. Regulation of isoprenoid pheromone biosynthesis in bumblebee males. ChemBioChem 17 (3), 260–267. doi:10.1002/cbic.201500415.

Raudvere, U., Kolberg, L., Kuzmin, I., Arak, T., Adler, P., Peterson, H., Vilo, J., 2019. g: profiler: a web server for functional enrichment analysis and conversions of gene lists (2019 update). Nucleic. Acids. Res. 47 (W1), W191–W198.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., Smyth, G.K., 2015. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic. Acids. Res. 43 (7), e47. doi:10.1093/nar/gkv007.

Rivera-Perez, C., Nouzova, M., Lamboglia, I., Noriega, F.G., 2014. Metabolic analysis reveals changes in the mevalonate and juvenile hormone synthesis pathways linked to the mosquito reproductive physiology. Insect Biochem. Mol. Biol. 51, 1–9.

Robinson, M.D., McCarthy, D.J., Smyth, G.K., 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics 26 (1), 139–140. doi:10.1093/bioinformatics/btp616.

Sadd, B.M., Barribeau, S.M., Bloch, G., de Graaf, D.C., Dearden, P., Elsik, C.G., … Worley, K.C., 2015. The genomes of two key bumblebee species with primitive eusocial organization. Genome Biol. 16, 1–32. doi:10.1186/s13059-015-0623-3.

Schal, C., Sevala, V., Cardé, R.T., 1998. Novel and highly specific transport of a volatile sex pheromone by hemolymph lipophorin in moths. Naturwissenschaften 85 (7), 339–342.

Slessor, K.N., Kaminski, L.A., King, G., Borden, J.H., Winston, M.L., 1988. Semiochemical basis of the retinue response to queen honey bees. Nature 332 (6162), 354–356.

Slessor, K.N., Kaminski, L.A., King, G., Winston, M.L., 1990. Semiochemicals of the honeybee queen mandibular glands. J. Chem. Ecol. 16 (3), 851–860.

Smith, A.A., Holldober, B., Liebig, J., 2009. Cuticular hydrocarbons reliably identify cheaters and allow enforcement of altruism in a social insect. Curr. Biol. 19 (1), 78–81. doi:10.1016/j.cub.2008.11.059.

Smith, A.A., Millar, J.G., Hanks, L.M., Suarez, A.V., 2013. A conserved fertility signal despite population variation in the cuticular chemical profile of the trap-jaw ant Odontomachus brunneus. J. Exp. Biol. 216 (Pt 20), 3917–3924. doi:10.1242/jeb.089482.

Soroker, V., Hefetz, A., 2000. Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. J. Insect Physiol. 46, 1097–1102.

Starkey, J., Brown, A., Amsalem, E., 2019. The road to sociality: brood regulation of worker reproduction in the simple eusocial bee *Bombus impatiens*. Anim. Behav. 154, 57–65. doi:10.1016/j.anbehav.2019.06.004.

Steiger, S., Stokl, J., 2018. Pheromones Regulating reproduction in subsocial beetles: insights with references to eusocial insects. J. Chem. Ecol. 44 (9), 785–795. doi:10.1007/s10886-018-0982-9.

Steitz, I., Ayasse, M., 2020. Macrocyclic lactones act as a queen pheromone in a primitively eusocial sweat bee. Curr. Biol. 30 (6), 1136–1141. doi:10.1016/j.cub.2020.01.026, e1133.

Steitz, I., Brandt, K., Biefel, F., Minat, A., Ayasse, M., 2019. Queen recognition signals in two primitively eusocial halictid bees: evolutionary conservation and caste-specific perception. Insects 10 (12). doi:10.3390/insects10120416.

Teerawanichpan, P., Qiu, X., 2010. Fatty acyl-CoA reductase and wax synthase from Euglena gracilis in the biosynthesis of medium-chain wax esters. Lipids 45 (3), 263–273. doi:10.1007/s11745-010-3395-2.

Tengö, J., Hefetz, A., Bertsch, A., Schmitt, U., Lübke, G., Francke, W., 1991. Species specificity and complexity of Dufour's gland secretion of bumble bees. Comp. Biochem. Physiol. 99B (3), 641–646.

Treanore, E., Derstine, N., Amsalem, E., 2021. What can mechanisms underlying derived traits tell us about the evolution of social behavior? Ann. Entomol. Soc. Am. 114 (5), 547–561.

Tupec, M., Bucek, A., Janousek, V., Vogel, H., Prchalova, D., Kindl, J., … Pichova, I., 2019. Expansion of the fatty acyl reductase gene family shaped pheromone communication in Hymenoptera. Elife e39231. doi:10.7554/eLife.39231.

Valterová, I., Martinet, B., Michez, D., Rasmont, P., Brasero, N., 2019. Sexual attraction: a review of bumblebee male pheromones. Z. Naturforsch. C J. Biosci. 74 (9–10), 233–250. doi:10.1515/znc-2019-0003.

Warnes M.G.R., Bolker B., Bonebakker L., Gentleman R., & Huber W. (2016). Package 'gplots'. Various R programming tools for plotting data.

Wenning, L., Yu, T., David, F., Nielsen, J., Siewers, V., 2017. Establishing very long-chain fatty alcohol and wax ester biosynthesis in Saccharomyces cerevisiae. Biotechnol. Bioeng. 114 (5), 1025–1035.

Wickham, H., 2011. ggplot2. Wiley Interdiscip. Rev. Comput. Stat. 3 (2), 180–185.

Wilson, E.O., 1971. The insect societies. Harvard University Press [Distributed by Oxford University Press], Cambridge, Massachusetts, USA.

Wu, T., Hu, E., Xu, S., Chen, M., Guo, P., Dai, Z., … Yu, G., 2021. clusterProfiler 4.0: a universal enrichment tool for interpreting omics data. The Innovation 2 (3), 100141. doi:10.1016/j.xinn.2021.100141.

Yan, H., Liebig, J., 2021. Genetic basis of chemical communication in eusocial insects. Genes Dev. 35 (7–8), 470–482.

Yang, P., Zhu, J.Y., Gong, Z.J., Xu, D.L., Chen, X.M., Liu, W.W., … Li, Y.F., 2012. Transcriptome analysis of the Chinese white wax scale Ericerus pela with focus on genes involved in wax biosynthesis. PLoS One 7 (4), e35719.

Yen, C.L.E., Monetti, M., Burri, B.J., Farese Jr, R.V., 2005. The triacylglycerol synthesis enzyme DGAT1 also catalyzes the synthesis of diacylglycerols, waxes, and retinyl esters. J. Lipid Res. 46 (7), 1502–1511.

Yew, J.Y., Chung, H., 2015. Insect pheromones: an overview of function, form, and discovery. Prog. Lipid Res. 59, 88–105. doi:10.1016/j.plipres.2015.06.001.