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Conservation and modification of genetic and physiological toolkits underpinning diapause in bumble bee queens

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Abstract

Diapause is the key adaptation allowing insects to survive unfavourable conditions and inhabit an array of environments. Physiological changes during diapause are largely conserved across species and are hypothesized to be regulated by a conserved suite of genes (a 'toolkit'). Furthermore, it is hypothesized that in social insects, this toolkit was co-opted to mediate caste differentiation between long-lived, reproductive, diapause-capable queens and short-lived, sterile workers. Using *Bombus terrestris* queens, we examined the physiological and transcriptomic changes associated with diapause and CO_2 treatment, which causes queens to bypass diapause. We performed comparative analyses with genes previously identified to be associated with diapause in the Dipteran *Sarcophaga crassipalpis* and with caste differentiation in bumble bees. As in Diptera, diapause in bumble bees is associated with physiological and transcriptional changes related to nutrient storage, stress resistance and core metabolic pathways. There is a significant overlap, both at the level of transcript and gene ontology, between the genetic mechanisms mediating diapause in *B. terrestris* and *S. crassipalpis*, reaffirming the existence of a conserved insect diapause genetic toolkit. However, a substantial proportion (10%) of the differentially regulated transcripts in diapausing queens have no clear orthologs in other species, and key players regulating diapause in Diptera (juvenile hormone and vitellogenin) appear to have distinct functions in bumble bees. We also found a substantial overlap between genes related to caste determination and diapause in bumble bees. Thus, our studies demonstrate an intriguing interplay between pathways underpinning adaptation to environmental extremes and the evolution of sociality in insects.

Keywords: caste differentiation, diapause, genomics, reproduction, social insects

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Introduction

How organisms adapt to survive in varied environmental conditions is a fundamental question in ecological genetics, which can now be examined at the genomewide scale across ecologically diverse species (Nadeau & Jiggins 2010; Stapley *et al.* 2010). Diapause is the key adaptation allowing insects to survive adverse conditions and inhabit a vast array of environments (Denlinger 1986; Hahn & Denlinger 2011; Sim & Denlinger 2013). It involves a suite of synchronized behavioural and physiological changes that modulate development, longevity, starvation resistance, stress tolerance and reproduction (Denlinger 2002; MacRae 2010). Although some of the

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mechanisms regulating diapause are species specific and different species may vary in the degree of dormancy they present (reduced activity or complete developmental arrest), the life stage in which diapause takes place (larval, pupal or adult), and the environmental conditions associated with dormancy, many of the mechanisms regulating the maintenance of diapause appear to be conserved (Sim & Denlinger 2013). It has been hypothesized that a conserved suite of genes, 'the insect diapause genetic toolkit', mediates the common behavioural and physiological changes associated with the maintenance of the diapause state across insect species (Poelchau *et al.* 2013a). However, the majority of studies using genomewide gene expression approaches to characterize the insect diapause genetic toolkit have focused only on dipteran species (Robich & Denlinger 2005; Kankare *et al.* 2010; Ragland *et al.* 2010, 2011; Williams *et al.* 2010; Poelchau *et al.* 2011, 2013a,b; Yamada & Yamamoto 2011; Gong *et al.* 2013; Sim & Denlinger 2013), and thus, it remains to be determined whether these mechanisms are conserved across other taxa. Intriguingly, it has also been hypothesized that in social insect species, diapause-related genes were co-opted to generate caste differences between reproductive, long-lived, diapause-capable queens and sterile, short-lived workers (Hunt & Amdam 2005; Hunt *et al.* 2007), and thus, diapause-related genes may underpin this major transition in the evolution of sociality.

Here, we examine these two hypotheses (conservation of a diapause genetic toolkit and co-opting of diapause-related genes for caste differentiation) using queens of the bumble bee *Bombus terrestris* (Apidae; Hymenoptera). *B. terrestris* is an economically important pollinator which is widespread in temperate zones, with queens entering a state of complete dormancy during a winter diapause prior to establishing a social colony (for a comprehensive review of bumble bee biology, see (Amsalem *et al.* 2015). Thus, comparisons of the genes underpinning the maintenance of diapause in *B. terrestris* and in the previously studied dipteran species will be a key test of the 'diapause genetic toolkit' hypothesis, because these species experienced distinct evolutionary histories and diapause in different developmental and physiological states. Furthermore, bumble bees show intermediate levels of sociality, with a solitary phase of the queen (diapause, colony founding) followed by a highly eusocial phase where the queen is the primary reproducer and workers take over all other colony duties. Thus, bumble bees can be effectively used to study the mechanisms regulating the transition from solitary to social behaviour (Amsalem *et al.* 2015), such as the co-opting of diapause genes to mediate caste differentiation, which has thus far only been examined using a handful of physiological markers in social wasps (see below for further discussion and Hunt *et al.* 2007).

Diapause in insects can generally be divided into three stages: prediapause (induction, preparation and initiation), diapause (maintenance) and post-diapause (termination and reproduction; Kostal 2006). During the prediapause phase, a time-keeping mechanism is hypothesized to receive cues from the environment and to translate these into changes in hormones and other signalling molecules, such as ecdysteroids, juvenile hormone (JH) and insulin (Sim & Denlinger 2008, 2013). These hormonal changes drive a variety of behavioural and physiological changes, including increased food consumption, synthesis of storage proteins and accumulation of nutritional stores in the fat body (Denlinger *et al.* 2012). As the insect enters diapause, metabolism is reduced or suppressed, longevity is increased, and the insect exhibits greater resistance to stress. Exit from diapause is often associated with changes in resource allocation and transition to reproduction. JH in particular is involved in regulating the transitions between these three stages in adult insects in many species: its absence often correlates with the onset of diapause, JH is maintained at low levels during diapause, and increased synthesis of JH is usually associated with the termination of diapause (Larrere *et al.* 1993; Hartfelder 2000; Tatar & Yin 2001; Denlinger 2002).

During her annual life cycle, the bumble bee queen transitions through several distinct phases, including diapause (reviewed in (Amsalem *et al.* 2015). In late summer, shortly after emerging as an adult, the virgin queens leave the colony to mate and enter a winter diapause, during which the queens are completely dormant in an underground cavity for 6–9 months (Alford 1969). Diapause terminates in the spring, likely due to temperature and light cues, although the exact trigger is unknown. After emerging from diapause, an individual queen searches for a nest site, fully activates her ovaries and forages to provision her developing offspring (Michener 1974). Upon emergence of the first adult worker, a reproductive division of labour is established, where the queen specializes on egg laying and the workers take over the other colony tasks. The queens and workers differ greatly in their behaviour and physiology, although in terms of gross morphology, they are very similar except that the queens are much larger, and thus, caste differences in bumble bees are considered to be relatively primitive (Amsalem *et al.* 2015).

In bumble bees, the molecular and physiological changes associated with diapause have been investigated mainly in *B. terrestris*, and there are several differences from what is observed in dipteran species. *B. terrestris* queens accumulate nutritional stores prior

to entering diapause, which are critical for their survival (Alford 1969; Fliszkiewicz & Wilkaniec 2007). Although this process is presumably regulated by JH levels (Roseler & Roseler 1988), JH regulation of diapause in bumble bees appears to be distinct from JH regulation of diapause in Diptera: levels of both JH and ecdysteroids are low in queens before and during diapause and increase only several days after the termination of diapause (i.e. after the queen emerges from dormancy and resumes normal activity), and ovaries are activated only about a month later, just prior to colony founding (Larrere *et al.* 1993; Geva *et al.* 2005). Within Diptera, the insulin signalling pathway, heat-shock proteins, hexamarins and general metabolic pathways such as AMPK (AMP-activated protein kinase, regulating energy and stress tolerance) appear to be a key regulator of the diapause phenotype (Yocum *et al.* 2005; MacRae 2010; Sim & Denlinger 2013). However, little is known about the genes that regulate diapause in bumble bee queens. Several of the genes that are differentially regulated between queens vs. males and workers are involved in nutrient storage (e.g. hexamarins) and fatty acid biosynthesis (Colgan *et al.* 2011) and thus may play a role in diapause. Expressed sequence tag analysis of diapausing queen in *Bombus ignitus* revealed a handful of genes associated with physiological processes (Kim *et al.* 2006). Additionally, expression levels of several heat-shock protein genes vary in a tissue-specific manner during diapause (Kim *et al.* 2008) and emphasize the complexity associated with diapause. Overall, there is an indication that genes involved in metabolism, nutrition storage and stress response mediate the diapause phenotype in bumble bees. However, it is unclear whether the general diapause genetic toolkit identified in dipteran species is operating in this system, and there appear to be clear differences in the role of JH.

Diapause in bumble bees can also be bypassed by treating the newly mated queens with CO_2, a practice which is commonly used in commercial bumble bee rearing operations (Roseler 1985; Tasei 1994). In *B. terrestris* queens (and also in *Apis mellifera* honeybee queens), CO_2 induces oogenesis, stimulates egg laying, inhibits the formation of fat reserves and increases the size of the corpora allata, which is the source of JH in insects (Roseler & Roseler 1984; Roseler 1985; Niño *et al.* 2011, 2013; Vergoz *et al.* 2012). The long-term impacts of CO_2 treatment vs. diapause on bumble bee queen survival and performance have not been examined. However, previous studies have demonstrated that increasing the duration of diapause (which is nonexistent in CO_2-treated queens) resulted in reduced survival and shorter pre-oviposition period (i.e. the post-diapause period until first egg laying (Beekman *et al.* 1998), smaller cohort size of first and second brood, earlier switch of the queen from laying diploid female to haploid male eggs and the production of more males and less queens (Duchateau *et al.* 2004). Examining the impacts of CO_2 treatment vs. diapause on bumble bee queens can provide insights into the mechanisms mediating the transition from mated queens to founders (i.e. post-colony establishment), as well as to highlight any further effects of CO_2 on queen physiology and health.

Finally, in social insects, it has been hypothesized that genes associated with diapause were co-opted to generate queen vs. worker castes (Hunt & Amdam 2005; Hunt *et al.* 2007), which is a fundamental transition in the evolution of eusociality (Wheeler 1986). The two castes differ in their reproductive potential, nutritional stores and longevity (Smith *et al.* 2008), all parameters that are also regulated during diapause. While there have been several studies identifying genes associated with queen–worker caste differentiation in a number of social insect species (Pereboom *et al.* 2005; Barchuk *et al.* 2007; Feldmeyer *et al.* 2014), the only studies seeking to identify genes associated with diapause in Hymenoptera examined either solitary bees (in the pupal stage) or only examined a handful of genes in a restricted time frame (Yocum *et al.* 2005, 2006; Kim *et al.* 2008; Colgan *et al.* 2011) and the extent to which genes regulating diapause and caste differentiation overlap has not been examined.

Here, we characterized multiple physiological traits (nutritional stores, JH levels and ovary activation) in bumble bee queens (*B. terrestris*) as well as genome-wide expression patterns generated by transcriptome sequencing of the queen fat bodies to examine the mechanisms underpinning the diapause phenotype in bumble bees. We focused on fat body (the primarily tissue for nutrient storage, metabolic processes and immunity in insects) because we aimed to characterize the downstream regulatory mechanisms that operate during diapause rather than the processes that trigger the entry or exit from diapause (which may differ more substantially between species). We hypothesized that the genomic and physiological mechanisms underpinning the diapause phenotype will be largely conserved and similar to those found in solitary insects, indicating the existence of an insect diapause genetic toolkit. Additionally, if diapause-related pathways were indeed co-opted to regulate social behaviour in social insects, key genes involved in the evolution of sociality should exhibit distinct expression patterns and the diapause genes should overlap significantly with genes associated with caste differentiation in bumble bees. We further predicted that CO_2-treated queens will exhibit changes equivalent to the changes occurring when queens exit diapause, but may also show a

trade-off at the transcriptional or physiological level between reproduction and health.

Materials and methods

Overview of bumble bee rearing and collections

Queens of *Bombus terrestris* (n = 171 in total, 16–29 per treatment, eight source colonies taken from five distinct genetic lines, see Table S1, Supporting information) were generated at Bizbee, EinYahav, Israel. Bizbee is a commercial bumble bee rearing and production facility and thus has extensive expertise and facilities for creating these sample groups using standard commercial practices. Six different treatment groups were created (i) virgin, (ii) mated, (iii) CO_2-treated, (iv) diapause, (v) founder post-diapause and (vi) founder post-CO_2. A diagram explaining the protocol and timing for treatment, queen age and sampling is provided together with Table S1 (Supporting information). Further detailed explanations of the treatment and collection regimes are provided below (see 'Detailed description of treatments').

Queens were maintained in the laboratory in nest boxes at a constant dark, temperature of 28–30 °C and 50% relative humidity and supplied with unlimited food (50% sucrose and fresh honeybee collected pollen) during their entire developmental period (except during diapause) until sampling. Grouping of queens was performed prior to treatment (for the purpose of mating, CO_2 treatment or diapause) and post-treatment (for the purpose of colony establishment) and was standardized across treatments. Treatments were performed in groups of ~30 queens after which queens were kept in groups of 2–3 per cage. Queens sampled for gene expression analyses were flash-frozen with dry ice and were immediately transfer to −80 °C until further analyses. Dry ice is preferentially used when ovarian tissue must be dissected and analysed, because freezing in liquid nitrogen may cause ovaries to shatter. We have previously conducted several transcriptomic studies using samples frozen on dry ice (e.g. Niño *et al.* 2011).

Queens were evaluated for JH titres in the hemolymph, nutritional stores (weight, glycogen and lipids) in fat body tissue (eviscerated abdomens with attached fat bodies), ovarian activation and gene expression in fat body tissue (RNA-Seq and qRT–PCR) as described in the individual experimental sections below. Sample sizes for each of the analyses are shown in Table S1 (Supporting information). Results of statistical tests are shown in Table S2 (Supporting information).

The choice of queen groups aimed to characterize the general changes queens experience before, during and post-diapause rather than to address specific regulatory mechanisms underlying the entrance or the exit from diapause. These sample groups allowed us to compare queens during their natural life cycle (virgin, mated, diapause, founder post-diapause queens), as well as examine the short- and long-term effects of bypassing diapause with CO_2 (CO_2-treated vs. mated and founder post-CO_2 vs. founder post-diapause queens, respectively).

Detailed description of treatments

See Table S1 (Supporting information) and associated diagram for an overview of the sample generation and collection. Virgin, mated and CO_2-treated queens were all collected when they were 5-10 days old (after adult emergence). The other three groups were all mated and matured for additional periods of time as follows: diapause queens were given another 9–10 weeks of diapause; founder post-diapause queens were given 9–10 weeks of diapause and another month post-diapause to lay eggs (thus, were approximately 4 months old); founder post-CO_2 queens were given another month post-CO_2 treatment to lay eggs (thus, were approximately 1.5 months old). All ages are defined from adult emergence.

Mating. At 5–10 days of age, virgin queens were grouped with unrelated males and were observed until mating took place (Plowright & Jay 1966). Mating couples remain connected for 15–30 min and thus could be transferred into a second cage where they were supplied with unlimited food until sampling or assigning to one of the other treatment groups. Mated queens were sampled approximately 24 h post-mating. Queens assigned to the two other treatments (CO_2 or diapause) were treated on the same day of mating.

CO_2 treatment. Mated queens were grouped in a sealed cage on the day of mating and treated with pure CO_2 until they were anesthetized (for approximately 1 min, (Roseler 1985). Queens subsequently were kept in a sealed cage for 30 minutes post-treatment and were CO_2 treated again 24 h later. The cage was ventilated between treatments. CO_2-treated queens were collected approximately 24 h after the second treatment. Founder post-CO_2 queens were grouped with several callow workers to stimulate them to lay eggs. Queens were collected after egg laying was observed, approximately 1 month later.

Diapause. Mated queens were grouped in sealed carton boxes according to their colony of origin on the day of mating and kept in the dark at 4 °C for 9–10 weeks. By the end of this period, the boxes were opened and

diapause queens were immediately collected into dry ice. Prior to sampling, we confirmed that the collected queens had survived diapause by searching for signs of life (leg twitching or abdominal movements); these movements were observed within a period of seconds. In founder queens post-diapause, diapause was terminated by exposing the queens to room temperature (~25 °C) and daylight. Under these conditions, diapause queens display rapid wing movements within 20 minutes. These queens were kept in pairs and were sampled after egg laying was observed, approximately 1 month later. Only the reproductively dominant queen (determined by ovary dissection, see below) in each pair was collected.

Analysis of juvenile hormone titres

Hemolymph of individual queens (n = 28, 5-6 queens per treatment, five source colonies, see Table S1, Supporting information) was sampled for analysis of JH-III titres in five time points (virgins were not included). For hemolymph extraction, the queens were anesthetized with ice and under a stereoscope binocular. Hemolymph was sucked by capillary action into a pulled glass needle that was inserted under the fourth abdominal segment. Ten microlitres of hemolymph from each individual was mixed with 90 μL of HPLC grade methanol. Samples were stored at −20 °C until shipping to the USDA-ARS laboratory in Gainesville, Florida, where they were processed according to a previously described protocol (Amsalem *et al.* 2014b). Immediately after hemolymph extraction, the queens were frozen on dry ice. Queens that were used for hemolymph extraction were not used for any genomic analysis. JH titres in queens were compared using Kruskal–Wallis test because the data were not randomly distributed (tested using Kolmogorov–Smirnov test for normality, $P < 0.05$, Results of statistical tests are shown in Table S2, Supporting information).

Analysis of queen body mass, glycogen and lipids in the fat body

Prior to dissections, the total body mass and abdomen mass were measured using an electronic scale. Glycogen and lipid percentages in the abdominal fat body were measured in individual queens (n = 84, six time points, 9–16 queens per treatment, eight source colonies, Table S1, Supporting information) using a modified version of a protocol derived from (Kaufmann & Brown 2008) and (Van Handel & Day 1988). For the separation of glycogen and lipids, the entire abdomen was homogenized using 500 μL 2% sodium sulphate. Two hundred microlitres of the homogenate was added to 2.8 mL chloroform/methanol mix (v:v 1:1) and the samples were centrifuged to achieve separation between the precipitate (glycogen) and the rest of the fraction. The two layers were separated and glycogen was measured with the hot anthrone reaction (5 mL anthrone/sample). The remaining fraction was mixed with 2 mL of distilled H_2O to achieve separation between the upper aqueous fraction (sugars) and the lower organic fraction (lipids). The two layers were separated and lipids were quantified by a vanillin-phosphoric acid reaction (5 mL vanillin/sample). A standard curve for carbohydrates was developed using five different concentrations of 0.1% anhydrous glucose diluted in distilled H_2O. A standard curve for lipids was developed using five different concentrations of 0.1% canola oil diluted in chloroform. Absorbance values (OD 525 for lipids and OD 625 for glycogen) for each sample were measured with a ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and converted to micrograms per queen, based on a formula calculated from a regression line derived from the standard sample values. The amount of glycogen and lipids are presented as percentages of abdomen mass to normalize for any differences in queen size and mass. Before performing the statistical tests, the data were converted to proportions and transformed using arc sin. We then used a parametric test (one-way ANOVA) to compare the glycogen and lipid data as well as the data of body mass after ensuring the data are normally distributed (Table S2, Supporting information).

Analysis of ovarian activation

Individual queens (n = 33, 6–8 queens per treatment, eight source colonies, Table S1, Supporting information) were dissected under a stereo-microscope on dry ice. The length of the terminal oocyte in the three largest ovarioles (at least one ovariole per ovary; queens possess four ovarioles per ovary) was measured with a scaled ocular. Mean terminal oocyte length for each bee was used as an index of ovarian activation, as in (Amsalem & Hefetz 2010, 2011). RNA was extracted from the remaining abdominal fat body tissue of these queens (see details below). Twenty-five samples were used for RNA-Seq analysis (five queens per treatment), and all the 33 samples were used for qRT–PCR. Ovarian activation in queens was compared using Kruskal–Wallis test because the data were not normally distributed (tested using Kolmogorov–Smirnov test for normality, $P < 0.05$, Table S2, Supporting information).

RNA-Seq analysis

Total RNA samples were extracted from the abdominal fat bodies of 25 individual queens (five queens per

treatment, five source colonies/genetic lines, Table S1, Supporting information) using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series Accession no. GSE73009; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73009). Previous studies indicated that bumble bee colonies are highly variable in pheromonal identity (Rottler *et al.* 2013), hibernation survival, colony foundation and response to inbreeding (Gerloff & Schmid-Hempel 2005), and colony size and number of sexual (Duchateau *et al.* 2004). We therefore used five different genetic lines (the different distinct genetic lines are kept in Bizbee since 2006) in our analysis, and from each line, we sampled all groups (Table S1, Supporting information).

RNA quantity and quality were assayed with a ND-1000 Spectrophotometer (NanoDrop Technologies) and a 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Sample preparation and sequencing was performed by the Genome Core Facility at Penn State according to standard RNA sequencing protocol. Libraries were constructed from 100 ng of total RNA using the TruSeq Stranded mRNA Sample Prep Kit according to the manufacturer's protocol (Illumina). Libraries were sequenced using 100-nt single-read sequencing on an Illumina HiSeq 2500 in Rapid Run Mode. All 25 samples were split across two lanes to control for a bias between lanes. The average number of reads (± SE) generated per sample was 7 850 812 ± 877 427. About 12% of the reads were trimmed during the analyses (including adaptor sequences, low quality and/or short reads) resulting in 6 840 050 ± 338 616 reads, on average, per sample. Transcriptome sequencing reads were preprocessed using Trimmomatic (Bolger *et al.* 2014) removing adaptor sequences, low-quality reads and reads shorter than 36 bp after trimming. The transcriptome sequencing reads were aligned to the *Bombus terrestris* genome build 1.1 (Sadd *et al.* 2015) using TOPHAT v2.0.10 (Trapnell *et al.* 2009). The resulting alignments were used to assemble transcripts using the standard CUFFLINKS v2.1.1 protocol (Trapnell *et al.* 2012). The read counts per assembled transcript were imported into R statistical software (www.r-project.org) for further analyses. Transcripts with low read counts (<5 reads per transcript) were removed. The data were normalized using a trimmed mean of M-values (TMM) method (Robinson & Oshlack 2010). The TMM was used to generate a normalization factor for each sample, which was then converted into a size factor for use with DESEQ. The DESEQ package in R, which is based on a negative binomial distribution, was used to identify significantly (FDR < 0.05) differentially expressed transcripts (DETs) (Anders & Huber 2010). Transcripts were annotated using BLAST v2.2.26+ (Zhang *et al.* 2000), and a generalized linear model was used to determine the differentially expressed transcripts in each of the pairwise comparisons.

The principle component analysis (PCA) and the clustering analyses were performed using a complete-linkage clustering method and were generated from normalized read counts for each significant transcript (significance was determined using a generalized linear model and corrected for multiple testing, FDR < 0.05). The PCA was run on a covariance matrix among treatments and the output is a matrix of variable loadings containing eigenvectors, which are represented on the *y*-axis for each variable and principal component. The length of the PCA bars represents the strength of the correlation with the principal component and the direction (positive or negative) represents either a positive or negative correlation with that principal component. The eigenvalues that were generated from the PCA were used to calculate the percentage of variance that is explained by each principal component.

For the gene ontology (GO) analysis, the list of *Bombus terrestris* transcripts were mapped to Flybase IDs using a reciprocal BLAST approach (so each one had to match the best blast hit to the other) with e-value cut-off of 10^{-5} and a default BLASTN parameters using BLAST v2.2.26+ (Zhang *et al.* 2000) and uploaded to DAVID Bioinformatics Resources 6.7 (http://david.abcc.ncifcrf.gov/). The overrepresented GO terms of significantly differentially expressed genes (with FLYBASE matches) in the different queen groups were determined using a background list of all genes expressed among the samples. To refine the list of GO terms, the significant terms (Benjamini correction <0.05) that were generated using DAVID Bioinformatics Resources were imported into REVIGO (Supek *et al.* 2011), which clusters the GO terms using semantic similarity measures.

qRT–PCR analysis

To validate the RNA-Seq results, we evaluated the expression differences of 11 genes in queens taken from five time points as described above (n = 33 queens, 6–8 queens per treatment, eight source colonies) using quantitative real-time PCR (qRT–PCR). These genes (see the Table attached to Fig. S2, Supporting information) were selected based on their biological relevance to diapause and the RNA-Seq results. Design of forward and reverse primers for each gene (see Table attached to Fig. S2, Supporting information) was performed using PRIMER3 v 0.4.0 (http://frodo.wi.mit.edu/).

RNA was extracted from the fat body samples of the individual queens as described above. Synthesis of

cDNA was performed according to the manufacturer's instructions using 200 ng of RNA with reverse transcriptase (Applied Biosystems). The first-strand cDNA reaction was diluted by adding 35 μL of ultra-purified water and stored at −20 °C until use. Two microlitres of diluted cDNA were combined with 5 μL SYBR Green (Bioline, Luckenwalde, Germany), 0.2 μL of each forward and reverse primer and 2.6 μL DEPC-water. To control for PCR efficiency and individual differences across samples, we used two housekeeping genes: *arginine kinase* (AK) and *phospholipase A2* (PLA2). These genes were found to be stable in *Bombus terrestris* fat body (Hornakova *et al.* 2010), were used in a previous study (Amsalem *et al.* 2014a) and were also found to be stable in the current qRT–PCR analyses (data not shown). Expression levels were determined using qRT–PCR on an ABI Prism®7900 sequence detector with SYBR Green detection method. Negative control samples (cDNA reaction without RT enzyme) and a water control were also present on each plate. PCR product quality and specificity was verified using melt curve analysis. A standard curve was performed for each set of primers using five different concentrations of cDNA to determine the r^2 and efficiency (see Table attached to Fig. S2, Supporting information). Triplicate reactions were performed for each of the samples and averaged for use in statistical analysis. Expression levels of candidate genes were normalized to the geometric mean of two housekeeping genes using the $2^{-\Delta Ct}$ technique. Expression levels (in Fig. S2, Supporting information) were normalized to the group with the lowest expression level for each gene. Expression levels between the different queen groups were compared using Kruskal–Wallis test.

Results and discussion

Physiological parameters associated with diapause and CO_2 treatment in queens

Ovary activation levels were significantly and similarly higher in founders (either post-CO_2 or diapause) compared to all other groups, indicating that an experience of 9–10 weeks of diapause or CO_2 treatment is equally effective at inducing oogenesis (Fig. 1a; Table S2, Supporting information). However, 24 h after CO_2 treatment, ovaries were not activated and thus the effect of CO_2 on ovary activation takes time to manifest. Corresponding with ovary activation, JH titres were significantly higher in the two founder groups vs. mated or diapause queens (Fig. 1b). JH is a gonadotropin in *Bombus terrestris* (Roseler 1977; Amsalem *et al.* 2014b; Shpigler *et al.* 2014) and rising JH levels lead to ovary activation and the increased fat body metabolism of queens (Roseler & Roseler 1988). However, JH titres were also significantly higher in CO_2-treated queens that had inactivated ovaries.

Results of ovary activation are consistent with our predictions and previous studies (Alford 1969; Michener 1974; Geva *et al.* 2005) and indicate that ovaries are only activated 1–2 weeks after the termination of diapauses (i.e. after the queen emerges from dormancy and resumes normal activity) or CO_2 treatment. Our JH results are consistent with a previous study demonstrating that the rates of JH biosynthesis are low during pre-diapause and diapause, and corpora allata reactivation occurs only several days after diapause termination (Larrere *et al.* 1993). Previous studies in *B. terrestris* also demonstrated that CO_2 causes a sharp increase in JH titres, similar to that found in founders (Fuzeaubraesch *et al.* 1982; Buhler *et al.* 1983; Nicolas & Sillans 1989) but

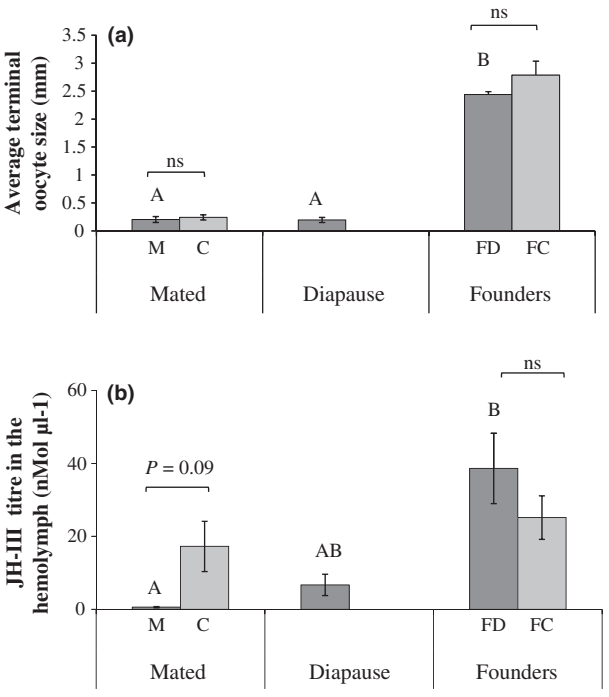

Fig. 1 Ovarian activation (a) and juvenile hormone titres (b) in *Bombus terrestris* queens during their natural life cycle and after CO_2 treatment. Average terminal oocyte size and JH titre were measured in five groups of the following: mated (M, n = 8,5 for ovaries and JH, respectively); CO_2-treated (C, n = 8,5); diapause (D, n = 7,6); founder after 9–10 weeks of diapause (FD, n = 6,6); and founder post-CO_2 treatment (FC, n = 7,6) queens. Samples were taken from individual queens originated from five distinct genetic lines (see Methods and Table S1, Supporting information for details). Different letters above columns represent statistical differences during the natural life cycle at $\alpha = 0.05$. P-values represent statistical differences for CO_2-treated queens vs. the corresponding controls. Data are presented as means ± SE.

see (Larrere *et al.* 1993). Thus, overall our data and others (Larrere *et al.* 1993) suggest that while rising JH titres are critical for diapause termination in other species (Denlinger *et al.* 2012), a change in JH levels is not causally associated with diapause termination in bumble bee queens.

Body mass was measured in six groups of queens (n = 171, 23–32 queens per treatment, Table S1, Supporting information). Virgin queens were the lightest with an average weight of 897.5 ± 23.4 mg (n = 32). Queens gained weight during the first days of their life, reaching their highest mass at the time of mating (mated queens, 996 ± 23.3 mg, n = 32). A significant weight loss was recorded in diapausing queens (896.9 ± 13.6, n = 29) and founder post-diapause queens, who had initiated egg laying (825.5 ± 21.7 mg, n = 23) (Table S2, Supporting information). CO_2-treated queens (941.2 ± 11.9, n = 31) were significantly lighter than mated queens, although both groups were collected at the same time point (24–48 h post-mating) and were supplied with unlimited food. Thus, differences in body mass are likely to reflect either change in the CO_2 queens' behaviour (decreased food consumption) or shift in their metabolism. Finally, founder post-CO_2 queens (820.5 ± 16, n = 24) did not significantly differ from founder post-diapause queens (Table S2, Supporting information).

One of the conditions necessary for the queens to pass into a state of diapause and survive diapause is the accumulation of an appropriate quantity of fat body (Alford 1969; Roseler & Roseler 1986; Fliszkiewicz & Wilkaniec 2007). Fat bodies store glycogen and triglycerides, the main energy reserves in animal cells (Arrese & Soulages 2010). Glycogen was the lowest in virgin queens and reached its peak levels in mated queens, with little variation as a function of diapause. However, a significant decrease in glycogen was recorded in founder post-diapause queens (Fig. 2, Table S2, Supporting information). CO_2 treatment did not affect the glycogen content in the fat body either in the short (mated vs. CO_2) or in the long term (founder post-CO_2 vs. founder post-diapause).

Lipids accounted for an average 19.5% of the total abdomen weight (12.4% of the total body mass) in mated, prediapause, queens (also in (Alford 1969), and there were no differences in lipid content in fat bodies of virgin, mated, diapause or founder post-diapause queens. However, founder post-CO_2 queens had significantly lower percentages of lipids in their fat bodies compared to founder post-diapause queens. No differences in lipids were found in the short term between mated and CO_2-treated queens (Table S2, Supporting information). A previous study where queens were sampled from natural hibernacula at various times

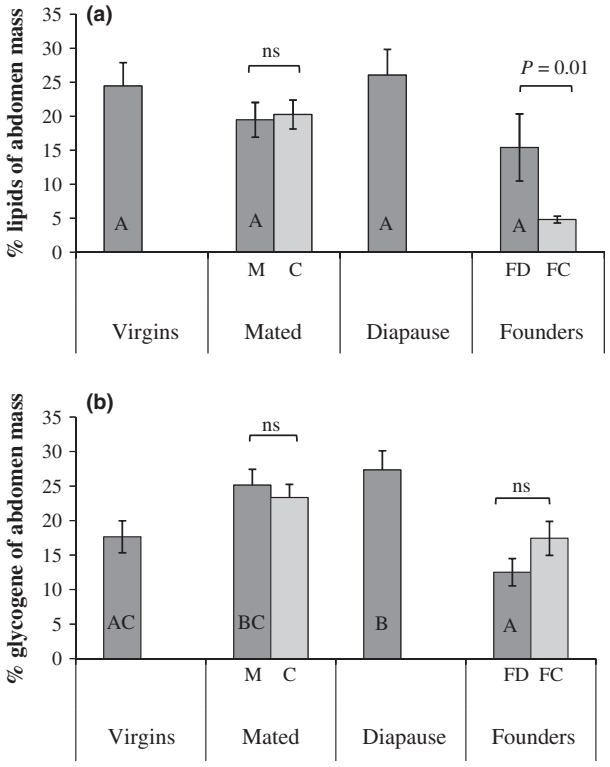

Fig. 2 The percentages of glycogen and lipids in fat body of queens during their natural life cycle and after CO_2 treatment. Percentage of lipids and glycogen were measured in fat body of six groups of queens: virgins (V, n = 16); mated (M, n = 16); CO_2-treated (C, n = 16); diapause (D, n = 13); founder after 9–10 weeks of diapause (FD, n = 9); and founder post-CO_2 treatment (FC, n = 14) queens. Samples were taken from individual queens originated from five distinct genetic lines (see Methods and Table S1, Supporting information for details). Different letters in columns represent statistical differences between groups during the natural life cycle at $\alpha = 0.05$. P-values represent statistical differences for CO_2-treated queens vs. the corresponding controls. Data are presented as means ± SE.

during diapause found that 80% of abdominal lipids were depleted during diapause (Alford 1969), while the queens in our study lost none (Fig. 2). Glycogen reserves too were not significantly different in queens at the onset and termination of diapause (mated vs. diapause, Fig. 2). This may indicate that the artificial diapause regime that we used (which is standard in laboratory settings, (Beekman & Stratum 2000; Kim *et al.* 2006; Gosterit & Gurel 2009; Amin *et al.* 2011) puts fewer physiological challenges on queens due to a combination of a shorter diapause period (9–10 weeks compared to up to 9 months in natural conditions), constant diapause temperatures (4 °C in the current study, as opposed to the fluctuations in temperatures under natural conditions) and optimal nutrition prior to the onset of diapause. As queens did not lose any

lipids or glycogen during diapause but still lost a significant proportion of their body mass, we conclude that under relatively short artificial diapause, they are likely to lose mostly water (that forms 60-70% of body mass before the onset of diapause) and to utilize honey stomach contents (Alford 1969). The observed effects of CO_2 are consistent with previous studies where glycogen and lipids did not accumulate in the fat bodies of *B. terrestris* queens treated with either CO_2 or JH (Roseler & Roseler 1984 in (Roseler 1985). Interestingly, however, our studies indicated that, over the long-term, founder post-CO_2 queens had lower lipid mass (Fig. 2). This difference is not likely to be the result of age effect between the two founders because it was associated with the younger founders (post-CO_2 queens, 1.5 months old) and not with the older founders (post-diapause, 4 months old) that are more likely to lose more of their reserves. However, this may be caused by the CO_2 treatment interfering with the queens' ability to regulate their metabolism, or to a potential trade-off between the transition to reproduction and other life traits. Both founder groups were collected about a month after egg laying, but if founder post-CO_2 queens laid eggs at a higher rate, they could have depleted their lipid stores more rapidly.

Global gene expression patterns associated with diapause and CO_2 treatment in queens

We performed a whole transcriptome sequencing analysis in five groups of queens (all groups but the virgins; $n = 25$ libraries, five queens per treatment, Table S1, Supporting information). Of the 26 610 transcripts that passed quality control measures (see Methods), 4515 were differentially regulated at FDR < 0.05 (the full transcript list is provided in Table S3a, Supporting information). Based on a hierarchical clustering analysis using the significantly differentially expressed transcripts (DETs), the five sampled groups clustered together according to their social and reproductive status: the two founder groups clustered together, while the mated, CO_2-treated and diapause queens clustered together, with a close association between the mated and CO_2-treated queens (Fig. S1, Supporting information). The first principal component analysis (PCA) of the DETs that accounted for 50.96% of the variation gives a similar pattern: it primarily represents variation between the nonreproductive groups (mated, CO_2-treated and diapause) vs. the (reproductive) founder groups (Fig. 3). Interestingly, the second PCA that accounted for 30.66% of the variation in transcript levels primarily represents variation that is associated with CO_2 treatment, with similar patterns in CO_2-treated and founder post-CO_2 groups. Overall, gene expression patterns

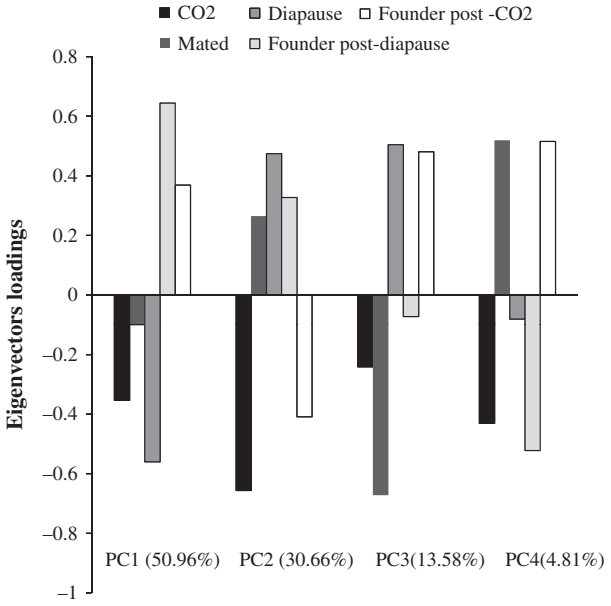

Fig. 3 Principal component analysis of the significantly DETs in queens during their natural life cycle and after CO_2 treatment. Twenty-five libraries were obtained for five groups of queens (five individuals per group).

were most affected by the transition to the laying, founder state, while CO_2 had additional effect on queens in both the short and the long term.

Genes and processes associated with diapause. To characterize the transcripts that are most important in the transition from diapause to reproduction in *B. terrestris* queens, we identified all the DETs between diapause and founder post-diapause queens (1432 and 1064 that were down- and upregulated during diapause, respectively, see Table S3a, Supporting information). We performed gene ontology (GO) analysis using the DETs that have *Drosophila melanogaster* orthologs with FlyBase annotations (Fig. 4a). Among the GO categories upregulated in diapause queens, 38 GO terms were significantly enriched at $P < 0.05$ (Table S3b, Supporting information), and these included development, morphogenesis, metabolism, cell communication and reproduction. Among the GO categories downregulated in diapause queens, 14 GO terms were significantly enriched at $P < 0.05$ (Table S3c, Supporting information). These included cellular components, cell, intracellular and metabolism (Fig. 4b). Additionally, two KEGG pathways (MAPK, mitogen-activated protein kinase and progesterone-mediated oocyte maturation) were marginally significantly enriched in the transition from diapause to reproduction (downregulated in diapause vs. founder post-diapause queens). However, they did not pass the Benjamini correction ($P = 0.06$ and 0.08 for

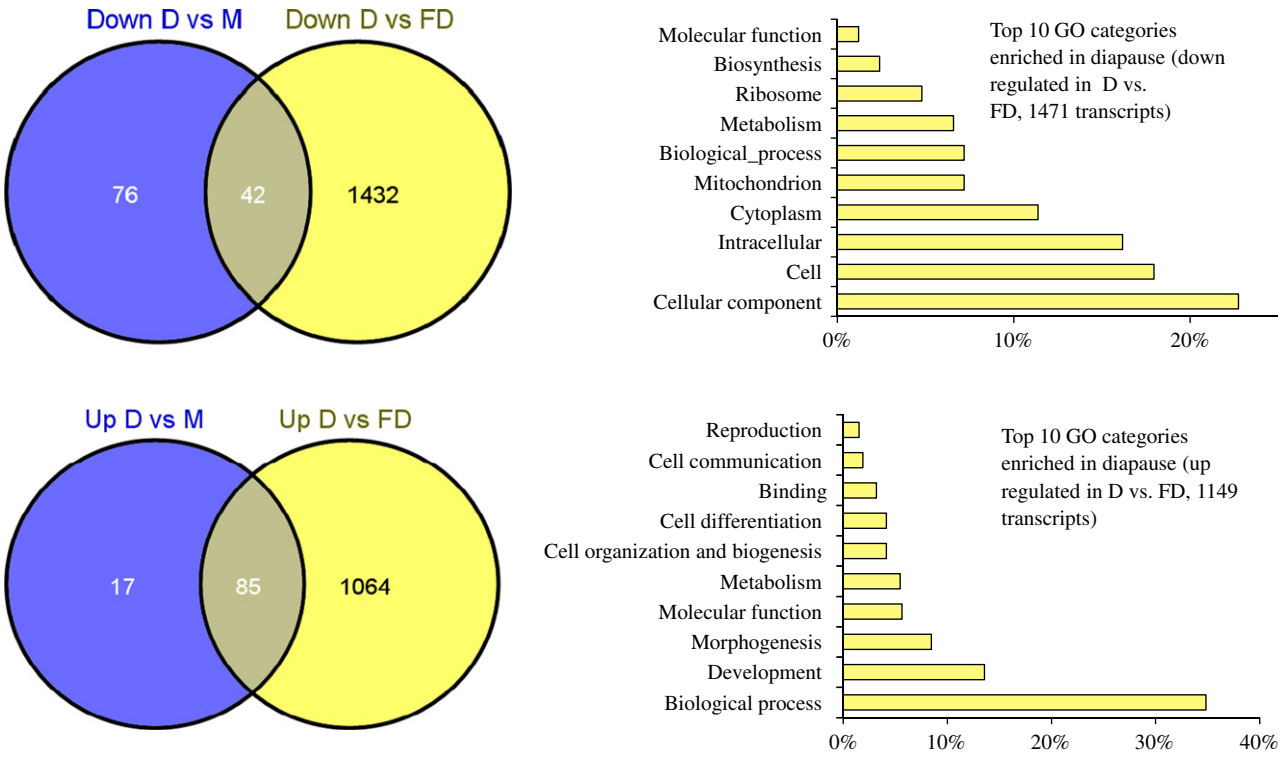

Fig. 4 Transcripts and functional categories regulated during diapause. To the right: Gene ontology (GO) categories regulated during the transition from diapause (D) to founding a colony (FD). We identified the DETs that were significantly downregulated or upregulated in diapause relative to founder post-diapause queens. GO analysis was performed on these sets of transcripts. Only the top 10 GO categories that were significantly overrepresented (Benjamini < 0.05) are included. To the left: Transcripts regulated exclusively during diapause. We identified transcripts that were downregulated in diapause queens (D) relative to both mated (M) or founder post-diapause (FD) queens (42 genes), or were upregulated in diapause queens relative to both groups (85 genes).

MAPK and progesterone-mediated oocyte maturation, respectively).

While transcripts that were regulated between diapause and founder post-diapause queens may reflect the processes involved in the overall transition to reproduction, transcripts that are uniquely regulated in diapause vs. *both* mated and founder post-diapause queens (i.e. upregulated in diapause and downregulated in the two other groups and vice versa) may describe specific processes that are important for maintaining the diapause state. To identify these transcripts, we performed pairwise comparisons between mated vs. diapause queens and between diapause vs. founder post-diapause queens and then overlapped the two groups to identify shared transcripts (Fig. 4b). This overlapped list of transcripts should exclude any transcripts that are differentially expressed as a function of reproductive status, age or any process that is independent of diapause. A total of 42 and 85 transcripts were exclusively down- and upregulated, respectively, in diapause queens relative to the two other states indicating a unique gene expression pattern during diapause (Table S3d, Supporting information). In fact, more transcripts were exclusively upregulated (85) than downregulated (42) in diapause queens, reaffirming that diapause is not simply a cessation of transcriptional activity (Denlinger 2002). Surprisingly, given the widespread role of diapause in insect life history, 7% of these transcripts were annotated as 'hypothetical proteins' that may be unique to *B. terrestris* (i.e. these transcripts contained open reading frames where a protein should exist, but had no identifiable orthologs in other species, (Sivashankari & Shanmughavel 2006). Among *all* of the DETs that exhibited differential expression in diapause queens (either compared to mated or founder post-diapause queens, 2666 DETs), almost 10% were hypothetical. It has been hypothesized that such 'orphan' genes may be particular importance in caste and social evolution (Sumner 2014).

Is there a genetic toolkit regulating diapause across insect species?. To determine whether common genes and pathways are associated with diapause across Diptera and Hymenoptera, we compared the DETs that were significantly regulated during diapause in our study (compared to both mated and founder post-diapause)

with those transcripts identified in early-diapausing flesh flies *Sarcophaga crassipalpis* (compared to nondiapausing flies (Ragland *et al.* 2010). Note that as we only compared DETs that had *Drosophila* orthologs in both species, naturally, there were many more DETs in *S. crassipalpis* (4627, all the DETs that were identified in diapause) than in *B. terrestris* (1177, 44% of the genes identified in diapause). Comparisons were conducted using both up- and downregulated DETs and significantly overrepresented GO categories associated with those transcripts. The two species shared 236 and 86 down- and upregulated DETs, respectively (Fig. 5, Table S3e, Supporting information). This overlap was statistically significant according to Fisher's exact test (two-tail: $P < 0.001$ for both the up- and downregulated transcripts). Similar overlap was found also at the level of GO categories: 10 (of 34 and 105 for *S. crassipalpis* and *B. terrestris*) and 21 categories (of 96 and 30 for *S. crassipalpis* and *B. terrestris*) significantly overlapped in the up- and downregulated categories (Table S3f, Supporting information, Fisher's exact test, two-tail: $P < 0.001$ for up- and downregulated GO terms, details of the statistical test can be found in the legend to Fig. 5). Thus, despite the differences in the insect species (Diptera vs. Hymenoptera) and the stage where diapause takes place (pupae vs. adult), the two species have many shared elements on both the level of specific genes and gene functional categories that are regulated during diapause.

We examined the shared elements (transcripts and GO categories) in more details in order to characterize them. A handful of the shared transcripts matched with general processes that were associated with diapause in different species such as JH synthesis (*juvenile hormone epoxide hydrolase*), insulin signalling and metabolism (such as *glycogen-binding subunit 76A*) and stress response (such as *menin-like* and *stress response protein NST1*). However, the majority of the shared elements were transcripts regulating processes at the cellular level, mostly downregulation of ribosomal and mitochondrial activity and upregulation of nucleotide binding and protein activity (the list of shared transcripts and the corresponding GO categories can be found in Tables S3e and S3f, Supporting information). Thus, the 'diapause genetic toolkit' may primarily involve genes that maintain diapause at the cellular level.

At the level of specific genes, our results also corresponded well with data obtained in previous studies. For example, genes for metabolic enzymes (*sorbitol dehydrogenase* and *trehalase like*) showed the same patterns of expression during diapause in *B. terrestris* queens and in the orange wheat blossom midge (Gong *et al.* 2013). In both *B. terrestris* queens and *Drosophila montana* females, expression levels of *hsp-83* were downregu-

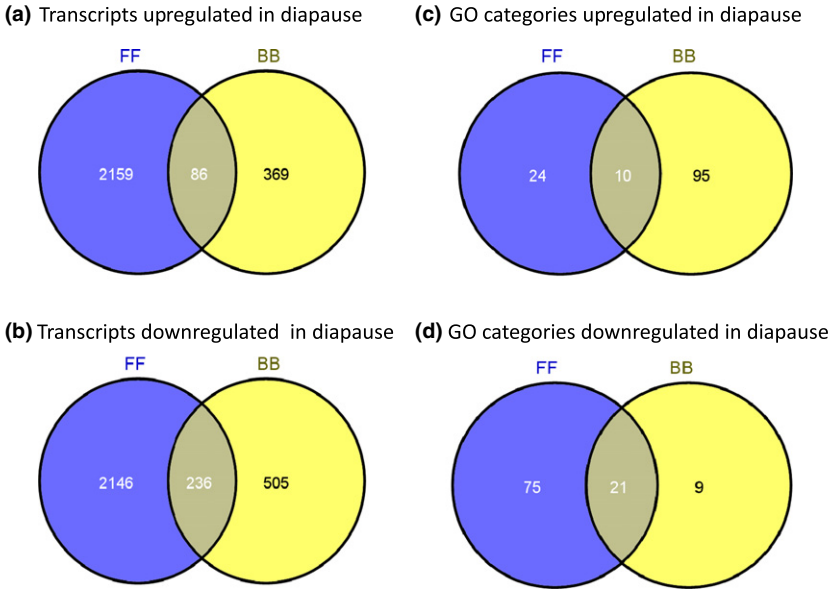

Fig. 5 Shared differently expressed transcripts and gene ontology (GO) categories that were up- and downregulated during diapause between *Bombus terrestris* (BB) and the flesh fly *Sarcophaga crassipalpis* (FF). Fisher's exact test was conducted based on the number of transcripts/GO categories that were exclusively differentially expressed in *B. terrestris*, exclusively differentially expressed in *S. crassipalpis*, overlapped between the two species and the total number of transcripts or GO categories that were identified in both species (8777 transcripts and 1932 GO categories). All four comparisons overlapped significantly according to Fisher's exact two-tail test as follows: a. table [86,369,2159,6163] $P < 0.001$; b. table [236,505,2146,5890] $P < 0.001$; c. table [10,95,24,1781] $P < 0.001$; d. table [21,9,75,1827] $P < 0.001$.

lated in diapause vs. post-diapause (Kankare *et al.* 2010). Similarly, expression levels of *Pepck* and *PCNA* (genes that were suggested to have a universal role in diapause) decreased in prediapause vs. diapause in both *B. terrestris* queens and the Asian tiger mosquito, *Aedes albopictus* (Poelchau *et al.* 2013a). Many more specific examples exist, suggesting that regardless of the species or the developmental stage at which diapause takes place, core genetic mechanisms underlining the maintenance of diapause are conserved across different taxa.

Candidate genes and processes associated with diapause. We identified several candidate genes from the RNA-Seq analysis, as well as others from studies of diapause in other species (all the genes listed below are also provided in Table S4, Supporting information with their corresponding transcript/s in the current study). We tested the expression of eleven of these genes using a larger sample size of queens with qRT–PCR (Table S5; Fig. S2, Supporting information, n = 6–8 individuals/treatment).

Insulin signalling genes, such as *foxo* and the insulin receptor *InR*, are key regulators of diapause in other species (Sim & Denlinger 2008, 2013; Kubrak *et al.* 2014). Low insulin levels (either due to starvation, diapause or maturation) upregulate *foxo*, which in turn upregulates *InR*, thus allowing the cells to respond rapidly to changes in nutrition by turning on the mechanisms that stimulate metabolism (Puig *et al.* 2006). Levels of both *foxo* and *InR* were significantly upregulated in diapause and mated vs. founder post-diapause queens, suggesting that young mated queens are already programmed to diapause. The insulin signalling pathway has a positive association with JH levels (Mirtha *et al.* 2014). Accordingly, JH levels were low and *foxo* expression levels were high in mated and diapause queens. JH titres increase and *foxo* decreases only after the transition to reproduction in the founder post-diapause queens. In addition, the sharp increase in JH levels right after CO_2 treatment may stimulate the insulin signalling pathway and may explain why CO_2 queens are metabolically compromised in the long term, after colony founding.

Diapausing queens are also undoubtedly affected by the progressive decrease in temperature that they experience. Translation of temperature cues to physiological changes in insulin and JH signalling pathways may be mediated by heat-shock proteins. In *Drosophila melanogaster*, the heat-shock protein *hsp-83* binds to and stabilizes the JH receptor complex, allowing it to activate expression of key JH-responsive genes, including the transcription factor *Kr-h1* (He *et al.* 2014), which has been associated with JH-mediated reproductive dominance in bumble bee workers (Shpigler *et al.* 2010). Diapause queens show downregulation of *hsp-83* that is consistent with the maintenance of low JH signalling during diapause. Several other heat-shock proteins (*shsp, hsc70* and *hsp90*) are differentially regulated in a tissue-specific manner throughout diapause in *B. terrestris* queens (Kim *et al.* 2008), of these, *hsc70* was found to be significantly upregulated in diapause vs. founder post-diapause queens in the current study.

Neurotransmitters also play a key role in regulating diapause. Dopamine, for example, is an important regulator of diapause induction in several insect species (Puiroux *et al.* 1990; Noguchi & Hayakawa 1997, 2001; Denlinger 2002). Dopamine and other biogenic amine levels are modulators of the corpora allata activity (Hartfelder 2000) – the tissue that produces JH – and thus may help transduce environmental cues into changes in hormones and physiology. In our study, diapause queens displayed low levels of *alpha-methyldopa hypersensitive (amd*), a gene that is directly involved in regulating the levels of DOPA decarboxylase, the enzyme responsible to dopamine synthesis. This decreased expression is consistent with the reduced JH titres found in diapause queens. Gamma-aminobutyric acid (GABA) is another predominantly inhibitory neurotransmitter that shows increased activity in response to environmental stresses. We found that a GABA receptor-associated gene (*Gamma-aminobutyric acid receptor)* was significantly downregulated in diapause queens compared to either mated or founder post-diapause queens. A decreased GABA signalling in the diapause state is consistent with previous studies showing that GABA inhibitors can influence diapause in *Sarcophaga bullata* (Webb & Denlinger 1998) and *Bombyx mori* (Hasegawa & Shimizu 1990).

Changes in stress response and immune function are also commonly associated with the diapause phenotype. Expression levels of two key immune genes were downregulated in diapause queens: *phenoloxidase subunit A3-like* (*PO*), which is involved in wound healing (Zufelato *et al.* 2004; Colgan *et al.* 2011), and the antimicrobial peptide (AMP) *defensin* (though note that a qRT–PCR for *defensin* revealed a nonsignificant trend, see Fig. S2, Supporting information). Expression levels of two other genes were upregulated in diapause queens: the AMP *hymenoptacaein* (also tested using qRT–PCR, Fig. S2, Supporting information), and *menin*, a global regulator of stress response (Papaconstantinou *et al.* 2005). Additionally, as lysozymes are important defence mechanisms in *B. terrestris* (Erler *et al.* 2011; Richter *et al.* 2012), we tested the expression levels of two additional lysozyme genes using qRT–PCR. *Lys-3* (but not *Lys-1*) was significantly reduced in founders compared to mated and diapause queens (Table S5; Fig, S2, Support-

ing information). Altogether, these results suggest that immune-related genes are subjected to a complex modulation during diapause, rather than a simple up- or downregulation.

Expression of several metabolic genes related to fatty acid and lipid metabolism was regulated as well. Both *lipid storage protein 1* (*Lsd-1*) and *long-chain-fatty acid-CoA ligase ACSBG2-like* (an ortholog to *bubblegum* in *Apis mellifera*) showed an intermediate level of expression in diapause queens between mated (highest) and founder post-diapause queens (lowest). *Lsd-1* is involved in activating triacylglycerides (TAG) metabolism in *Drosophila melanogaster* (Arrese *et al.* 2008) and may be related to changes in lipid content in bumble bee queens. Interestingly, in *Apis mellifera*, expression levels of both of these genes are responsive to immunostimulation and correlate with changes in cuticular hydrocarbon patterns (Richard *et al.* 2012). *Lsd-2* favours the accumulation of TAG and is hypothesized to show an opposite expression pattern to *Lsd-1* (MacRae 2010); expression levels were indeed the opposite with high levels in founder post-diapause vs. mated queens, with diapause queens, again, displaying intermediate levels. Altogether, the findings point to a continuous and progressive regulation of TAG that starts before entering diapause. Another gene, *putative fatty Acyl-CoA*, was found to be involved in the biosynthesis of wax esters (Teerawanichpan & Qiu 2010) and was downregulated in diapause queens. Wax esters function as reserve fuels and the enzyme that synthesizes them is predicted to be higher at times when reserves are accumulated. Thus, downregulation of *putative Acyl-CoA reductase* in diapause queens is consistent with the diapause maintenance period, where reserves are used rather than accumulated.

Finally, the protein encoded by the conserved *couch potato gene* (*cpo*) was identified as a major genetic locus determining diapause phenotype in *D. melanogaster* (Schmidt *et al.* 2008) and in other insects (Kankare *et al.* 2010; Zhang & Denlinger 2011). Although *cpo* was not differentially expressed in the RNA-Seq analysis, its expression levels in qRT–PCR were significantly higher in mated and diapause compared to founder post-diapause queens (Fig. S2, Supporting information), in accordance with the *D. melanogaster* studies.

The effect of CO_2 on bumble bee queens. To characterize the effect of CO_2, we focused on the genomic changes CO_2 induced in the short term (between mated and CO_2-treated queens, before ovaries are activated) and the long term (post-colony founding, between the two founder queen groups). Although CO_2 treatment was responsible for 30.66% of the variation in gene expression between the queen groups (Fig. 3), expression levels of only a small number of transcripts were significantly differentially expressed due to CO_2 treatment in the long- and short term. Thus, while CO_2 treatment may have caused small changes in expression of many transcripts (a trend that is well pronounced in the qRT–PCR data, see Fig. S2, Supporting information), these changes only reached significance for a very small subset of transcripts.

Comparison of the mated and CO_2-treated groups identified 12 DETs in the short term (Table S3a, Supporting information). Among these were several key genes related to stress and immune response [*carbonyls vitellogenin-6* (Nakamura *et al.* 1999), *phenoloxidase subunit A3-li*ke, *e*, *Lys-1*, *hymenoptaecin*, *defensin*], reproduction [*serine protease inhibitor 3-like* (Harrison *et al.* 2015)] and metabolism [*facilitated trehalose transporter* (Kikawada *et al.* 2007), *headcase* (Weaver & White 1995), *alkyl glycerol monooxygenase-lik*e (Watschinger *et al.* 2010) and *putative fatty acyl-CoA reductase* (Teerawanichpan & Qiu 2010)]. Additionally, although metabolic-related genes that were tested using qRT–PCR in CO_2-treated queens showed an intermediate levels between mated and founder queens, the trend was not significant (*Lsd-1:* $P = 0.08$, *long-chain-fatty acid-CoA ligase ACSBG2-like :* $P = 0.08$; Fig. S2, Supporting information). Many genes in the CO_2-treated queens showed a similar trend (Fig. S2, Supporting information), suggesting that CO_2-treated queens become more founder-like immediately after CO_2 treatment.

Comparisons between the two founder groups and mated queens revealed the interacting effects between the transition to active egg laying and CO_2 treatment vs. diapause (Fig. 6). Comparison of the founder post-CO_2 and founder post-diapause groups identified only 6 transcripts whose expression levels were significantly impacted by CO_2 treatment in the long term. Two of the genes that were downregulated in founder post-CO_2 queens (*laccase* and *glucose dehydrogenase*) were previously found to be significantly upregulated in reproductive vs. nonreproductive females of *B. terrestris* (Harrison *et al.* 2015), suggesting again that founder post-CO_2 queens are metabolically/reproductively deprived compared to founder post-diapause queens. Approximately 400 DETs were shared between both founder groups compared to mated queens (transcripts were assessed independently in each founder group compared to mated queens and were then overlapped; Table S3a, Supporting information), indicating that these are likely associated with active egg laying; consistent with this, many of these DETs were classified into GO terms associated with reproduction, cell cycle and cell differentiation (Fig. 6). In addition to the 400 DETs that were shared between the founders, only 60 additional DETs were shared between founder post-

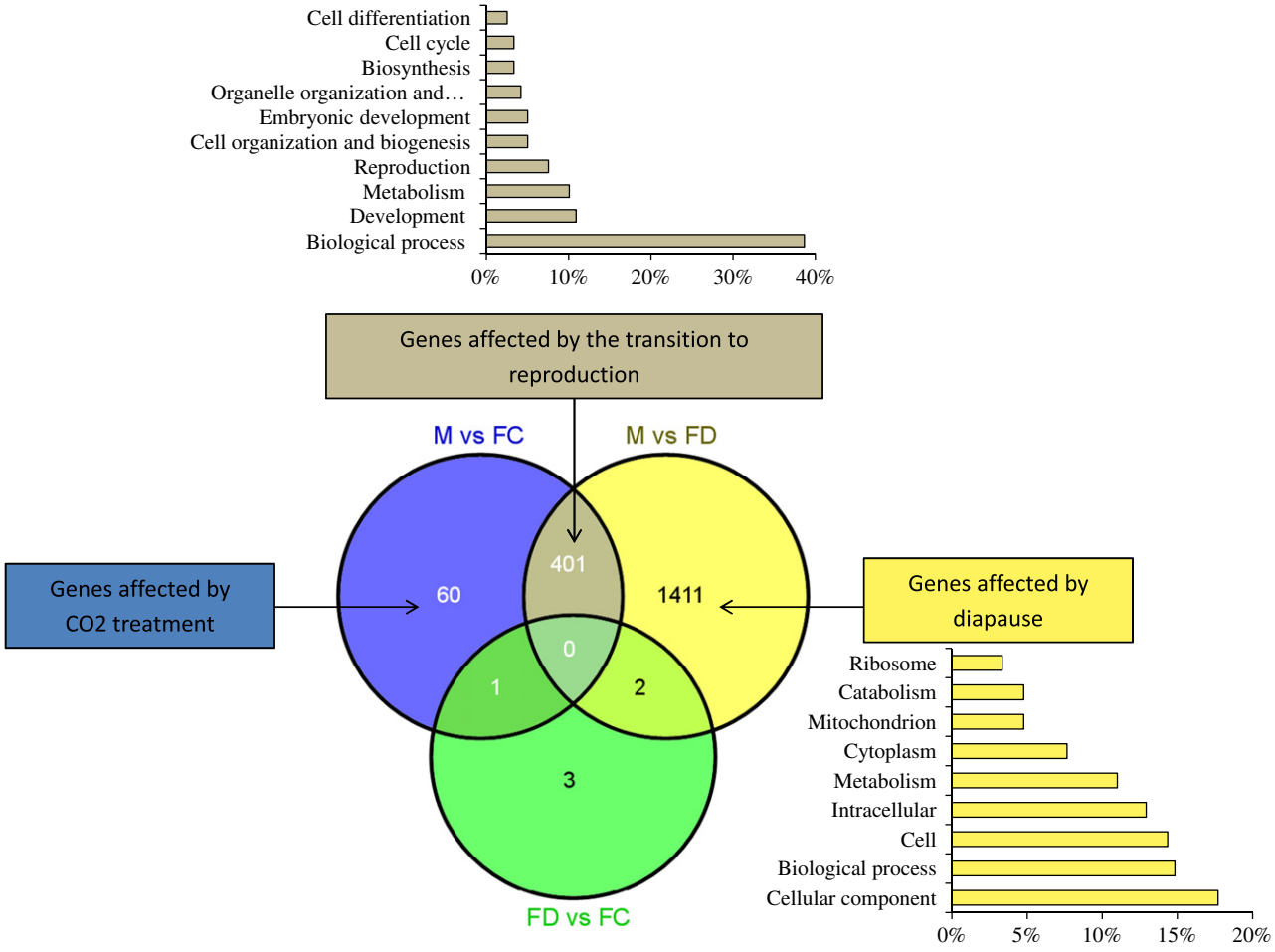

Fig. 6 Identification of transcripts and gene ontology (GO) terms exclusively regulated in the transition to diapause and reproduction in *Bombus terrestris* queens. We overlapped the DETs between the pairwise comparisons of mated vs. founder post-diapause and CO_2 treatment queens (M vs. FD and M vs. FC, respectively) and the two founder groups (FC vs. FD) and identified the transcripts that are regulated during reproduction, diapause only and CO_2 only. Gene ontology (GO) analysis was performed on these sets of transcripts. Only the top 10 GO categories that were significantly overrepresented (Benjamini <0.05) are included.

CO_2 and mated queens, while additional 1411 DETs were shared between founder post-diapause and mated queens (Table S3a, Supporting information). The transcripts influenced by diapause only classified to GO terms mostly involved in cellular and metabolic processes.

Overall, our results provide some indication that CO_2 treatment may have subtle negative effects on queen health. Founder post-CO_2 have lower lipid stores than founder post-diapause queens (Fig. 2), suggesting founder post-CO_2 queens may be more vulnerable to nutritional stress and have fewer resources to allocate to egg laying and/or lay eggs in a higher rate compared to founder post-diapause queens. After CO_2 treatment, JH levels rise rapidly, coincident with changes in expression in stress response and immunity genes. Finally, in the long term, the transition from mated to founder post-diapause queens is associated with many more changes in gene expression than the transition from mated to founder post-CO_2 queens: it remains to be determined whether the genomic differences in the transition of queens to founders via CO_2 or diapause are associated with physiological or behavioural changes.

Role of diapause genes in the evolution of social behaviour. It has been hypothesized that the genes underpinning diapause in solitary insects were co-opted in the evolution of social insects to generate queen vs. worker castes (Hunt & Amdam 2005; Hunt *et al.* 2007). Primitively eusocial insects that lack morphological differences between castes (such as bumble bees) may provide an insight into this transition. Previous study in the primitively eusocial paper wasp *Polistes metricus* demonstrated that a diapause signature (of extended maturation time and enhanced synthesis and sequestra-

tion of a hexameric storage protein) characterizes the development of gyne offspring and is regulated by JH, suggesting a shared regulatory mechanism for diapause and caste differentiation in early life (Hunt *et al.* 2007).

To examine this hypothesis using our data, we compared the list of DETs in diapause queens (vs. both mated and founder post-diapause queens) with the 16 transcripts found to differ significantly in expression levels between queen- and worker-destined bumble bee larvae in a subtractive hybridization study (Pereboom *et al.* 2005). Of these, four were not differentially expressed in Pereboom *et al.* study and have no hits to the current *B. terrestris* genome. Of the remaining 12 transcripts, seven are significantly regulated in diapause queens (Table S6, Supporting information). In a second study (Colgan *et al.* 2011) that identified DETs among all life cycle stages and castes (including larvae, pupae and males) in *B. terrestris*, we examined the 30 contigs with the highest *R*-values (Table 2 in Colgan's study). Of these, seven contigs do not match the annotated gene list from the *B. terrestris* genome, while 18 overlap with transcripts differentially regulated during diapause in our study (Table S6, Supporting information). The five contigs that did not overlap displayed no difference between queens and workers in Colgan's study. A third study (Harrison *et al.* 2015) that also examined transcriptional differences among all life cycle stages in *B. terrestris* identified 791 transcripts as 'fertility genes' (i.e. transcripts upregulated in reproductive workers compared to nonreproductive workers and that were also upregulated in mother queens compared to nonreproductive workers) and 40 transcripts as 'queen genes' (i.e. transcripts, upregulated in mother queens compared to both reproductive and non-reproductive workers). As the nucleotide sequences were not available, we used the transcripts that had matches to *B. terrestris* annotated gene list (the list of hits was provided by Harrison *et al.* 2015) which corresponded to 473 and 18 transcripts in the fertility and queen gene list, respectively. Among these, 86 fertility transcripts (58 unique hits) and six queen transcripts matched DETs in the current study. The comparison and list of genes that overlapped between the studies are provided on Table S6a (Supporting information). While a true test of the hypothesis that caste differentiation genes were co-opted from diapause genes would involve a comparison with a transcriptomic analysis of worker- vs. queen-destined larvae/pupae, overall these results indicate that there is indeed great overlap between caste and diapause-associated genes.

Another aspect of the transition from solitary to social life styles is the change in the role of key genes and signalling pathways related to reproduction. The JH–vitellogenin pathway in particular went through several modifications during the evolution of sociality, with JH changing its role from gonadotropin in most insects (including bumble bees) to a regulator of maturation and division of labour in honeybees and several other eusocial insects (Amsalem *et al.* 2014a). Vitellogenin also evolved distinct nonreproductive functions in honeybees and bumble bees such as regulating foraging or aggressive behaviours (Amdam & Page 2010; Amsalem *et al.* 2014a). Sociality is also associated with a shift in the pattern of interaction between JH and vitellogenin: expression levels of JH and vitellogenin are positively correlated in most insects (Hagedorn 1979), negatively correlated in the honeybee (Pinto *et al.* 2000) and apparently uncoupled in *B. terrestris*, which could represent an intermediate stage in the evolution of sociality (Amsalem *et al.* 2014a). Indeed, consistent with other studies in *B. terrestris* (Amsalem *et al.* 2014b; Shpigler *et al.* 2014), JH retained its ancestral state as gonadotropin (higher in founder queens with activated ovaries and lower in nonreproductive, mated and diapause queens), but its levels were not directly associated with entering or exiting diapause (Larrere *et al.* 1993). *Vitellogenin* expression levels showed no differences among mated, diapause and founder post-diapause queens (Fig. S2, Supporting information). Furthermore, other genes associated with JH and vitellogenin pathways (*vg-6*, *JH hydrolase*) showed distinct patterns (compared to other diapausing insects) that are either associated with reproduction but not with diapause, or not associated with either diapause or reproduction. As both JH and vitellogenin are associated with social roles in social insects, their modified regulation patterns during diapause may indicate that they were co-opted to function instead in caste differentiation and division of labour, early in the evolution of social insect groups.

Interestingly, our results also suggest that vitellogenin has evolved novel function related to queen behaviour in bumble bees. In a previous study, we found that vitellogenin is associated with dominance and aggressive behaviour in *B. terrestris* workers (Amsalem *et al.* 2014a). In the current study, we found that vitellogenin expression levels are increased in CO_2-treated vs. mated queens, and these queens also display increased activity (such as flying and aggression, personal observations, EA) prior to nest founding. Another vitellogenin transcript, *vg-6*, was also expressed at higher levels in young, sterile queens (mated, CO_2-treated and diapause queens) vs. old, reproductive queens (the two founders), consistent with its hypothesized role in ageing rather than in reproduction (Nakamura *et al.* 1999; Harrison *et al.* 2015). Thus, the role of these two vitellogenin transcripts in bumble bees needs to be further

examined with regard to activity levels and aggressive behaviour.

Conclusions

Together with other studies, our results provide a model for how diapause might be regulated in bumble bees (Fig. 7). Overall, the observed physiological and transcriptional changes associated with diapause in bumble bee queens indicate that this process is largely regulated by genes and pathways related to insulin signalling, JH, nutrient storage, stress resistance and core metabolic and cellular pathways. Furthermore, based on the transcriptome comparison with the flesh fly and qualitative comparisons with specific genes and pathways in other species, these genes appear to indeed form a diapause genetic toolkit that is shared across species regardless of the degree of dormancy they present, the stage in which diapause takes place, and the environmental conditions associated with the dormancy, although more species and orders should be examined. The shared elements are accompanied by modifications that are likely to be species specific or related to the social organization: there are clearly novel molecular mechanisms that evolved to regulate diapause in bumble bee queens, as evidenced by the large proportion of 'orphan' genes regulated in diapause queens and the uncoupling of JH and vitellogenin levels from the entry to/exit from diapause. While our study evaluated the major transitions that occur during the queen's life, future studies, using shorter time period or different tissues, are necessary to evaluate the mechanisms that mediate entry or exit from diapause in response to environmental cues, and initiation of egg laying upon nest founding.

Bypassing diapause using CO_2 treatment resulted in overall fairly subtle effects, including changes in JH levels and expression of immunity and stress genes in the short term and lipid mass in the long term. Overall, these results suggest that bypassing diapause with CO_2 treatment (a practice commonly used in commercial bumble bee rearing operations) may have negative consequences on queen health, but these need to be further investigated, particularly given the importance of bumble bees to pollination services for a large variety of agricultural crops (Velthuis & van Doorn 2006).

Finally, we found substantial overlap between the genes associated with diapause and those differentially expressed between developing and adult bumble bee queens and larvae. Thus, the mechanisms regulating diapause may indeed have been co-opted to generate nonreproductive, nondiapausing workers and fecund, long-lived, diapausing queens. Genomic data using a broader array of solitary and social insects can provide information about how conserved this toolkit is and how it was modified during the evolution of social behaviour.

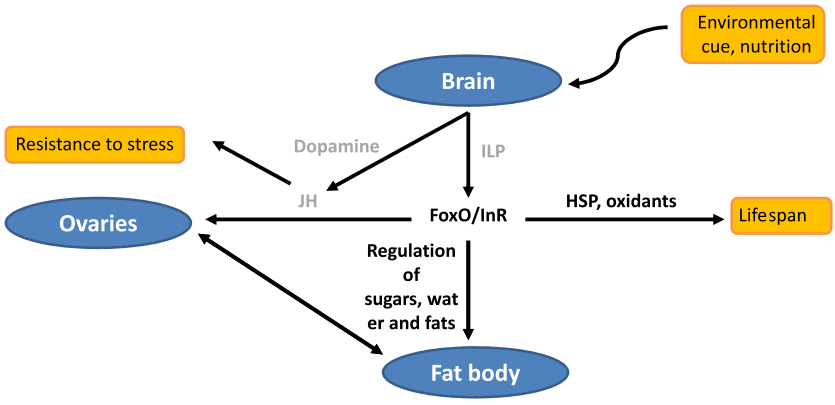

Fig. 7 Simplified schematic representation of the pathways regulating diapause and their interacting effects on bumble bee queens. An environmental cue, most likely a change in temperature and/or nutrition, is predicted to trigger diapause-associated change in the brain. Nutrition-sensing pathways such as TOR or MAPK (the latter was downregulated in diapause queens) or heat-shock proteins are most likely to be effected, leading to a change in hormones, metabolism and longevity. The first change in the brain is likely to be a reduction in levels of insulin and neurotransmitters such as dopamine that inhibit the synthesis of JH in the corpora allata. JH and insulin have pleiotropic effects on fat storage, ovarian activation immunity and ageing. Increase in JH levels shifts the fat body tissues from accumulating fats to generating resources for ovarian activation and egg production, ultimately resulting in colony establishment. This shift is regulated by broad metabolic changes particularly in genes related to the biosynthesis and transport of lipids. Utilizing lipids and the regulation of heat-shock proteins are associated with decreased longevity and immune functions (also regulated by the fat body tissue). For a detailed overview of the pathway components, the reader is referred to the text. ILP, insulin-like peptide; *InR*, insulin receptor; *foxo*, forkhead box-related transcription factors, class O, HSP, heat-shock proteins; JH, juvenile hormone.

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E.A. designed and performed research, analysed data and wrote the manuscript. D.A.G. analysed RNA-Seq data, J.C. provided the queens and performed treatments (mating, CO_2, diapause), P.E.A.T. performed the juvenile hormone analysis. C.M.G. designed research and wrote the manuscript along with E.A.

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 GSE73009; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73009). Transcript counts, normalized reads for PCA, RT–PCR gene expression, and all phenotypic raw data including ovarian activation, JH levels, body mass, lipid and glycogen content are available on Dryad: doi:10.5061/dryad.td887.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sample size of *B. terrestris* queens that were used in the current study and a diagram explaining the sampled queen groups, point of sampling and treatments.

Table S2 Statistical test results obtained for ovarian activation, Juvenile hormone, body mass, glycogen and lipid content in queen fat body.

Table S3a List of all significantly expressed transcripts in the current study.

Table S3b List of significant GO terms up regulated in diapause vs. founder post diapause queens.

Table S3c List of significant GO terms down regulated in diapause vs. founder post diapause queens.

Table S3d List of significantly expressed transcripts exclusively regulated in diapause queens (vs. both mated and founder post diapauses queens).

Table S3e List of significantly expressed transcripts shared by *Bombus terrestris* and *Sarcophaga crassipalpis*.

Table S3f List of significantly GO terms shared by *Bombus terrestris* and *Sarcophaga crassipalpis*.

Table S4 List of genes of interest and their corresponding transcript ID in the current study.

Table S5 List of genes and primers for qRT-PCR and the number of reads in the RNA-Seq analysis corresponding to the genes that were tested using qRT-PCR.

Table S6 Comparison between caste specific genes in larvae and adults of *B. terrestris* in previous studies and diapause specific genes in the current study.

Table S6a Comparison between fertility and queen genes in Harrison *et al.* 2015 study and diapause specific genes in the current study.

Fig. S1 Heatmap and clustering analysis of the DETs in *B. terrestris* queens fat body.

Fig. S2 Validation of RNA-seq results using qRT-PCR.